

Understanding the Role of MicroRNA-31 in Regulating Cellular Sensitivity to Chemoradiotherapy in Pancreatic Ductal Adenocarcinoma



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DECLARATION

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Abstract

Pancreatic adenocarcinoma (PDAC) is a lethal malignancy with a poor survival rate. One main challenge regarding PDAC is resistance to chemotherapy and radiotherapy. Therefore, there is an urgent requirement to characterise the mechanisms underpinning chemo-resistance and radio-resistance in PDAC. Recent evidence has revealed that microRNAs (miR) play a pivotal role in resistance to chemotherapy in other cancer types by controlling drug trafficking and sequestration. Additionally, it is well established that miRs can modulate radio-resistance by altering levels of oxidative stress. MiR-31 has previously been demonstrated to regulate sensitivity to chemoradiotherapy in other cancer types, although it remains largely unexplored in PDAC. Here, we investigated the biological role and potential mechanisms of miR-31 in PDAC chemo-resistance and radio-resistance.

Overexpressing miR-31 in BxPC-3 cells significantly promoted clonogenic resistance to platinum-based chemotherapeutics, particularly cisplatin. Reciprocally, suppressing miR-31 in Panc-1 cells enhanced cisplatin sensitivity. Although miR-31 increased chemo-resistance, paradoxically, inductively coupled plasma mass spectrometry (ICP-MS) revealed a higher relative intracellular accumulation of platinum. This was associated with a significantly decreased intranuclear concentration of platinum which may explain the differences in DNA damage induction. *In silico* analysis displayed ATOX1, a vital drug transporter, as a predictive target of miR-31, may play an essential role in shuttling cisplatin to the nucleus. Overexpressing ATOX1 in PDAC cells displayed increased cisplatin sensitivity and presents as a useful target for modulating chemo-resistance in PDAC.

Moreover, it was found that manipulating miR-31 altered radiosensitivity in PDAC cells by regulating oxidative stress and DNA damage. Glutathione peroxidase 8 (GPx8) is an anti-oxidant enzyme that plays an important role in the elimination of reactive oxygen species (ROS). Using online

bioinformatics algorithms, we identified the 3'UTR of GPx8 as a predictive target of miR-31. Our study demonstrates that manipulating miR-31 alters GPx8 expression for the first time, thereby regulating ROS detoxification and promoting either a radioresistant or radiosensitive phenotype.

Our study demonstrates the potential mechanisms underlying the chemo-resistance and radio-resistance of PDAC cells mediated by drug trafficking and oxidative stress by miR-31, indicating promising targets and therapeutic strategies in PDAC.

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Chapter One

General Introduction

1.1 Pancreatic Ductal Adenocarcinoma

1.1.1 Overview

Despite recent advances in understanding the disease, pancreatic cancer remains one of the world's deadliest malignancies. Although the disease accounts for only 3% of all cancers, it is the seventh leading cause of cancer-related deaths worldwide [1]. In addition, pancreatic cancer has an overall five-year survival rate of less than 9%, with most patients dying within three to eight months post-diagnosis. GLOBOCAN estimates showed approximately 495,773 diagnoses and 466,003 deaths from pancreatic cancer globally in 2020 [2]. Pancreatic cancer affects almost 600 people in Ireland each year [2]. A significant concern is that this mortality rate is continuing to match the increasing incidence in the Western world; pancreatic cancer is expected to surpass breast cancer to become the second most common cause of cancer-related death in the United States by the year 2030 [3], a trend paralleled in Europe. Because pancreatic ductal adenocarcinoma (PDAC) accounts for over 90% of pancreatic cancer cases, pancreatic cancer will therefore be referred to as PDAC.

Symptoms are often described as non-specific and may be overlooked by patients and physicians [4]. For this reason, as well as the lack of biomarkers for early detection, PDAC presents with an inferior prognosis. Many patients are diagnosed at the inoperable metastatic stage at initial presentation, and even in the minority of patients who undergo surgery (15%), most will relapse and succumb within two years. One significant obstacle to PDAC treatment is that patients are typically unresponsive to chemotherapy and radiotherapy and achieve only modest prolongation in overall survival with conventional therapies, frequently at the cost of significant side effects and a negative impact on quality of life [5]. As such, there is a considerable need to characterize resistance mechanisms to chemotherapy and radiotherapy in PDAC, identify predictive biomarkers

to guide the choice of cytotoxic therapy, and develop new therapeutic approaches.

1.1.2 Risk Factors

As with many other cancers, the risk of developing PDAC increases with age and is predominantly a disease that affects older individuals [8]. The mean age of patients diagnosed with early-stage cancer is around 2.3 years younger than those with advanced-stage cancer, suggesting that it takes approximately 12-24 months for PDAC to advance from the early to the late stage [9]. It is well established that the age at which incidence peaks differ between countries. For example, in India, the incidence of PDAC becomes more common in the sixth decade of life, whereas in the United States, it is the seventh decade of life [10].

Worldwide, the incidence of PDAC is more significant in males than females [1], although incidence rates differ among men and women in developed and developing countries. Where males are twice as likely to develop PDAC than women in India. Despite the sex difference, a systematic review of 15 studies concluded that reproductive factors are not associated with PDAC in women [11]. Therefore, this suggests that the differences in PDAC point towards environmental or undiscovered genetic factors as alternative explanations for male predominance.

It has been proposed that approximately 5-10% of PDAC has a familial basis [12], and patients with familial risk factors have an increased risk of developing PDAC than those with no family history. A first-degree relationship is described as a parent-child or sibling-sibling relationship. One epidemiological study found that Icelanders with a first-degree family relative with PDAC had a 2.33-fold increased risk of developing the disease themselves [13]. Another relatable study extended these analyses by following thousands of patients with a family history of PDAC and showed that patients with two first-first-degree relatives with PDAC

have a 6-fold increased risk of developing the disease, and patients with three or more first-degree relatives with PDAC have up to a 32-fold increased risk [14]. Furthermore, a meta-analysis of nine studies demonstrated that having one affected relative resulted in an 80% increased risk of PDAC [15]. In familial PDAC, the risk rises exponentially with the number of first-degree relatives involved, and BRCA2 and PALB mutations are the most common inherited mutations [16, 17].

The relationship between diabetes and PDAC has long been recognized since 1833, when a patient presented with diabetes and died six months later from PDAC [18]. Since then, many epidemiological studies have displayed that diabetes (predominantly type II) occurs more frequently in PDAC patients and is a well-established risk factor for the disease [19]. In addition, insulin resistance, associated hyperglycaemia, and hyperinsulinemia have all been proposed to be the underlying mechanisms for developing diabetes-associated PDAC [6, 20].

The epidemiological evidence for associating alcohol consumption and its increased risk of developing PDAC is generally mixed [21]. Results from meta-analyses and pooled analyses consistently displayed that daily consumption of ≥ 30 g of alcohol (the equivalent of >3 glasses of any alcoholic drink per day) can increase an individual's risk of developing PDAC by 20% [22]. Cigarette smoking has been undeniably identified as the most important modifiable risk factor for PDAC, with numerous individual and combined studies displaying a positive correlation. The international Pancreatic Cancer Case-Control Consortium (PanC4) study analysed 12 case-control studies, including 6507 pancreatic cases and 12,890 controls, where the results confirmed that current cigarette smoking is associated with a two-fold increased risk of PDAC, and the risk increases with the number of cigarettes smoked and duration of smoking [23].

Chronic pancreatitis (CP) is a progressive inflammatory condition of the pancreas resulting in pathological fibrosis and destruction of acinar cells. Therefore, it is responsible for most of the burden of exocrine pancreatic disease. The overall incidence ranges from 2-14/100,000 in the United States population [24]. Even though there is a strong association between CP and PDAC, over 20 years, only 5% of patients with CP will develop PDAC compared to other more prevalent factors [25]. However, a meta-analysis study displayed a 13-fold increased risk of developing PDAC in these patients compared with controls [25], so ideally, CP patients could be a potential target group for PDAC screening.

1.1.3 Pathogenesis

The classical and well-characterized precursor lesions of PDAC display a ductal phenotype, implying this tumour type's ductal cell of origin. The three best-characterized precursors of this malignancy include pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN), and mucinous cystic neoplasms (MCN) [26]; and it is proposed that these three precursors derive from PDAC stem cells (CSC) [27]; where each precursor has its own unique clinical, pathological, and molecular characteristics.

Pancreatic Intraepithelial Neoplasia (PanIN)

PanIN is non-invasive, microscopical (generally less than 5 mm) mucinous-papillary lesions in the small pancreatic ducts. These lesions were first grouped in 2001 and were initially graded from 1-3 [28]. Lately, a two-tiered system has been introduced to simplify classification, suggesting that the historical grades of 1A/1B and 2 be classified as low-grade PanIN. The original PanIN 3 was revised to a high grade [29]. Concrete data shows that, in general, PanIN 3 is more prevalent in older individuals, patients with PDAC, and patients at high risk for PDAC (e.g., from CP) [30, 31]. Peters *et al.* [32] designed a microsimulation model and

estimated the lifetime probability of progressing from PanIN 1 to detectable PDAC to be 1.5% for men and 1.3% for women. It was also estimated that the progression from PanIN 1 to detectable PDAC took 33.6 years and 35.3 years, respectively, and PanIN 3 to detectable PDAC took 11.3 and 12.3 years [30]; this indicates a possible window for screening preceding the development of invasive PDAC.

Intraductal Papillary Mucinous Neoplasms (IPMN)

IPMN is a cystic pancreatic lesion deriving from intraductal growth of mucin-producing cells and was first described in 1980. It accounts for 1-2% of all pancreatic exocrine tumours and up to 50% of all cystic tumours [33]. Based on location and extent, three subtypes can be identified: main-duct (MD-IPMN), branch-duct (BD-IPMN), and mixed-type IPMN (MT-IPMN). MD-IPMN is perceived as dilation of the main pancreatic duct of greater than 5 mm and is frequently located in the pancreatic head (~65%) and accounts for up to 21% of the IPMNs; additionally, MD-IPMN has the highest risk of exhibiting malignant progression (28-81%) [34, 35]. BD-IPMN is defined as a grape-like cyst (>5 mm) that accounts for up to 64% of IPMNs and can develop multifocally (where more than one tumour can occur) throughout the pancreas. BD-IPMNs have the least risk of malignant progression (7-42%), although their multifocality (40%) is subtle [34, 35]. Finally, MD-IPMN is a crossover between MD-IPMN and BD-IPMN and is seen in up to 38% of all IPMN cases, of which 20-65% are malignant [34, 36].

Mucinous Cystic Neoplasms (MCN)

MCN also represent premalignant lesions in the pancreas where they account for 25% of pancreatic cysts, are more predominant in women, and are defined as mucin-producing and septated cyst-forming epithelial neoplasia of the pancreas with an idiosyncratic ovarian-type stroma [37]. MCNs are often remote; their sizes range from 5 – 35 cm, consisting of a

thick fibrotic wall, and show no clear communication with the ductal system [38]. A study by Crippa *et al.* [35] revealed that of 163 patients with resected MCN, 12% and 17.5% had invasive carcinoma and malignant MCNs, respectively.

1.1.4 Histological subtypes

Most PDACs are believed to arise from PanIN, and the histopathological features help with categorization [39]. PanIN-1A and PanIN-1B are tall columnar cells with basally located small oval nuclei and abundant supranuclear mucin. PanIN-1A and PanIN-1B differences include the flat epithelium and papillary (and micropapillary) architecture, respectively. PanIN-2 presents mainly papillary epithelium with mild to moderate cytological atypia. PanIN-3 is distinguished by typically papillary or micropapillary proliferations of cells with notable cytological atypia.

Histologically, IPMNs can be categorized as gastric-foveolar, intestinal, pancreatobiliary, or oncocytic types established on the direction of differentiation of the neoplastic epithelium [40]. Gastric-foveolar IPMNs are lined by the foveolar epithelium of the gastric mucosa. The neoplastic epithelial cells consist of apical mucin with minute basally orientated nuclei. The flattened epithelium comprises a single layer of cells, but the neoplastic epithelium may often form papillae. Mitosis is scarce, and lesions mostly display low-grade dysplasia. Gastric-foveolar IPMNs can frequently be mixed with intestinal and pancreatobiliary type epithelium. Finally, invasive carcinomas are uncommon but tend to be classified as ductal carcinomas when present. Intestinal IPMNs mirror villous adenomas of the gastrointestinal tract. The neoplastic cells have elongated nuclei and are often described as being pseudostratified. Intestinal IPMNs generally have moderate-to high-grade dysplasia [41]. Pancreatobiliary IPMNs are frequently high-grade lesions with intricate architecture, crib forming papillae, and bridging. They consist of cuboidal neoplastic cells and present atypical nuclei with visible nucleoli. Lower-grade dysplasia is uncommon but, when presented, is characterized by

mild atypia with hyperchromasia and enlarged nuclei [41]. Oncocytic IPMNs are morphologically the most complex lesions and present with intricately branched papillae, cribriform formations, and solid cell nests. They commonly harbour high-grade dysplasia.

Microscopically, MCN cysts are lined by a columnar mucin-producing neoplastic epithelium [42]. Its ovarian-type stroma consists of densely packed spindle cells with round to elongated nuclei and a little cytoplasm. The stromal cells generally express oestrogen, progesterone, and inhibin receptors. It is common to observe fibrotic stroma within the lesions. The degree of dysplasia in MCN is erratic and may change precipitously from minimal to severe. The majority of MCNs are presented as low-grade dysplasia [42].

1.1.5 Clinical Presentation

Due to the location of the pancreas, the initial growth of cancer is silent; therefore, displayed symptoms are often a sign of advanced disease. The presenting symptoms of PDAC are mainly dependent on tumour location. Most tumours are discovered on the pancreatic head; signs and symptoms include the right-upper quadrant or epigastric pain (79%) and jaundice, which affects around 56% of patients as tumours can obstruct the biliary system. Other common manifestations include nausea or vomiting (51%) and diarrhoea (43%). Cancer in the pancreatic body or tail often presents with new or worsening back pain (49%), as PDAC has an affinity for pancreatic nerves [6-7]. Systemic manifestations may include rapid weight loss (85%) and anorexia (83%). Tumours that advance beyond the pancreas may cause duodenal obstruction or gastrointestinal bleeding. Finally, laboratory study abnormalities can include elevated liver function studies, hyperglycemia, and anaemia [6-7].

1.1.6 Staging

The staging of PDAC is based on the tumour-node-metastasis classification (**Table 1**). The size of the tumour and its association with the

main blood vessels are considered when classifying the tumour from TX to T4; nodal classification is defined by the magnitude of lymph node involvement which ranges from NX to N1, and finally, the presence or absence of metastatic cancer which has spread to distant organs, defines the metastatic category as M0 or M1, respectively [43]. Staging for PDAC is a tremendously difficult task, although it influences survival data to appropriately stratify patients into different treatment categories (**Table 2**).

Recent changes in the TNM classification for the T parameter have extended the number of patients compliant with surgical resection [44]. Due to current advantages in surgical techniques, particularly in the venous interposition grafts, the tumours of patients with limited superior mesenteric vein involvement are now deemed resectable. Before the change in TNM classification, these patients would have been considered to have T4 disease. However, the newer TNM classification describes such cases as T3 disease [44].

Table 1. A representation of TNM classification for PDAC.

Primary tumour (T)	DESCRIPTION
TX	Primary tumour is inaccessible.
T0	Primary tumour is undetectable.
T1	Tumour is limited to the pancreas only. Tumour length ≤ 2cm
T2	Tumour is limited to the pancreas only. Tumour length > 2cm
T3	The wide spread of the tumour to tissues, blood vessels, and nerve cells surrounding the pancreas.
T4	Tumour has metastasised to distant organs. Unresectable primary tumour.
Regional Lymph nodes (N)	DESCRIPTION
NX	Regional lymph nodes are inaccessible.
N0	No tumour spread to regional lymph nodes.
N1	Tumours spread to regional lymph nodes.
Distant metastasis (M)	DESCRIPTION
M0	No distant metastasis
M1	Distant metastasis

Table 2. TNM staging and appropriate treatment of PDAC.

Stage	T	N	M	TREATMENT EXPLOITED
IA	T1	N0	M0	Surgery, Postoperative chemotherapy, Postoperative chemoradiotherapy
IB	T2	N0	M0	Surgery, Postoperative chemotherapy, Postoperative chemoradiotherapy
IIA	T3	N0	M0	Surgery, Postoperative chemotherapy, Postoperative chemoradiotherapy
IIB	T1, T2, T3	N1	M0	Surgery, Postoperative chemotherapy, Postoperative chemoradiotherapy
III	T4	N0 or N1	M0	Chemotherapy, Chemoradiotherapy, Palliative surgery
IV	T1, T2, T3, T4	N0 or N1	M1	Palliative therapy, Chemotherapy

1.1.7 Biomarkers for Early Detection

Biomarkers are essential in managing patients with invasive cancers [45]. PDAC remains to have an abysmal prognosis even with the tremendous effort invested in identifying accurate biomarkers, ideally present in the timeframe between carcinogenesis onset and tumour invasion, to allow diagnosing PDAC in early curable stages to improve overall survival [46]. The ideal biomarker should be easily detected in this setting with adequate sensitivity and specificity. However, no such biomarker exists. Consequently, there is an urgent unmet need to identify ideal biomarkers in PDAC.

Serum carbohydrate antigen (CA) 19-9 is the only biomarker approved by the United States Food and Drug Administration (US FDA) [47]. Although CA-19-9 is regarded as the most common and validated biomarker and has been widely used in diagnosing PDAC for a long time, it remains to have a poor predictive value in asymptomatic patients [48]. There are two main challenges facing the use of CA-19-9 alone as a biomarker for the early detection of PDAC. Firstly, only 65% of patients with resectable PDAC have elevated serum CA 19-9 levels, so CA-19-9 testing would result in false-negative results and deceive the diagnosis [49]. Secondly, elevated CA-19-9 has been observed in other medical conditions, including chronic pancreatitis and acute cholangitis, and gastrointestinal malignancies, including gastric and colorectal cancer [50]. Numerous other carbohydrate antigens have been considerably studied, including CA-242, CEA, and CA-125, where they have all been found to be overall less sensitive than CA-19-9 [51].

Mutations of K-ras remain the predominant genetic characteristic in PDAC patients. They can be effectively detected in the serum, pancreatic juice, and faeces, even in patients with premalignant pancreatic lesions [53]. However, K-ras mutations are not exclusively specific to PDAC and are frequently evident in other malignancies and chronic pancreatitis

[54]. One meta-analysis questioned whether K-ras mutation rates increased parallel to the grade of dysplasia in duct lesions. K-ras mutations were identified in 36%, 44% and 87% of PanIN-1A, 1B, and 2-3 lesions, respectively [55]. Mulcahy *et al.* [56] identified K-ras mutations in the plasma of all four patients suffering from chronic pancreatitis (4/4) and reported that all patients were later diagnosed with PDAC during follow-up; from 7.3 months to 16.7 months after the time of K-ras mutation testing. These studies are a useful indication that K-ras mutations are an early event during tumourgenesis; therefore, K-ras mutations can be considered potential biomarkers with appreciable sensitivity for the early diagnosis of PDAC, but further characterization and verification in larger cohorts are essential.

1.2 Treatment

Treatment of PDAC is generally multimodal, where patients will receive surgery, chemotherapy, and/or radiotherapy.

1.2.1 Surgery

Surgery alone remains the only potentially curative treatment. Unfortunately, only those diagnosed early are eligible for surgery, meaning less than 20% of patients have this option; amongst these candidates, the median postoperative survival is less than 20 months, with a five-year survival rate of approximately 20% [57]. Before 1935, pancreatic resections were deemed impossible, with surgery-mortality rates reaching as high as 30%. However, since then, pancreaticoduodenectomy, also known as the Whipple procedure, involves removing the pancreatic head, duodenum, and a portion of the common bile duct, gallbladder, and sometimes part of the stomach is carried out regularly, with surgical-mortality rates falling to below 3% [58].

1.2.2 Chemotherapy

In 1997, the first milestone study regarding palliative chemotherapy in PDAC patients was published, wherein gemcitabine was compared with weekly-dose-5-fluorouracil (5-FU) as the first-line treatment for patients diagnosed with locally advanced unresectable or metastatic PDAC [59]. The results revealed that gemcitabine treatment increased survival from 4.41 to 5.65 months. As gemcitabine therapy was shown to be more beneficial than 5-FU for a percentage of patients, gemcitabine monotherapy became the standard of care, despite only modest increases in overall survival.

A study published in 2011 by Conroy *et al.* [60] first introduced the FOLFIRINOX regimen – a combination of infusional 5-FU/folinic acid, irinotecan, and oxaliplatin – into the treatment of metastatic PDAC. More recently, FOLFIRINOX improved overall survival compared with gemcitabine when administered as a first-line treatment in patients with metastatic PDAC [61]. A phase III trial was instigated to investigate the efficacy of modified-FOLFIRINOX, compared with gemcitabine, as adjuvant therapy after resectable PDAC. Here, Conroy *et al.* [61] showed that median disease-free survival was 21.6 months in the modified-FOLFIRINOX group and 12.8 months in the gemcitabine group. The three-year disease-free survival rates for the modified-FOLFIRINOX group and the gemcitabine group were 39.7% and 21.4%, respectively. The overall survival rate at three years was 63.4% in the modified-FOLFIRINOX group compared to 48.6% in the gemcitabine group. A randomized phase III trial successfully used gemcitabine-free combination therapy for the first time [61].

Furthermore, germline and somatic genetic profiling of PDAC is a new trend in modern research for treatment options [62]. From the perspective of the clinical and therapeutic influence of mutations in PDAC, the BRCA1 and BRCA2 genes are the most studied. This is because BRCA1/2 plays a pivotal role in the homologous recombination (HR)

pathway [63]. Tumours that are BRCA1/2 deficient are deemed defective in DNA repair by HR and are sensitive to DNA crosslinking agents, such as cisplatin. Therefore, these agents are logical options for treating BRCA1/2- deficient tumours and have been clinically effective [64].

1.2.3 Radiotherapy

The role of radiotherapy in PDAC continues to be investigated, and its use in the adjuvant setting remains controversial [65]. Generally, neoadjuvant radiotherapy is accepted in borderline resectable diseases, though prospective data are scarce. Neoadjuvant radiotherapy, with or without chemotherapy, may downstage the illness and increase the chances of complete resection [66]. Interestingly, neoadjuvant radiotherapy can eliminate the need to treat hypoxic tumour tissue, which is often a result of the surgical disruption of a blood supply to the tumour cells. Additionally, extreme surgical stress and postoperative complications significantly increase the release of perioperative cytokines, a phenomenon known as surgical oncotaxis, which has been shown to enhance tumour metastasis, resulting in a poor prognosis for cancer patients [67]. Surgical oncotaxis can adversely affect the efficacy of adjuvant treatment, which can be avoided by neoadjuvant radiotherapy.

One particular meta-analysis that included 111 trials with 4,394 patients investigated the effects of neoadjuvant chemotherapy and radiotherapy on tumour response, resectability, and patient survival [68]. Neoadjuvant chemotherapy was given in 96.4% of the studies with the primary agent's gemcitabine and FOLFIRINOX therapy. Neoadjuvant radiotherapy was applied in 93.7% of the studies with doses ranging from 24 to 63 Gy, where most patients were 1.8 Gy/fraction, 2 Gy/fraction, or 3 Gy/fraction. The studies were divided into two groups: group 1 included patients whose tumours presented as resectable on preoperative examination, and group 2 included patients whose tumours presented as borderline resectable or unresectable. Additionally, Gillen *et al.* [68]

revealed that similar percentages of the tumours in both groups responded to neoadjuvant therapy by shrinking tumours. In group 1 and group 2, approximately 75% and 33% of the tumours were resectable post-neoadjuvant therapy, respectively. After resection, the average overall-survival time for group 1 patients was 23.3 months. The average survival time for group 2 patients after resection was 20.5 months. Therefore, it should be considered that patients with locally non-resectable tumours should be included in neoadjuvant regimes and later re-evaluated for resection.

To date, randomized trials have been unsuccessful in resolving the debate surrounding the role of adjuvant radiotherapy in resectable PDAC. In the 1980s, the Gastrointestinal Tumour Study Group (GITSG) conducted the first randomized trial to evaluate adjuvant chemoradiotherapy's role in resecting PDAC [69]. Patients also received bolus 5-FU chemotherapy during radiotherapy. Unfortunately, despite the trial demonstrating an overall survival benefit between the treatment arm and the observation arm (20 vs. 11 months, respectively), only 43 patients were accrued over eight years. Furthermore, the GITSG study was heavily criticized over slow accrual, small sample size, and suboptimal radiotherapy with a low dose delivered in a split-course fashion, so the trial was forced to close early.

The European Organisation for Research and Treatment of Cancer (EORTC) study consisted of 218 patients, where the objective was to investigate the overall-survival benefit of adjuvant radiotherapy and infusion 5-FU versus observation alone after surgery in patients with PDAC [70]. This study did not show significant overall-survival benefits with the addition of chemoradiotherapy to surgical resection. Median overall-survival rates for the chemoradiotherapy arm as compared to the observation arm were 17.1 months and 12.6 months, respectively. Like the GITSG study, the EORTC study was criticized due to suboptimal RT, small sample size, the high proportion of patients preceding assigned

therapy, and the inclusion of patients with positive surgical margins without stratification deemed as study design flaws.

Further research is needed to define optimal chemotherapy and radiation doses and techniques and to assess the effects of chemotherapy and radiation more closely – not only on survival but also on local disease control and quality of life.

1.2.4 Targeted therapies

Poly (ADP-ribose) polymerase 1 (PARP) enzymes primarily repair single-strand DNA breaks and play crucial roles in DNA damage repair [71]. Accumulation of single-strand DNA breaks in the presence of PARP inhibitors results in the formation of double-strand breaks, which require homologous recombination to repair [71]. Tumours that are deficient in DNA damage repair mechanisms such as BRCA mutants respond better to platinum-based chemotherapies. Interestingly, the phase III POLO trial showed a near doubling of progression-free survival compared with a placebo in advanced PDAC when using the PARP inhibitor, Olaparib [72]. As a result, the US Food and Drug Administration (FDA) approved olaparib as a maintenance treatment for germline BRCA mutated advanced PDAC that has not progressed on platinum-based chemotherapy.

The KRAS gene are the most mutated oncogenes in cancers, resulting in abnormal proliferation and tumorigenesis [73]. Certain tumours are more dependent on KRAS mutation, especially PDAC. The most predominant KRAS mutation site in PDAC occurs at codon 12; most commonly G12D (45%), followed by G12V (35%), and G12R at 17%. Other mutations such as G12C and G12F occur at a lower frequency [74]. Significant progress is now being made in the G12D space with the development of numerous compounds that can bind to and inhibit KRAS G12D, most particularly MRTX1133 [75].

1.2.5 Chemoradiotherapy resistance

The majority of patients with cancer will receive chemotherapy or radiotherapy as a curative treatment or during palliative care [76]. Undoubtedly, the poor prognosis can be partially attributed to the inherent resistance that PDAC appears to have toward chemotherapy and radiation. Resistance to chemotherapy and radiation, the ability of cancer cells to evade or cope with the presence of therapeutics, is a critical challenge that oncology research seeks to understand and overcome [77]. Either having refractory or resistant disease defines patients [78]. Patients with innate resistance to chemoradiotherapy, also termed refractory disease, are inherently resistant to the treatment. Patients who initially respond but relapse have acquired resistance [79].

Chemotherapeutic agents work through multiple mechanisms, generally inhibiting physiological DNA processes and targeting abnormal proliferating cells [80]. For example, cisplatin intercalates double-stranded DNA, and 5-FU prevents the synthesis of DNA nucleotides; all inhibit DNA replication and thus prevent cell growth and encourage apoptosis. If cells can nullify these effects by stimulating their growth and inhibiting apoptosis, then resistance is achieved.

Numerous mechanisms have been established that play a key role in chemoresistance [80, 81]. Firstly, alterations to drug transport across the cell membrane are one of the primary examples of chemoresistance. The decreased influx and increased efflux of drugs restrict drug accumulation within the cell, thus limiting the drug cytotoxicity. The influx copper transporter CTR1 has been displayed as one of the significant transporters for drug accumulation, and any defects have been strongly associated with chemoresistance, especially resistance to cisplatin [82]. The ATP-binding cassette (ABC) transporter family is also associated with chemoresistance. These transmembrane proteins can reduce the intracellular concentrations of drugs via an increase in the efflux of drugs and the redistribution of drugs away from the site of action [83]. In

addition to the accumulation of the drugs within the cell, the localization of the drugs at the site of action is also essential to exceeding the sublethal threshold of cytotoxicity. Since platinum-based chemotherapeutic agents exert their cytotoxic effects in the nucleus, the localisation of platinum in the cytoplasm sequesters the drug from the nucleus and promotes resistance [84]. Intracellular pH can also alter cellular sensitivity to chemotherapeutics, such as cisplatin, which is inactivated by high intracellular pH [85].

It is well established that DNA damage is induced by chemotherapeutic agents or reactive oxygen species (ROS) by limiting their accessibility to the nucleus [86]. Glutathione (GSH) is the most abundant nonprotein antioxidant in eukaryotic cells. Interestingly, GSH detoxification neutralises the DNA-damaging molecules produced by drugs and radiation [87, 88]. Moreover, elevated GSH levels are observed in various tumours, making them more resistant to chemotherapy [89]. Therefore, it is not surprising that targeting GSH presents an attractive approach for medical intervention against resistance to anti-cancer treatment.

Various endogenous and exogenous DNA-damaging agents, such as ionizing radiation and chemotherapeutic agents, can lead to DNA lesions, including single-strand breaks (SSBs) and double-strand breaks (DSBs) [90]. To prevent this, cells have evolved a series of mechanisms called DNA damage response (DDR) to deal with such lesions [91]. The increase in DNA repair activity is also thought to be means of resistance, especially to that of a platinum-based agent [92]. The central DNA repair pathways in the cell are direct repair (DR), base excision repair (BER), nucleotide excision repair (NER), mismatch repair (MMR), homologous recombination (HR), and non-homologous end-joining (NHEJ).

DR is defined as eliminating DNA and RNA damage using chemical reversion that does not require conventional DNA repair properties, such as a nucleotide template, breaking the phosphodiester backbone, or DNA synthesis. In mammalian cells, the DR is utilized to repair specific types of

DNA damage caused by ubiquitous alkylating agents by the alkylguanine DNA alkyl-transferase protein (AGT) [93]. Alkyl and methyl DNA adducts, such as those formed by the platinum-based agents, are quickly and efficiently repaired by AGT.

The BER pathway recognises and removes DNA bases damaged by alkylation, ROS, or radiation [94]. Initially, a DNA glycosylase recognizes and releases a damaged base that cleaves the N-glycosidic bond between the base and the DNA backbone, generating an AP site. NER is involved in the recognition and repair of several different bulky DNA-helix distorting lesions, which may potentially block DNA replication or transcription [95]. MMR repairs mismatching of base pairs and insertions or deletions that may arise during replication or recombination and some damage caused by ROS [96]. Mismatches are recognised by one of two mutS homolog (MSH) heterodimer protein complexes, which generally comprise MSH2 and MSH6, depending on the type of damage. Another heterodimer comprised MLH1, and postmeiotic segregation-increased 1 (PMS1) forms a complex with the MSH heterodimer to initiate repair. Deficient or alterations in MMR have been revealed to promote resistance to chemotherapeutics, including cisplatin and topoisomerase II inhibitors because MMR typically recognises DNA adducts and induces apoptosis [97, 98].

The HR and NHEJ are responsible for the repair of dsDNA breaks which are the most cytotoxic form of DNA damage caused by anti-cancer treatment [99, 100]. Briefly, HR favourably uses the sister chromatid as a repair template, which allows the high-fidelity restoration of the sequence at the break, resulting in error-free repair. The sister chromatid is only present after replication, and HR is primarily active in the S/G2 phases of the cell cycle [101]. The main steps in HR repair are mediated by the single-strand binding protein replication protein A (RPA), Rad51, Rad52, and Rad54, and the Rad51 paralogs XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D [102]. Conversely, NHEJ directly re-ligates the two

broken DNA strands using a template-independent mechanism. Because it does not require a homologous template, NHEJ is not restricted to a particular cell cycle phase. NHEJ is the primary pathway responsible for the repair of IR-induced DSBs [103].

Eukaryotic cells have four phases within the cell cycle. G1, S, G2, M, and one phase outside the cell cycle, G0 [104]. Both the cell position within the cell cycle and the activation of cell cycle checkpoints following exposure to anti-cancer treatment play a role in determining cellular sensitivity to chemotherapy and radiation [105, 106]. Generally, cells in the M and G2 phase are most sensitive to radiation; cells in the G1 phase are more resistant, while cells in the S phase are most resistant. In comparison, 5-FU is only cytotoxic to cells during the S phase of the cell cycle [107]. In contrast, cisplatin DNA adduct formation is cell cycle independent but is most cytotoxic in the late stages of G1 immediately before the S phase [108]. However, the ability of cells to acquire and maintain resistance to chemotherapy in PDAC remains both a clinical and scientific challenge. Overall, the investigation into the affected molecular pathways and how these pathways are modulated helps improve prognosis and survival times in patients who, unfortunately, with this disease, have limited options.

1.3 Tumour Biology

In 2000, Hanahan and Weinberg published their prominent review: the hallmarks of cancer [109], where they attempted to organize the sophisticated complexities of cancer biology into six significant hallmarks. In 2011, four more hallmarks were added [110]. More recently, in 2022 [111], the original six hallmarks noted in 2000 and ten hallmarks noted in 2011 was developed to incorporate fourteen aspects contributing to cancer (**Figure 1**).

1.3.1 Sustaining Proliferative signalling

Actively proliferating cells are fundamental to the development of oncogenesis [112]. It is well established that cancer cells can become hyper-responsive to proliferation signals by increasing the expression of growth factor receptors on the cell surface and upregulating the production of growth factors to stimulate autocrine proliferation [113]. Interestingly, the MAPK and PI3K-Akt signalling pathways are the most frequently activated signalling pathways in PDAC (approximately 90%). Hence significant efforts have been made to develop pharmacological agents targeting these signalling pathways. Briefly, the MAPK pathway consists of a kinase cascade, where K-Ras activates Raf kinases, activating MEK1/2 [114]. In pre-clinical models, MEK inhibitor PD325901 reduced tumour burden and prolonged survival time and showed a synergistic effect with Akt inhibitor GSK690693 [115]. Additionally, efforts have been made to develop pharmacological agents targeting MAPK and PI3K-Akt signalling pathways. PI3K and Akt inhibitors were tested in pre-clinical models in ovarian cancer, revealing compelling anti-cancer interest [116]. These findings indicate that targeting K-Ras downstream pathways is a potential therapeutic option for PDAC patients harbouring K-Ras mutations.

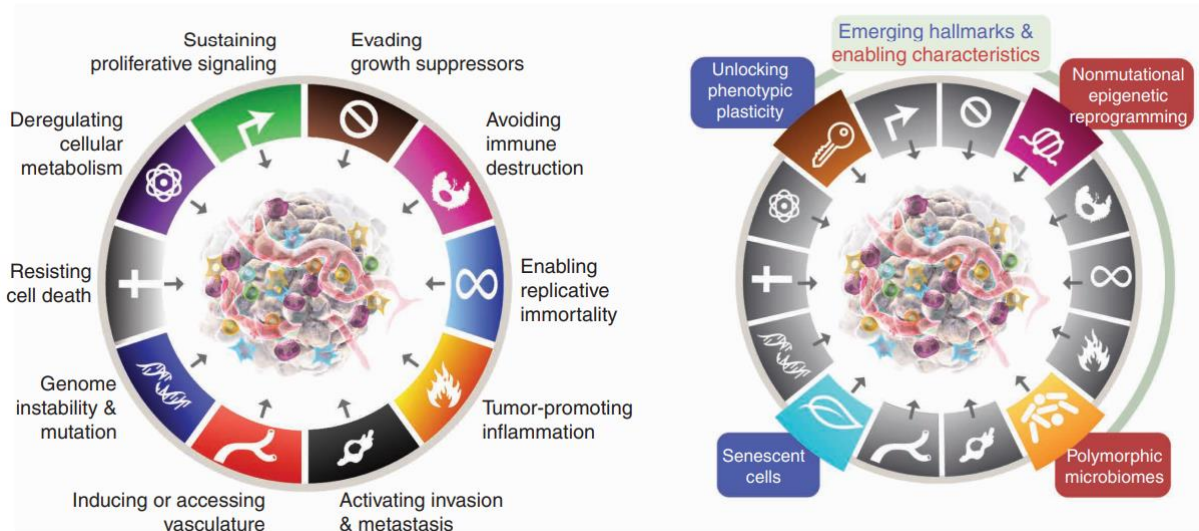


Figure 1. Hallmarks of cancer. Left, the Hallmarks of Cancer currently embody eight hallmark capabilities and two enabling characteristics. In addition to the six acquired capabilities—Hallmarks of Cancer—proposed in 2000 [109], the four provisional “emerging hallmarks” were introduced in 2011 [110]. Right, a conceptual diagram illustrating the fourteen revised hallmarks of cancer adapted from [111].

1.3.2 Evasion of Growth suppressors

In addition to enhancing the growth factor-mediated pathways, cancer cells can repress anti-proliferative growth by suppressing signalling by altering the tumour suppressors such as the retinoblastoma protein (RB) and tumour protein (TP53) and are considered the gatekeepers of cell cycle progression [117, 118]. The RB and TP53 suppressor proteins are additional targets in PDAC due to their documented inactivation in PDAC [119, 120]. A previous study by Thomas *et al.* [121] displayed that targeting RB phosphorylation by activating phosphatase presents a rational strategy to inhibit PDAC cell growth. TP53 suppressor gene mutations are one of the most frequently observed abnormalities in cancer and are mutated in 70% of PDAC patients [122]. One recent study assessed TP53 mutations and mRNA expression in 57 patients with PDAC, and it was demonstrated that patients with low TP53 mRNA expression were associated with a worse prognosis ($p = 0.032$) [123]. This evidence suggests that RB and TP53 could be ideal therapeutic targets and biomarkers for prognosis and therapy prediction.

1.3.3 Deregulating cellular metabolism

Cancer cells reprogram their energy metabolism pathways to meet the high demand inflicted by increased cell proliferation [124]. In a normal cell, aerobic respiration occurs through glucose processing to pyruvate via glycolysis. In anaerobic conditions, glycolysis is primarily utilised, a primitive method of energy metabolism, which is an inefficient pathway that yields 2 molecules of ATP per glucose, whereas 38 molecules of ATP are generated in complete aerobic oxidation and is also known as oxidative phosphorylation [125]. In tumours, glucose uptake dramatically increases, and lactate is made, even in oxygen and fully functioning mitochondria. This process is known as the Warburg Effect [126], and to date, there is no conclusive explanation as to why the cancer cell prefers glycolytic metabolism. To facilitate increased glucose uptake for energy metabolism, the cancer cell upregulates the glucose transporter GLUT1.

Recently, Boira *et al.* [127] showed that GLUT1 could be related to higher aggressivity in PDAC and could be used as a prognostic marker. Additionally, a previous study revealed that the maintenance of glycolysis activity is vital for the survival of PDAC cells, where blocking glycolysis using 2DG, a derivative of glucose, results in apoptosis [128].

1.3.4 Resisting cell death

Apoptosis is a mechanism of programmed cell death essential for maintaining tissue homeostasis by eliminating damaged or abnormal cells to conserve normal cells [129]. Within the context of cancer, cells can avoid apoptosis regardless of multiple mutagenic events and the dysregulation of critical cellular pathways. The Bcl-2 family is the best-characterized group of apoptosis-mediating factors [129, 130]. It can be divided into two groups according to their functional properties: anti-apoptotic proteins like Bcl-xL and Bcl-2 and pro-apoptotic proteins, including Bax and Bak. High levels of Bcl-1 have been found in various cancers [131], but interestingly, Bcl-2 has been reported to be decreased in PDAC cells [132]. Bcl-2 antisense constructs, such as G3139, have been used alongside chemotherapeutic agents to treat various cancers [133]; however, if this is a therapeutic approach that would be successful in PDAC displaying decreased levels remains uncertain.

1.3.5 Evading immune destruction

Unsurprisingly, avoiding immune cell-mediated destruction is fundamental to cancer development and progression [134]. Cancer cells produce immunosuppressive factors to evade immune destruction, including TGF- β , which can paralyze cytotoxic T lymphocytes and natural killer cells [135]. Interestingly TGF- β plays contradicting roles in PDAC, where it behaves as a tumour suppressor in early-stage PDAC by promoting apoptosis and inhibiting cell cycle progression [136]. However, it acts as a tumour promoter in the late stage by immune evasion and metastasis [136, 137]. The microenvironment in PDAC is highly

immunosuppressive and composed of T regulatory cells, which block cytotoxic T lymphocyte's duties in tumour recognition and destruction [138].

1.3.6 Genomic instability

One main characteristic of cancer cells is genomic instability [139]. Genetic integrity is closely monitored by several surveillance mechanisms, DNA damage checkpoints, DNA repair responses, and mitotic checkpoints. Hence a defect in regulating any of these mechanisms often results in genomic instability.

ATM has been frequently mutated in PDAC and presents as one of the essential mediators of DNA damage response and repair pathway alongside ATR [140]. Briefly, double-strand DNA breaks activate the cascade of ATM and BRCA1/BRCA2 to launch DNA repair [141]. BRCA1/BRCA2 are two vital proteins in the activation and operation of homologous recombination (HR) in double-strand DNA break repair. BRCA1/BRCA2 interact with HR, initiating proteins such as RAD51 and recruiting DNA repair assembly [141, 142]. PALB2 performs a significant role in recruiting DNA repair machinery to activate HR, and interestingly, its mutations are related to an increased risk of PDAC [143]. Compared to other DNA damage repair systems, HR uses homologous DNA to repair the breaks, yielding error-free and high-quality outcomes [144]. A switch from HR to different DNA repair pathways confers an increased risk of alterations in DNA sequence, which creates frequent deleterious changes in genetic material due to low-fidelity DNA repair and genetic rearrangements [145].

PARP1 is a crucial nuclear enzyme essential for the activation of DNA damage response, particularly in single-stranded DNA break repair (SSBR) [146]. Spontaneous single-stranded DNA breaks are recognized by PARP1, and recruitment of X-ray Repair Cross-Complementing Protein 1 (XRCC1), which functions as a scaffolding protein in SSBR, is mediated by

PARP1 [146, 147]. Additionally, evidence suggests that PARP1 is involved in NHEJ [148]. Recently, PARP inhibitors have demonstrated clinical activity in patients with PDAC and pathogenic variants in BRCA1, BRCA2, and PALB2, improving the efficacy with combination therapies, particularly platinum-based agents [149, 150].

1.3.7 Angiogenesis

The vascular system within the body delivers essential nutrients and oxygen to both normal and cancer cells. The endothelial cells forming the systemic vasculature vessels are generally quiescent. However, the generation of new vasculature is switched on in cancerous cells, which is needed for sustaining tumour growth and allows cancer cells to metastasize away from the site of the primary tumour [151]. The angiogenic switch is supported by various progressive and suppressive factors, including VEGF and TSP-1; these proteins are often dysregulated within cancer, leading to problems with vasculature and potentially an increase in new vessels forming in the tumour and its microenvironment [152]. VEGF gene expression can be modulated by several factors, including posttranscriptional regulation, hypoxia, and oncogene signalling [153]. PDAC is a hypovascular tumour in a hypoxic microenvironment. The prominent pathological feature is the high levels of fibrosis, termed desmoplasia, which generates excessive interstitial fluid pressures at primary and metastatic tumour sites [154]. Desmoplasia results in vasculature collapse that promotes cancer development and inhibits drug penetration and uptake, inducing cancer resistance to target therapy [155]. Therefore, anti-angiogenic treatment is effective in PDAC to inhibit blood vessel growth and prevent cancer cell growth. Axitinib and Sorafenib are effective drugs used to target growth factors and their corresponding receptors [156].

1.3.8 Activating invasion and metastasis

As tumour growth increases and a vasculature supply is acquired, the cancer cells undergo an epithelial-to-mesenchymal transition (EMT) [157]. Interactions between the cancer cells, stromal tumour cells, and the dysregulated intracellular signalling pathways induce the EMT phenotype. One broadly observed alteration in cell-to-environment interaction in PDAC involves E-cadherin and plays a critical role in coupling adjacent cells by E-cadherin bridges. Loss of the cell-cell adhesion molecule E-cadherin and the loss of cell-matrix attachment proteins are associated with migration and EMT and is commonly observed in PDAC tumours [158]. E-cadherin downregulation is modulated by a complex network of signalling pathways and transcription factors, such as Slug and Snail [157-159]. Consequently, it leads to decreased cell-cell adhesion, enables the separation of individual cells from the primary tumour mass, and represents an essential characteristic in carcinoma progression.

However, one recent study revealed elevated levels of expression of E-cadherin and functional adherent cell junction cultured PDAC cells, although they exhibited a highly invasive and metastatic potential [160]. These results indicate the importance of studies that definitively clarify the role of E-cadherin and EMT in PDAC development and progression.

1.4 MicroRNAs

1.4.1 MicroRNAs: Biogenesis and Functionality

MicroRNAs (miRNAs) are a class of non-protein-coding RNAs represented by short, single-stranded RNA approximately 18–24 nucleotides in length [161]. They are described as master regulators of gene expression at the post-transcriptional level [162]. Emerging evidence has revealed that miRNAs are vital for normal animal development and are engaged in many biological processes [163]. In 2001, numerous small non-coding RNAs were identified and were collectively termed miRNA; since then,

thousands of miRNAs have been discovered, and there are currently over 2,500 annotated miRNAs in the human genome, but the functional roles of many miRNAs remain unclear, and an attractive area of research [164].

MiRNA biogenesis (**Figure 2**) begins by being frequently transcribed in the nucleus by RNA polymerase II, producing a single-stranded RNA transcript 1–7 kb in length [165]. Then, this primary miRNA folds onto itself and is processed into precursor-miRNA (pre-miRNA) by Drosha and DGCR, where the 3'-UTR and 5'-UTR ends are cleaved and are subsequently exported into the cytoplasm by the exportin-5/RanGTP complex in the nuclear membrane [166, 167]. In the cytoplasm, the pre-miRNA is further processed by Dicer and TRBP, which involves the removal of the terminal loop, forming the miRNA duplex miRNA-5p/miRNA-3p, which is to become the mature strand or the passenger strand depending upon the thermodynamic stability and directionality of the duplex. Finally, the mature strand will be incorporated into the RISC (RNA-induced silencing complex), while the passenger strand is subsequently degraded [165-168].

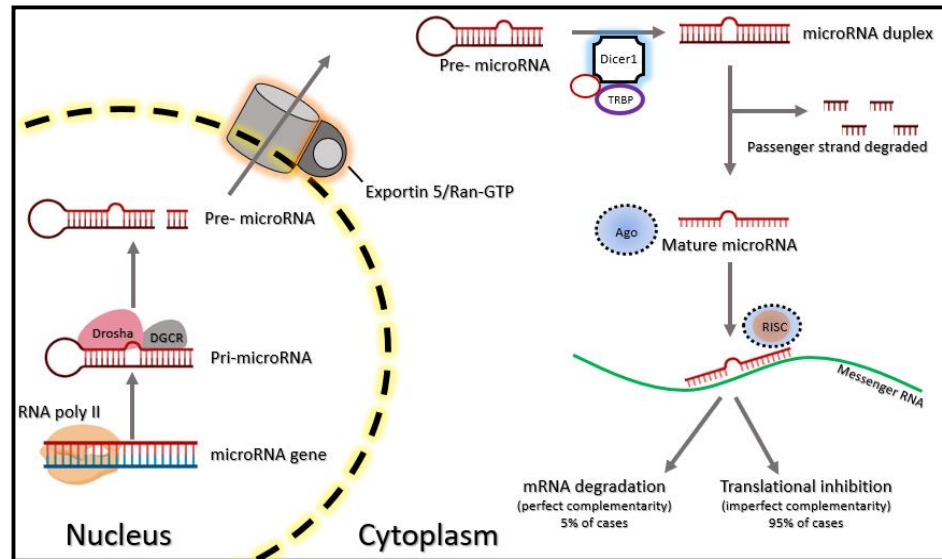


Figure 2. The biogenesis and functionality of miRNAs. RNA polymerase II transcribes a specific microRNA gene producing a primary (pri) microRNA strand, which subsequently folds onto itself. Primary miRNA is processed by Drosha and DGCR, producing a precursor (pre) miRNA that is transported into the cytoplasm via the exportin/Ran-GTP network. In the cytoplasm, the precursor miRNA is further processed by Dicer and TRBP, and the hairpin loop is removed. The microRNA duplex is formed, containing one mature strand and one passenger strand. The passenger strand is degraded, and the mature strand is incorporated into the RNA-induced silencing complex (RISC), which also consists of an argonaute (Ago) protein. Finally, the mature miRNA can now bind by either perfect or imperfect complementarity to the messenger (m) RNA, resulting in degradation or inhibition, respectively. Diagram was designed using biorender.com.

The major functional elements of the RISC are composed of argonaute (Ago) proteins. Of the four family members identified in mammals (Ago1–4), only Ago2 can directly cleave the mRNA target [169]. Ago proteins directly bind to the mature miRNA strand and seek target mRNAs that have complementarity to the miRNA. The ‘seed’ region within the target mRNA is a 2–7 nucleotide sequence fundamental for miRNA binding [170]. The ‘seed’ regions are commonly found at the 3’-end of the mRNA, as the RISC has less competition with the ribosomal units and translational machinery [170, 171]. Additionally, regulated target mRNA usually has longer 3’-UTR, which presents multiple miRNA binding sites. In animals, miRNA-mRNA binding is generally a result of imperfect complementarity, where the RISC can tolerate base wobbles and mismatch binding. In contrast, near-perfect complementarity is commonly seen in plants [172]. This imperfect relationship aids a single miRNA in targeting numerous mRNA targets, whereas a single mRNA can be targeted by multiple miRNAs [170-172].

How the mature miRNAs downregulate gene expression at the post-transcriptional level remains unknown. However, mRNA degradation and translational repression are the two most plausible mechanisms to describe this phenomenon [173]. The identity of the target determines the choice of mechanism. Once incorporated into the RISC, the mature miRNA will specify degradation if the mRNA has sufficient complementarity to the mature miRNA or results in translational repression if the mRNA does not have sufficient complementarity to be degraded [174].

MiRNAs account for approximately 1% of the genome and are estimated to regulate between 25%–35% of genes and are an essential element in various cellular pathways [175]. Different cell types have specialized functions, express a precise set of genes related to cell function, and are thus reflected in tissue-specific miRNA expression profiles [176]. For

example, dysregulated miRNA expression is a common feature in human diseases, especially in cancer [177].

1.4.2 MiRNAs in cancer biology

MiRNAs are involved in numerous pathways and cellular processes within the cell. Hence it is not surprising that miRNA dysregulation is viewed as a fundamental feature of cancer and is considered instrumental in the acquisition of the hallmarks of cancer, such as invasion, angiogenesis, and evasion of apoptosis. In 2002, Calin and colleagues first highlighted the link between miRNA and cancer [178]. They observed a common deletion and downregulation of chromosomal region 13q14 in B-cell chronic leukaemia (CLL). Interestingly, this region encoded the genes miR-15 and miR-16 and was revealed to be deleted in 68% of CLL patients. Moreover, Calin *et al.* [179] found that approximately 50% of miRNAs are located on fragile sites. They also revealed that miRNAs located in the deleted regions display lower expression in various cancer types.

Compared to normal tissue, the miRNA expression in cancer tissue is globally downregulated [180]. For example, the expression of miR-200a, miR-200b, and miR-200c was significantly higher than that in normal tissues, whereas miR-199a, miR-140, miR-145, and miR-125b1 displayed low expression in ovarian cancer tissues [181]. One potential explanation for this global decrease in miR expression may be the inhibition of DICER, as shown by Wilczynski *et al.* [182]. Intriguingly, miRs can also distinguish and characterise the different tumoural subgroups. For example, it has been revealed that miRs are differentially expressed in the different renal cancer subtypes and can be used to determine the subclass [183].

1.4.3 MiRNA Manipulation Therapy

It is suggested that cancer-associated miRNAs present with either oncogenic (oncomiR) or tumour suppressive (tsmiR) activities [184].

OncomiRs are commonly overexpressed in human cancers and require inhibition, which may help re-establish the normality of expression, thus

restoring the function of tumour suppressor genes [185]. AntagomiRs are generally used to inhibit miRNA expression; such inhibitors are single-stranded oligonucleotides complementary to the mature miRNA target. Subsequently, the mature miRNA cannot effectively be incorporated into the RISC, and its function is lost [186]. Other practical approaches for inhibiting miRNA expression include small inhibitors or decoys known as miRNA sponges and masking [187].

In contrast, tsmiRs are frequently under-expressed, so miRNA mimics have been designed to restore the normal function of tumour suppressive miRNAs by replacing or substituting the lost miRNA by using a synthetic miRNA-like molecule [188]. These small molecules resemble the relevant miRNA and can be incorporated into the RISC to achieve the downstream inhibition or degradation of target mRNAs. Furthermore, miRNAs can be restored using DNA plasmids containing specific miRNAs that epigenetically alter endogenous expression [189].

Depending on the miRNA and cancer type, suppressing or reintroducing miRNAs provides a potential and effective strategy for targeting specific pathways in cancer, including PDAC. The different methods used to manipulate miRNA expression in human diseases are summarised in **Figure 3**.

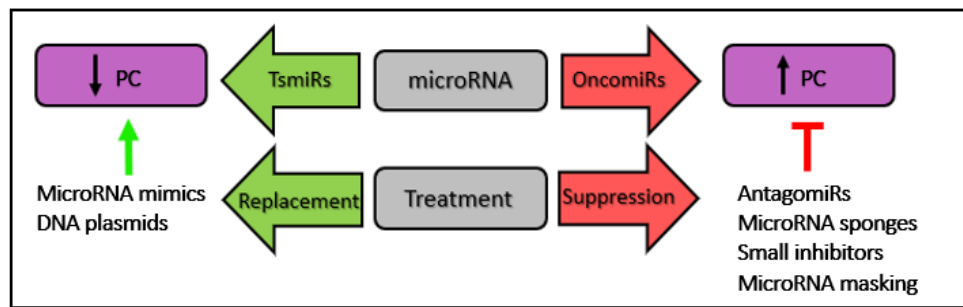


Figure 3. Strategies for the manipulation of miRNA expression in PDAC. Specific miRNAs are classified as either oncogene (oncomiRs) or tumour suppressors (tsmiRs). OncomiRs are often overexpressed in pancreatic cancer (PC), whereas tsmiRs are often underexpressed. Restoring tsmiRs is possible via the use of miRNA mimics and DNA plasmids containing genes for miRNA. Suppressing oncomiRs is also possible using miRNA inhibitors, including antagomiRs, miRNA sponges, small inhibitors, and miRNA masking. Diagram was designed using Biorender.com.

1.4.4 MiRNA Delivery

MiRNAs are relatively small with low molecular weight; they have been appropriately formulated into an effective delivery system, making them suitable for developing into clinical cancer therapy [190]. Additionally, miRNAs are sufficiently stable, can be readily chemically modified, and are less immunogenic than plasmid DNA-based gene therapy and protein-based drug molecules. The effective delivery strategies include viral vectors, inorganic nanoparticles, polymeric vectors, lipid-based vectors, cell-derived membrane carriers, and 3D scaffolds, all summarised in **Figure 4**.

Viral Vectors

Viral vectors have emerged as desirable vehicles for efficiently delivering genes, including miRNAs, particularly target cells, and causing long-term gene expression. Viruses possess different characteristics, and their mechanisms of action vary; therefore, a specific vector may be more suitable than others. The properties of four popular viral vector systems that can facilitate high-level transgene and miRNA expression are adenovirus, adeno-associated virus, retrovirus, and the subclass lentivirus. These viral vectors can be employed to effectively deliver therapeutic miRNAs to treat various types of cancer, including PDAC [191].

Adenoviruses belong to the *Adenoviridae* family, which are non-enveloped viruses that present with an icosahedral protein capsid that comprises a linear duplex DNA genome of approximately 36 kb. To date, over 400 gene therapy trials have been, or are currently being, conducted with adenoviral vectors, and most of these trials are for cancer treatment [192]. Adenoviral vectors are highly advantageous, as they can be grown into high titer stable stocks, are highly efficient, can effectively infect dividing or non-dividing cells, and have a large insert capacity of

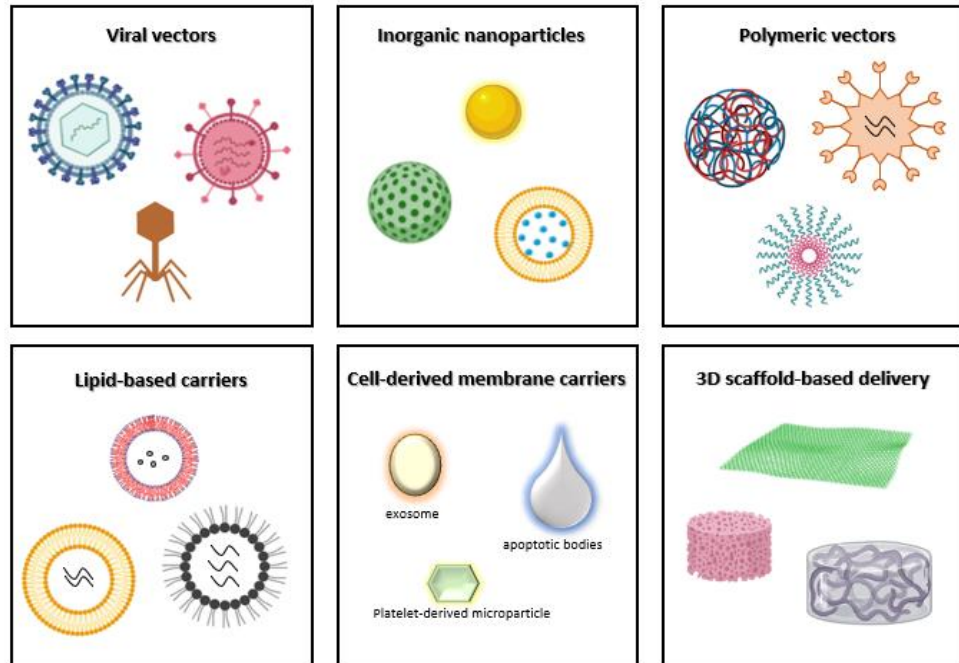


Figure 4. Different strategies used for the delivery of miRNAs. The most effective strategies for delivering miRNAs to target cells include viral vectors, inorganic nanoparticles, polymeric vectors, lipid-based carriers, cell-derived membrane carriers, and 3D scaffolds. Diagram was designed using Biorender.com.

approximately 8 kb. However, due to its strong immunogenicity *in vivo*, adenoviral vectors have been stripped away from their viral protein-coding sequences, creating the first helper-dependent adenovirus vectors [193]. This consequently reduced immunogenicity and improved efficiency, making their use an excellent strategy for delivering therapeutic genes.

Adeno-associated viruses also belong to the *Adenoviridae* family. They are non-enveloped viruses comprising single-stranded DNA genomes. Adeno-associated viruses can transduce both dividing and non-dividing cells; they are considered non-pathogenic (thus have relatively low toxicity) and have a low risk of insertional mutagenesis [194]. However, adeno-associated viruses depend upon helper viruses for replication, which is quite time-consuming and expensive. Furthermore, the insert capacity is less than that of an adenovirus – approximately 4 kb. Nevertheless, the absence of pathogenicity in humans makes adeno-associated viruses an attractive therapeutic vehicle. Furthermore, in contrast to other viral vectors, the adeno-associated virus is the only vector system whose wild-type virus is not associated with human malignancies [195].

Retroviruses belong to the *Retroviridae* family. They have enveloped viruses containing single-stranded RNA genomes. Retroviral vector-mediated gene transfer has been fundamental to the development of gene therapy [196]. Retroviruses have several distinct advantages over other vectors, one of the most important of which is their ability to transform their single-stranded RNA genome into a double-stranded DNA molecule that stably integrates into the target cell genome, allowing permanent modification of the target cell's nuclear genome [196]. Additionally, retroviral vectors present an inadequate immune response in the host and have a similar insert capacity to the adenoviral vectors. Unfortunately, the ability of retroviruses to integrate into the host cell's chromosome also raises the possibility of insertional mutagenesis and

oncogene activation [196]. Finally, retroviral vectors have low vector titers with the potential of infecting dividing cells only.

Lentiviruses represent a subgroup of the *Retroviridae* family, and – like retroviruses – they stably insert genetic material into their host cells, resulting in stable gene expression [197]. However, transcriptional silencing may occur over time. The main benefit of lentivirus over retrovirus vectors is that the former can transduce dividing and non-dividing cells. Additionally, lentiviruses are considered safer than retroviruses due to their new generation of self-activation. However, the challenge of an increased risk of insertional mutagenesis remains [197, 198].

Inorganic Nanoparticles

Due to its demonstrably reduced pathogenicity, low cost, and simplicity of production, non-viral vectors have significant safety advantages over viral approaches; and the ultimate goal, of course, is to facilitate more effective and patient-friendly treatment regimens by reducing drug concentration and dosing frequency and by offering easier administration [199]. In addition, inorganic nanoparticles offer exceptional prospects for cell-specific controlled delivery of miRNAs for therapeutic responses; these include gold, mesoporous silicon, graphene oxide, and iron oxide-mediated nanoparticles [200].

Gold nanoparticles are promising as a feasible and appealing technology. Furthermore, due to their unique physiochemical properties, their functional groups – including amines and thiol groups – can be easily modified on their surfaces, thus making suitable vectors for effectively delivering miRNAs to sites of interest [201].

Mesoporous silica nanoparticles are solid materials and are composed of a honeycomb-like porous structure comprising channels that have the potential to absorb or encapsulate relatively large quantities of bioactive molecules [202]. Such vectors thus present many advantages as drug

delivery vehicles, including uniform and tuneable pore size, easy independent surface functionalization, and enhanced thermal stability.

Graphene oxide has also become a convenient drug and gene delivery agent. Because of its small size, intrinsic optical properties, large specific surface area, low cost, and valuable non-covalent interactions with aromatic drug molecules, graphene oxide is an encouraging new material for biological and medical applications [203]. However, the negative charge of both miRNAs and graphene oxide may cause electrostatic repulsion between them [204].

Iron oxide nanoparticles, with their superparamagnetic properties, low toxicity, sizeable surface-area-to-volume ratio, and easy separation method, are used for numerous biological and medical applications, including drug delivery [205]. Interestingly, due to its polycation polymer-functionalized mesoporous structure, the miRNA-loading ability and tumour cell uptake efficiency of the nano-complex have been significantly increased [206].

Polymeric vectors

Polymeric vectors have recently gained significant attention owing to their low cytotoxicity and immunogenic properties; thus, they represent another attractive nanoscale strategy for therapeutic delivery [207]. Among these polymers, polyethyleneimine, polymeric molecules composed of positively charged repeating amine groups, can effectively bind to miRNAs to form nano-sized complexes, preventing degradation, endorsing cellular uptake, and efficient intracellular release [208]. Polyethyleneimine is subdivided into either linear or branched types. A recent study demonstrated that linear polyethyleneimine has the potential to modify its geometry more quickly as compared to branched polyethyleneimine. This means linear polyethyleneimine is more adaptable at a specific target site [207, 208]. Nevertheless, branched

polyethyleneimine is more effective at miRNA delivery than Lipofectamine 2000 [209].

Polymeric micelles are particularly well suited for miRNA delivery due to their inherent and modifiable properties [210]. In addition, polymeric micelles share similar capabilities with nanoparticles, liposomes, and other nano-carriers in their capacities as therapeutic delivery agents. Moreover, polymeric micelles are among the most extensively studied delivery platforms; the unique opportunities offered by the wide choice of hydrophilic corona and hydrophobic core make them ideal for drug delivery and targeting, especially for anticancer drugs [211].

Chitosan is a linear disaccharide made of D-glucosamine and N-acetyl-D-glucosamine. It is derived from the deacetylation of the naturally abundant chitin found in the cell walls of fungi. Chitosan displays encouraging biological properties, including low cytotoxicity, biocompatibility, and biodegradability [212]. It is also competent at delivering active compounds to specific sites. It has been widely investigated over the last few decades for potential application as a carrier of various therapeutic agents, including miRNAs [213].

Lipid-based Carriers

The use of lipids as drug delivery systems offers several advantages, including improved pharmacokinetics, increased absorption, and facilitated targeting [214]. Liposomes were first discovered in the mid-1960s, but not until years later were they considered an ideal candidate for anticancer drug delivery [215]. Additionally, liposomes are the most frequently used transfection reagents *in vitro*. It should be noted however, that safe and efficacious delivery *in vivo* is rarely attained due to toxicity, non-specific uptake, and unwanted immune response [215].

Solid lipid nanoparticles are a new generation of colloidal drug carrier systems consisting of solid surfactant-stabilized lipids at room and body temperature [216]. Solid lipid nanoparticles are advantageous because of

their low production cost, relatively low cytotoxicity, biodegradable composition, and stability against aggregation [217]. Furthermore, they offer protection to the entrapped therapeutic agents and prolonged therapeutic release from the matrix. They are just as valuable as liposomes and polymeric nanoparticles [217]. Nonetheless, solid lipid nanoparticles still present some limitations, including unpredictable gelation and low incorporation due to the crystalline structure of solid lipids.

Therefore, a new generation was developed by regulating the mixture between solid lipids with liquid oil, known as a nanostructured lipid carrier. These were designed to resolve the problems raised by solid lipid nanoparticles, which included limited drug-loading capacity and drug expulsion during storage [218]. Nanostructured lipid carriers are highly favourable for use as carriers of toxic chemotherapeutic agents, taking advantage of their minute particle size and ability to passively or actively target tumour sites to enhance their delivery and reduce side effects [219].

Cell-derived Membrane Carriers

Exosomes are small vesicles with a diameter ranging from 50–100 nm secreted by various cell types and tissues [220]. Exosomes can enter recipient cells via two fundamental mechanisms: interaction with receptors on the target cell and activation of signalling pathways, and release of their content after endocytosis or fusion with the plasma membrane, leading to gene expression and protein translation alterations [220]. Due to their high stability in circulation and the intrinsic ability for cargo transfer, exosomes have been investigated as a novel drug delivery system for many diseases, including cancer [221]. Furthermore, it has been demonstrated that tumour cells release exosomes containing miRNAs, which indicates that exosomes possess such capabilities as natural carriers of miRNAs and can thus be exploited as an ideal therapeutic delivery system [222].

Apoptotic bodies are extracellular vesicles that are formed during programmed cell death. Apoptotic bodies have the widest diameter, ranging from 50–500 nm [223]. Because of their lipid membranes, signals arrive undiluted and are protected from, for example, enzymatic degradation while traveling. There are numerous biomedical applications of these extracellular vesicles, including drug delivery using apoptotic bodies' delivery capabilities to target therapeutics to specific cells and tissues [223].

Once activated, platelets release tiny, membrane-bound micro-particles (0.1–1 μ M) containing bioactive proteins and genetic materials from their parent cells that may be transferred to and exert potent biological effects in recipient cells of the circulatory system. Therefore, these small lipid vesicles possess the capability to be therapeutic carriers by delivering miRNAs to target cells [224].

3D Scaffold-based Delivery Systems

Hydrogels (polyacrylamide) are swollen, hydrophilic, crosslinked polymer networks that present an excellent potential for use in biomedical research. They can be made into different forms for immobilization, ranging from thin films to nanoparticles. Their softness, biocompatibility, and aptitude for rapid diffusion of molecules make them beneficial for drug delivery, cell culture, wound healing, and sensing applications [225]. Hydrogels are a simple substrate for therapeutic immobilization with other advantages, such as entrapment, controlled release, and therapeutic protection. Compared to different scaffolds, immobilization in hydrogels occurs in 3D, which allows the high loading capacity of bioactive materials. Furthermore, hydrogels' exemplary optical transparency provides a convenient visual detection strategy. Hernandez *et al.* [226] demonstrated the feasibility of encapsulating miRNA therapeutics in hydrogels to enhance delivery and efficacy in later *in vivo* applications.

1.4.5 MiRNAs Modulate Sensitivity to Chemoradiotherapy in PDAC.

Chemoradiotherapy represents a vital therapeutic strategy for many patients diagnosed with PDAC [69]. Unfortunately, many patients are unresponsive to such treatments due to the extreme aggressiveness of pancreatic tumours that display resistance to chemoradiotherapy, resulting in a poor prognosis [77-80]. Despite the intensity of research into the mechanisms underlying chemoresistance and radioresistance, the precise mechanisms of these phenomena remain poorly understood. Recent studies have indicated that miRNAs appear to be critical regulators of resistance to chemoradiotherapy in many cancer types, including PDAC. Furthermore, targeting miRNAs by either inhibiting or over-enhancing their expression has been revealed as an effective strategy for developing novel, more highly effective personalized therapies for improving responses to treatment [227].

Previous studies have suggested a variety of drug resistance mechanisms in PDAC. The ABC transporter superfamily members are essential mediators of drug efflux and multidrug resistance in many tumours [83]. Epithelial-mesenchymal transition (EMT) has been a potential mechanism for chemotherapeutic resistance. Recent studies have shown that many signalling pathways, including the Wnt, TGF- β , Hedgehog, Notch, and NF- κ B signalling pathways, were significant for EMT induction [158-160]. Cell cycle, proliferation, and apoptosis are also profoundly associated with resistance to chemotherapeutics agents, and targeting these mechanisms could help improve cancer therapy outcomes [104, 105]. MiRNAs have been demonstrated as essential mediators of chemoresistance in PDAC by playing a fundamental role in the above mechanisms. Dhayat *et al.* [228] revealed that miR-31, amongst others, was significantly downregulated in established gemcitabine-resistant PDAC cell lines by overexpressing the ABCC1 transporter. Wei *et al.* [229] demonstrated that miR-21 could confer drug resistance to 5-FU in PDAC cells by regulating the expression of tumour suppressor genes, including

PDCD4. Moreover, a recent study showed that the upregulation of Bcl-2 is directly induced by miR-21 and is linked with apoptosis and chemoresistance in PDAC cells [230].

Interestingly, a study by Xiong *et al.* [231] found that miR-410-3p enhanced chemosensitivity to gemcitabine via inhibiting HMGB1-induced autophagy in PDAC cell lines. A recent study found that inhibiting miR-21 and miR-221 in tumour-initiating stem-like cells significantly reduced chemoresistance to 5-FU in PDAC [232]. Nagano *et al.* [233] investigated the relationship between miR-29a expression and the response to gemcitabine in PDAC cell lines. The group demonstrated that miR-29a expression correlates significantly with the growth-inhibitory effect of gemcitabine and that activation of the Wnt/ β -catenin signalling pathway mediated the miR-29a-induced resistance to gemcitabine in these cell lines [233].

A previous study aimed to examine whether miR-429 was involved in mediating chemoresistance to gemcitabine in PDAC cells [234]. Firstly, a gemcitabine-resistant PDAC cell line (SW1990/GZ) was established from its original cell line (SW1990), where miR-429 expression was significantly lower in the SW1990/GZ cells. Interestingly, overexpression of miR-429 in this resistant cell line increased cellular sensitivity to gemcitabine through the regulation of PDCD4. Similar patterns were also demonstrated within a xenograft nude mice model [234].

MiRNAs also regulate cellular sensitivity to platinum-based chemotherapeutics in PDAC cells. Li *et al.* [235] demonstrated that overexpression of miR-100 inhibited proliferation in PDAC cells and increased sensitivity to cisplatin. Moreover, overexpression of miR-100 led to significant inhibition of tumour formation *in vivo* [235]. Schreiber *et al.* [236] found that overexpression of miR-374b in PDAC cells restored cisplatin sensitivity. Radioresistance has become the main obstacle to treating PDAC due to its limitations on the efficacy and outcomes of radiotherapy in clinical treatment [77]. Zhang *et al.* [237] generated a

radioresistant PDAC cell line and found that miR-216a was significantly downregulated compared to the control cell line. Overexpression of miR-216a was shown to inhibit cell growth and colony formation ability, and it promoted cell apoptosis of radioresistant PDAC cells when exposed to irradiation by inhibiting beclin-1-mediated autophagy. Wang *et al.* [238] revealed that radioresistant PDAC cell lines displayed reduced miR-23b and increased autophagy compared with non-radioresistant control cells. Restoring miR-23b inhibited radiation-induced autophagy, whereas a miR-23b inhibitor promoted autophagy in PDAC cells by targeting ATG12; they thus concluded that miR-23b has significant potential to increase the sensitivity of PDAC cells to radiation therapy [238].

Resistance to chemoradiotherapy remains an extreme challenge today, leading to relapse and metastatic spread in many cancer types. Therefore, new therapeutic strategies are desperately needed. Over the last decade, much evidence has revealed that miRNAs have opened a new avenue to understanding resistance to chemoradiotherapy (**Table 3**).

Table 3. MiRNAs are mechanistically associated with chemoradioresistance in PDAC.

Mechanism	Target	miRNA	Treatment	Reference
Transporter	ABCC1	miR-	Gemcitabine	[228]
	ABCC1	31/330/378	Doxorubicin	[242]
	ABCC5	miR-1291	Gemcitabine	[243]
	ATP7A	miR-210	Cisplatin	[236]
	Glut-1	miR-374b miR-520f	Gemcitabine	[244]
DNA Damage & Repair	ATM/ATR	miR-520f	Gemcitabine	[244]
	DNA-PKc	miR-101	Gemcitabine	[245]
	RRM1	miR-101-3p	Gemcitabine	[246]
	RRM2	miR-20a-5p	Gemcitabine	[247]
	PDCD4	miR-21	5-Fluorouracil	[229]
Epithelial-mesenchymal Transition (EMT)	Fbw7/Notch-1	miR-223	Gemcitabine	[248]
	ZEB1	miR-200/let-	Gemcitabine	[249]
	Fyn	7	Gemcitabine	[250]
	RHOF	miR-125a-3p	Gemcitabine	[251]
	Snail	miR-3656 miR-153	Gemcitabine	[252]
Cell Cycle & Cell Proliferation	Wee1/	miR-15a	Gemcitabine/5-	[253]
	Chk1/BMI-1/Yap-		Fluorouracil	
	1	miR-21/221	Gemcitabine	[254]
		miR-17-5p	Gemcitabine	[255]
	PTEN/RECK/p27	miR-21	Gemcitabine	[256]
	PTEN	miR-21	Gemcitabine	[257]
	PTEN/pAkt	miR-99b	Radiation	[258]
	PTEN/P13K-Akt	miR193a	Radiation	[259]
	mTOR	miR-620	Radiation	[260]
	TGF-Beta HPGD			
Autophagy/Apoptosis	Bcl-2	miR-21	Gemcitabine	[230]
	ATG12	miR-23b	Radiation	[238]
	Bcl-2	miR-181b	Gemcitabine	[261]
	RAB27B	miR-155	Gemcitabine	[262]
	Bcl-1	miR-216a	Radiation	[237]
	Integrin-β1	miR-760	Gemcitabine	[263]
	Bcl-2/BIRC3	miR-374b-5p	Gemcitabine	[264]

Furthermore, they present the ability to manipulate their expression via miRNA manipulation therapy as a remarkably efficacious potential therapeutic tool.

1.4.6 MiRNAs in Clinical trials

MiR-122 is an important host factor for hepatitis C virus (HCV) and promotes HCV RNA accumulation. Reduced levels of miR-122 were observed in patients with hepatocellular carcinoma, suggesting a potential role of miR-122 in the development of HCC. The locked nucleic acid, Miravirsen, works by targeting miR-122, resulting in a dose-dependent and prolonged decrease of HCV RNA levels in chronic hepatitis C patients [239]. It is the first-ever drug that targets the miRNA. It entered clinical trials and is now in phase II clinical trial undergoing assessment for its safety and effectiveness in the patients.

MRX34 targets miR-34a and can be used to treat a wide range of cancers, including NSCLC, ovarian cancer, and hepatocellular carcinoma. Currently, this molecule is in phase I clinical trial for the remedy of liver-based tumours [240].

MGN-4220 has been noted that cardiac fibrosis can be avoided by inhibiting miR-29. MiR-29 acts by activating the Wnt signalling pathway to support the pathological alteration of the heart. The MGN-4220 molecule targets miR-29 for the treatment of cardiac fibrosis [241]. This molecule is currently undergoing development at miRagen therapeutics.

Hypothesis

The overall aim is to initiate a new programme of pancreatic cancer research, using our previous findings in multiple cancers to begin understanding the biological influence of microRNA-31 on pancreatic cancer cell growth and aggressiveness and to determine if it can be used as a therapeutic target to augment PDAC responses to chemotherapy and radiotherapy.

Specific aims

- i. To determine the influence of microRNA-31 on the relative sensitivities of PDAC cell lines to chemotherapy, specifically 5-FU, leucovorin, oxaliplatin, and gemcitabine.
- ii. To determine the influence of microRNA-31 on PDAC cell line sensitivity to clinically-relevant doses of radiotherapy.
- iii. To delineate the molecular mechanisms underpinning microRNA-31-mediated alterations in sensitivity to chemotherapy and/or radiotherapy.

Chapter Two

Materials & Methods

2.1 Reagents and materials

2.1.1 Cytotoxic drug preparation

Unless stated otherwise, all cytotoxic drugs were purchased from Sigma-Aldrich [Missouri, USA]. The manufacturer's manual determined concentrations of drugs and was research grade. Cisplatin was solubilized in 1X phosphate-buffered saline (PBS) [Lonza Group Ltd., Switzerland] at a stock concentration of 3.3 mM. Carboplatin was solubilized in PBS at a stock concentration of 26.8 mM. Oxaliplatin was solubilized in PBS at a stock concentration of 12 mM. 5-Fluorouracil (5-FU) was solubilized in DMSO [Sigma-Aldrich, USA] at a stock concentration of 61.5 mM. Gemcitabine was solubilized in PBS at a stock concentration of 10 mM. Leucovorin was solubilized in DMSO at a stock concentration of 10 mM. Bafilomycin A1 was solubilized in DMSO at a stock concentration of 15 mM. Aliquots were stored at -20°C and thawed at 37 °C immediately before ensuring all drugs were in solution. Drugs dissolved in DMSO did not exceed concentrations of 0.1% for negative controls.

2.1.2 Radiation treatment

Irradiation was performed using an X-ray generator (CIX2) (XStrahl) at a dose rate of 1.87 Gray (Gy)/min [XSTRAHL, Surrey, UK]. Detailed dosimetry and warm-up cycles were performed regularly to ensure the irradiated dose was accurate. The irradiator was also regularly validated and calibrated.

2.2 Cell lines

Cell lines (**Table 2.1**) [American Type Culture Collection (ATCC), VA, USA] were sustained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium [Biosciences, CA, USA].

Table 2.1 Cell line characteristics utilised with the *in vitro* cell model of refractory PDAC.

BxPC-3	Panc-1
61-year-old Female	56-year-old Male
Non-metastatic	Metastatic
KRAS: Wild type	KRAS: 12 Asp
TP53: 220 Cys	TP53: 273 His
P16: Wild type	P16: Homozygous deletion
SMAD4: Homozygous deletion	SMAD4: Wild type
Low endogenous miR-31	High endogenous miR-31
Stably transfected to overexpress/reintroduce miR-31	Stably transfected to suppress / Zip down miR-31
BxPC-3 miR-VC (vector control)	Panc-1 Zip-miR-VC (vector control)
BxPC-3 miR-31 (miR-31 overexpression)	Panc-1 Zip-miR-31 (suppressing miR-31)

2.2.1 Cell culturing

Cells were supplemented with 10% heat-inactivated foetal bovine serum (FBS) [Biosciences, USA], 1% Penicillin/Streptomycin (P/S) [Biosciences, USA], now referred to as complete medium. Cell culture reagents were appropriately stored at 4°C and warmed to 37°C in a water bath for at least 30 min before use. Cells were maintained in 95% humidified incubators at 37°C and 5% CO₂ [Thermo Forma, USA]. The aseptic technique was adopted for all sterile procedures. Tissue culture was carried out in a Cleanair laminar airflow cabinet and was cleaned with ethanol (70% v/v). All reagents and equipment were sterilized before entering the laminar airflow unit with ethanol. Spent reagents and materials were decontaminated in a solution containing Haz-Tabs [Guest

Medical, UK] overnight out of the laminar flow area before flushing down the sink with running water.

2.2.2 Sub-culturing

All cell cultures were maintained in an exponential growth phase in vented T75 cm² tissue culture flasks [Sarstedt, Numbrecht, Germany] and sub-cultured at 70%-80% confluency. Spent media was discarded to waste (containing decontaminating Haz-Tabs), and cells were briefly washed with sterile PBS. Next, cells were detached from the plastic surface of the flask using trypsin-EDTA [Sigma-Aldrich, USA]. To promote enzyme activity and cell detachment, cells were incubated with trypsin-EDTA for 3-5 minutes at 37°C. Cells were checked for complete detachment using an inverted phase-contrast Nikon microscope [Nikon Corporation, Japan]. Trypsin-EDTA was inactivated with equal amounts of complete medium. The cell suspension was split into fresh and labelled T75 cm² flasks at a varying ratio of 1:2 to 1:8, depending on experimental needs. Further complete medium was added to each flask to ensure a total volume of 15 mL. Cells were passaged approximately 2-3 times weekly, with passage numbers recorded accordingly.

2.2.3 Frozen cell stocks

Cell lines were regularly frozen and stored at -80°C and within liquid nitrogen. Regular freezing ensured passage numbers were kept low and cell lines were abundant. Confluent (70-80%) T75 cm² flasks were washed with PBS, incubated with Trypsin-EDTA, and inactivated with complete medium, as described in *section 2.2.2*. Cells were then collected in 15 mL sterile tubes [Sarstedt, Germany] and centrifuged for 3 minutes at 300 X g to pellet cells. Spent media was discarded to waste. Cell pellets were resuspended in 1 mL freezing media (95% FBS, 5% DMSO v/v). Cryotubes [Sarstedt, Germany] were labelled appropriately, and 1 mL of the cell freeze mix suspension was added to each cryotube. Cell suspensions were stored at -80°C for at least 24 hours in a Mr. Frosty freezing container,

ensuring a gentle lowering of cellular temperature at a rate of 1 °C/min. Cryotubes were left at -80°C if cells were required within the next 6 months or moved into liquid nitrogen for long-term storage.

Cells were reconstituted by removal from -80°C or liquid nitrogen and quick thawing to 37°C. Once thawed, the suspension was added to the preheated complete medium and centrifuged for 3 minutes at 300 X g to pellet cells. Spent media was discarded to waste. Defrosted cells were resuspended in 1 mL complete medium and transferred into a fresh labelled T25 cm² vented tissue culture flask [Sarstedt, Germany]. An additional complete medium was added, and cells were incubated at 37°C, 5% CO₂. To remove any residual DMSO, cells were washed with PBS, and complete fresh medium was applied the following day.

2.2.4 Cell counting

Cells were subjected to a PBS wash, Trypsin-EDTA, and coincident inactivation. Cell pellets were collected by centrifuging cells for 3 minutes at 300 X g. Spent media was discarded into waste. Cells were then resuspended in 1 mL preheated complete medium, and 20 µL of cell suspension was added to a well from a 96-well plate [Sarstedt, Germany] containing 180 µL of Trypan blue [Biosciences, USA]. The Trypan blue cell solution was mixed with 9 µL and loaded onto a Bright Line hemocytometer [Neubauer glass hemocytometer 0.01 mm depth, Marienfeld-Superior] covered with a glass coverslip. The hemocytometer was checked for air bubbles and was positioned under the microscope. Cells that remained viable appeared white due to the exclusion of the dye from the cell membrane interface, whereas dead cells appeared blue. An average was taken, and the dilution factor was considered to calculate a feasible cell count.

2.2.5 Mycoplasma testing

Cell culture supernatant (1 mL) was taken from a confluent 75 cm² flask. The tube was centrifuged at 300 X g for 3 min to collect any cell debris. A

polymerase chain reaction (PCR) was set up in 0.2 mL tubes to contain per reaction:

- 25 µL of Green GoTaq (polymerase enzyme)
- 1 µL of sense primer (10 µM) (5'- GGGAGCAAACAGGATTAGATACCCT-3')
- 1 µL of anti-sense primer (10 µM) (5'- TGCACCATCTGTCACTCTGTTAACCTC-3')
- 22 µL of molecular biology sterile grade water
- 1 µL of cell culture supernatant

A mycoplasma PCR negative (molecular biology sterile grade water) and a mycoplasma PCR positive (cell culture supernatant from a mycoplasma contaminated cell line) were included. The PCR reaction was set up as an initialisation set at 95°C for 5 min, followed by 40 cycles of a denaturing step at 94°C for 30 s, an annealing step at 55°C for 30 s, and an elongation/extension step at 72°C for 1 min followed by a final extension step at 72°C for 10 min. A 2% agarose gel was made by dissolving 2 g of agarose in 100 mL of Tris-acetate EDTA (TAE) buffer and heating in a microwave until the agarose dissolved. SYBR Safe DNA gel stain (10 µL) was added to the agarose mix, the gel was poured into the gel 64 preparation tank, and a 15-well comb was inserted into the mixture. The gel was allowed to be set for 30 min at room temperature. Once the gel had been set, the gel was placed in the electrophoresis tank, and the comb was removed from the gel. TAE buffer was added to cover the gel. PCR-amplified products (18 µL) were added to the wells, and the gel was run at 100 V for 1 h. The gel was then imaged on a Fusion Fx imaging system.

2.3 Manipulation of gene expression

2.3.1 MiRNA (miR) plasmids

Overexpression and suppression of specific miR were achieved by transfection with miR plasmids purchased from Origene [Herford, Germany] (**Appendices 1.1 & 1.2**). Plasmids were CMV promoter-driven, including a bacterial resistance gene (ampicillin or kanamycin) and a GFP-reported sequence. The miR-31 overexpression plasmid (MI0000089) encoded a miR precursor, then processed to the mature miR by the normal cellular machinery. The vector control plasmid (pCMVMIR) coded a scrambled sequence. Additionally, the miR-31 suppression (zip-down) plasmid (MZIP31-PA-1) produced an antisense oligonucleotide to the miR-31 sequence, which locks miR-31 and irreversibly inhibits its activity. The vector control plasmid (MZIP000-PA-1) coded a scrambled sequence. The overexpression plasmid and its vector control equivalent had a mammalian selection marker, conferring geneticin [Gibco, Massachusetts, USA] resistance. The suppression (zip-down) plasmid and its equivalent vector control have a mammalian selection marker encoding puromycin [Sigma-Aldrich, Ireland] resistance.

2.3.2 Transfection of miR Plasmids in PDAC Cell Lines

Cells were collected from the log phase, seeded into a 100 mm tissue culture dish at 3×10^5 cell density, and left to adhere overnight. The spent medium was removed, followed by a gentle wash of PBS. Incomplete Opti-MEM was added to the tissue culture dish. Transfections were performed using diluted Lipofectamine 2000 (L2K) [Thermo Fisher Scientific, Gloucester, UK], and for every 1 μg of plasmid used, three parts of L2K were required. 12 μg plasmid vector and vector control equivalents were used to transfect the miR-31 overexpression or suppression plasmid mixed in incomplete Opti-MEM. The suppressed miR-31 cell lines, which were referred to as Panc-1 Zip-miR-VC and Panc-1 Zip-miR-31, were maintained under a 2.5 $\mu\text{g}/\text{mL}$ puromycin selection for 10 days. Hereafter, fresh puromycin was applied to every sub-culture. The overexpressed miR-31 cell lines, BxPC-3 miR-VC and BxPC-3 miR-31 were maintained under 450 $\mu\text{g}/\text{mL}$ geneticin sulfate selection for 21 days.

Similarly, fresh geneticin was applied to every sub-culture. After six months, all cell lines would be destroyed, and earlier passages would be cultured to ensure maximum transfection efficiency.

2.3.3 ATOX1 plasmids

Overexpression of the protein ATOX1 was achieved by transfection with ATOX1 encoding plasmids from Origene (**Appendix 1.3**). Plasmids were CMV promoter-driven and included a bacterial resistance gene (ampicillin). The ATOX1 overexpression plasmid (RC221067) was designed by adding the ORF subclone of RC221067 into the control untagged pCMV6-AC vector (PS100020). An ampicillin resistance gene was encoded for bacterial selection; a neomycin resistance gene was encoded for mammalian selection.

2.3.4 Transfection of ATOX1 in PDAC Cell Lines

According to the manufacturer's instructions, liposomal transfection was performed using the L2K reagent. Cells were collected from the log phase, seeded into a 100 mm tissue culture dish at 1×10^6 cell density, and left to adhere overnight. Subsequently, the spent medium was removed and discarded from the dishes. Cells were subjected to a PBS wash, and Opti-MEM reduced serum media was added to the dish. L2K was diluted, with 9 μ L of Lipofectamine added to 150 μ L Opti-MEM. 1 μ g of ATOX1 overexpressing plasmid (RC221067) and its vector control equivalent (PS100020) were added to 150 μ L Opti-Mem. The Lipofectamine and diluted plasmid stock solutions were then combined at a ratio of 1:1. Following incubation, the combined plasmid/transfection reagent was added to the 100 mm culture dish. Following 6 h of incubation with the plasmid/transfection reagent in the Opti-MEM filled wells, spent Opti-MEM was removed, and fresh complete media was added. Panc-1 ATOX1 cells and Panc-1 Vector Ctrl cells were maintained under a 600 μ g/mL neomycin sulfate [Gibco, USA] selection for 21 days. Hereafter, fresh neomycin was applied to every sub-culture.

2.3.5 Silencing Glutathione Peroxidase 8 (Gpx8) in PDAC cells

The BxPC-3 cells (3×10^5) were transfected with either the siRNA scramble control (4390843) or siRNA GPx8 (4392420), purchased from Origene, using the L2K reagent. The final concentration of siRNA was 10 nM. Transfections were performed using OptiMem as in *section 2.3.4*, and the cells were treated 48 h post-transfection.

2.4 Gene expression analysis

2.4.1 RNA extraction

Surfaces and equipment were cleaned using 70% ethanol before procedures involving RNA. Filtered, RNase, and DNase-free sterile pipette tips [Thermo Fisher Scientific, UK] were used throughout all RNA/DNA-based experiments. A T75 cm² flask of cells (70-80% confluency) was placed on ice with spent medium discarded, followed by a gentle wash of cold PBS. Cells were lysed by adding 1 mL Trizol reagent [Thermo Fisher Scientific, UK], ensuring the entire surface was covered. This homogenate can be stored at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. Cells were gently scraped using a cell scraper, and all the cell lysate was collected and transferred into a fresh labelled 1.5 mL Eppendorf.

A volume of 100 µL of 1-Bromo-3-chloropropane BCP [Thermo Fisher Scientific, UK] was added to the lysate, vortexed for 15 seconds, then stored at room temperature for 10 min. Samples were centrifuged at 12,000 x g for 15 min at 4°C using the mini centrifuge [Eppendorf]. After centrifugation, the mixture separates into a lower red phenol phase, white interphase, and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase, whereas DNA and proteins are in the interphase and organic phase. If centrifugation is performed at a higher temperature, a residual amount of DNA may sequester in the aqueous layer.

The aqueous layer was carefully transferred into a new 1.5 mL Eppendorf, and RNA was precipitated by adding 0.5 mL isopropanol and mixed gently. Samples were stored at room temperature for 8 min and centrifuged at 12,000 x g for 12 min at 4 °C. RNA precipitate is often visible before centrifugation, forming a gel-like or white pellet on the bottom of the Eppendorf.

The supernatant was removed, and the RNA pellet was washed by vortexing in 1 mL of 75% (v/v) ethanol. Samples were centrifuged at 12,000 x g for 12 min at 4 °C.

The ethanol was removed, and the RNA pellet was air-dried for 5 min. It is essential to avoid it entirely during the RNA pellet as this will significantly decrease its solubility. The pellet was resuspended in 20 µL of RNase-free water and pulled down. Isolated RNA can be temporarily stored at -20 °C.

2.4.2 RNA Quantification

RNA was quantified spectrophotometrically using a Nanodrop 1000 spectrophotometer [version 3.3.0, Nanodrop Technologies, USA]. The instrument was cleaned using deionised H₂O and then blanked using 1 µL RNase-free H₂O. A 1 µL volume of isolated RNA from each sample was loaded into the nanodrop pedestal. RNA was measured in ng/µL. DNA contamination was assessed by noting the Abs₂₆₀/Abs₂₈₀ ratio, with a ratio of above 1.8, indicating a relatively pure yield. The Abs₂₆₀/Abs₂₃₀ ratio was also determined, with an above 1.7 indicating that the sample was free of phenol contamination.

2.4.3 cDNA synthesis for miRNA

According to the manufacturer's instructions, reverse transcription for miRNA was completed using the miScript II RT kit [Qiagen, MD, USA]. Reverse transcription used the HiSpec buffer, which ensures miRNAs only are polyadenylated and reverse transcribed with oligo-dT primers,

allowing amplification of mature miRNA. Whereas the HiFlex buffer ensures all RNAs are amplified.

Previously extracted RNA was placed on ice alongside the reverse transcriptase mix. MiScript Nucleics Mix (10X), 5X miScript HiSpec buffer, and RNase-free water were thawed at room temperature. Master mix for cDNA synthesis was prepared on a volume-per-reaction basis. Briefly, 4 μ L 5X miScript HiSpec buffer, 2 μ L 10X Nucleics Mix, and 2 μ L miScript Reverse Transcriptase Mix were added to form a master mix (miScript RT MM). A volume of 8 μ L of miScript RT MM was then added to 0.5 mL flat-topped tubes with 2 μ g of RNA and RNase-free H₂O (made up to 12 μ L), giving a total reaction volume of 20 μ L for each sample. Samples were gently vortexed, and pulse centrifuged to pool contents. The whole reaction was heated at 37°C for 1 h and then at 95 °C for 5 minutes to inactivate the miScript RT MM using the thermo-cycler [Thermo Fisher Scientific, UK]. Reverse transcribed RNA, now termed cDNA, was either placed on ice ready for quantitative-Real Time polymerase chain reaction (qRT-PCR) or could be stored at -20 °C for future investigation.

2.4.4 Quantitative Real-Time PCR for miRNA

To assess miRNA expression, SYBR Green-based qRT-PCR was employed. The miScript SYBR Green PCR kit and miScript primer assays [Qiagen, USA] were used along with cDNA synthesized in *section 2.4.3*. The SYBR Green qPCR process binds the SYBR Green dye to double-stranded DNA (dsDNA). The PCR amplifies the target sequence with the SYBR Green binding to each new copy of the dsDNA product, therefore increasing the fluorescent signal, which relates to the amount of amplified product.

Each sample was plated in triplicate into 96-well qPCR plates [Thermo Fisher Scientific, UK] to limit the influence of outliers in the dataset. A volume of 1 μ L cDNA (equivalent to 100 ng) was added to each well of the 96-well plate. Two different qPCR reaction master mixes (qPCR MM) were prepared on a volume per reaction basis, one qPCR MM having the primer

assay of interest and the other having a PCR loading control primer assay. The reaction volumes included 10 μ L 2X QuantiTect SYBR Green Master Mix, 2 μ L 10x miScript primer assay (miR-31 or RNU-6), 2 μ L 10X miScript Universal Primer, and 5 μ L RNase-free water, making the total volume in the well 20 μ L. The qPCR plate was covered with a transparent adhesive film and sealed. The plate was then subjected to a short spin in a miniplate spinner [Fisher Scientific, UK] to pool contents. Quant Studio 5 real-time thermal cycler [Applied Biosystems, Thermo Fisher Scientific, USA] was programmed to run for 40 cycles after the initial activation at 95°C for 15 min. Each cycle was set to denature at 94°C for 15 s, annealing primers at 55°C for 30 s, and extending primers at 70°C for 30 s.

Data collection was collected during the primer extension step. qPCR data were analysed using the Livak method [265].

2.5 Protein expression analysis

2.5.1 Protein lysate preparation

Cells previously seeded into a T75 cm² flask had spent media discarded to waste and were washed with 5 mL PBS, this was then discarded, and cells were trypsinized and placed into sterile 15 mL tubes with 10 mL complete medium. Next, cell suspensions were centrifuged for 3 min at 300 X g to pellet cells. From this point onward, cell pellets and reagents were kept on ice.

The supernatant (5 mL PBS) was carefully removed from the tube, avoiding disturbance of the pellet. The cell pellet was then resuspended in cold 1X RIPA lysis buffer (50 mM Tris-HCl pH8, 1% Triton-X, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, with the addition of protease and phosphatase inhibitor tablets [Sigma-Aldrich, Arklow, Ireland]). The RIPA volume depends on the pellet's size; a range of 40 μ L - 80 μ L per pellet was used to ensure optimal protein concentration. Cells were resuspended in RIPA and transferred into a 1.5 mL Eppendorf tubes. Cells with RIPA were kept on ice for 20-30 min to ensure efficient lysis and then

centrifuged at 12,000 X g, 4°C for 5 min. The supernatant was then collected and transferred to a new 1.5 mL Eppendorf.

2.5.2 Protein quantification

The protein was quantified using Pierce bicinchoninic acid (BCA) assay [Thermo Fisher Scientific, UK]. Initially, each BCA assay runs created a standard curve using 2 mg/mL bovine serum albumin (BSA). The protein concentration of a sample was calculated using the standard curve. Next, serial dilutions of the concentrated BSA were completed. A series of 8 known protein concentrations were produced from 25 µg/mL to 2000 µg/mL (diluted in RIPA buffer, with RIPA only as a blank). In a 96-well transparent bottom plate, 10 µL standards were aliquoted in duplicate. For protein concentrations to be within range of the assay, sample protein was diluted 1 in 10 within the well. The BCA assay reagent was made in a 15 mL tube at 50 parts of reagent A to one-part reagent B. Once mixed, 200 µL of the BCA assay reagent was added to all standards and protein-containing wells. The 96-well plate was covered in foil and incubated at 37°C for 20 min. Finally, absorbance was measured at 562 nm on an Absorbance VersaMax microplate reader [Molecular Devices, CA, USA]. Using GraphPad Prism, a standard curve was plotted with BSA concentration on the x-axis and absorbance at 562 nm on the y-axis. The protein concentration within samples was therefore calculated using the equation of the standard line ($y=mx+c$). The dilution factor was also taken into consideration.

2.5.3 Protein sample preparation

Quantified samples were prepared (30-50 µg per sample) to be loaded onto a gel for SDS-PAGE protein separation. Samples were prepared with RIPA buffer and normalized to a total volume of 20 µL in 1.5 mL tubes. A volume of 6.25 µL loading buffer (3.3% SDS; 6 M Urea, 17 mM Tris-HCL pH7.5; 0.01% bromophenol blue and 0.07 M β-mercaptoethanol) was added to each sample, and samples pulse centrifuged to pool the

contents of the 1.5 mL tubes. Samples were then heated to 95 °C for 8 min, after which samples were cooled on ice and pulse centrifuged again before gel loading.

2.5.4 SDS-PAGE

Preparation proteins were loaded onto a polyacrylamide gel and electrophoresed to separate proteins according to the size within the gel matrix. Gels were hand-cast (6-20%) according to recipes (**Appendix 3**). The gel casting apparatus was assembled with a glass spacer plate sandwiched with a short plate and clamped into a casting plate. The plates were checked for leaks by introducing 1 mL distilled H₂O in between the plates using a Pasteur pipette [Biosciences, USA]. The resolving gel was prepared in a 50 mL tube [Sarstedt, Germany] and gently pipetted between the plates using a Pasteur pipette, stopping 2 cm from the top of the short plat to all for the stacking gel. An isopropanol layer was gently pipetted on the resolving gel to ensure an even gel and assist polymerization. Once the resolving gel had polymerized after 20-30 min, the isopropanol was carefully poured off, and the prepared stacking gel was pipetted on top. Once the top of the short plate had been reached with the stacking gel solution, a 10-well comb (1.0 mm thickness) was pushed between the plates ensuring no bubbles underneath the wells. The stacking gel was left for another 20-30 minutes to polymerize.

Gels were clamped into a gasket, and the gasket fitted into a tank, with short plates facing inwards toward the central buffer reservoir. A small amount of 1X running buffer (diluted from 10X stock: 30.3 g Tris base, 144g glycine, 100 mL 10% SDS, H₂O up to 1 L) was poured between plates to ensure no leakage. Further, 1X running buffer was added to the space between plates up to the top of the gasket. After removing the well combs, using a 1 mL syringe gauge needle, wells were flushed with 1X running buffer to endure residual polymerized stacking gel that did not interrupt the loading of the gel.

Gels were loaded with molecular weight markers for the first well (5 μ L 1kB PageRuler Plus Prestained Ladder [Thermo Fisher Scientific, UK]). Next, a volume of 20 μ L sample was added to each well. Gels were then electrophoresed [PowerPac Universal, Bio-Rad] for 120 min at 100 V to 120 V. Gels were stopped when the dye front became close to the bottom of the gel. Glass plates were carefully separated after electrophoresis was completed, and the wells cut from the gel. The gel was then transferred to a box tray containing 1X transfer buffer (diluted from 10X stock: 30.2 g Tris base, 144 g glycine, dH₂O up to 1 L) for 5 minutes until equipment and reagents for transferring were prepared.

2.5.5 Western blotting

Proteins separated by SDS-PAGE were transferred onto polyvinylidene fluoride (PVDF) 0.20/0.42 μ M membrane [Thermo Fisher Scientific, UK]. Briefly, proteins are probed on the PVDF membrane with specific primary antibodies directed against a protein of interest. After washing, the PVDF membrane is probed with horseradish peroxidase (HRP) linked secondary antibodies bind to the primary antibodies and can be detected through a chemiluminescent substrate.

Cassettes were assembled appropriately, and all components were thoroughly soaked in 1X transfer buffer, ensuring the clear side was facing down. Firstly, a sponge was placed onto the clear side of the cassette, followed by two filter papers. Next, the PVDF membrane was activated in methanol for 30 s, the polyacrylamide gel, two filter papers, and finally, another sponge. Finally, a roller was used to ensure no air bubbles were present. The sandwich cassette was then carefully closed, fastened, and inserted into the transfer gasket, with the black side of the cassette facing the black side of the gasket. The transfer gasket was slotted into the tank and filled to the marked level with 1X transfer buffer. The tank was then placed in a magnetic stirrer, and protein was transferred at 100 V for 1 h.

After the transfer was completed, the cassette was removed and carefully disassembled using forceps to minimize contact with the PVDF membrane. Filter papers and gel remains were discarded to waste. Notably, the orientation of the membrane was noted and using a scalpel, the top left-hand corner of the membrane was cut. Next, the PVDF was transferred into a 50 mL tube to block the membrane (thus preventing non-specific binding of antibody), which contained 5% non-fat dried milk [Marvel, UK] in 1X TBST (TBS diluted from 10X: 24 g Tris HCl [Melford, UK], 5.6 g Tris base, 88 g NaCl, up to 1 L dH₂O, with the addition of 0.1% Tween-20) solution.

Membranes were incubated at room temperature on a tube roller [Fisher, UK] for 1 h. After blocking, the blocking solution was discarded, and membranes were washed in TBST for 10 min with two changes. Then, membranes were incubated in primary antibody overnight at 4 °C on a tube roller. Primary antibodies were prepared in 50 mL tubes, with either 5% milk solution or 5% BSA solution – prepared with TBST – depending on manufacturer recommendations (**Table 2.2**). Membranes were washed in TBST for 30 min, with at least 4 changes of wash during that period. Secondary antibodies were also prepared in fresh 50 mL tubes in milk solution. Next, membranes were incubated in a secondary antibody for two h at room temperature on a tube roller. Membranes were then washed in TBST for 30 minutes with five wash changes.

The membranes were imaged through a chemiluminescent imaging system (Fusion FX; Vilber Lourmat). Firstly, SuperSignal™ West Pico PLUS chemiluminescence substrate [Thermo Fisher Scientific, UK] was prepared by combining the two reagents (1:1). Membranes were put onto a sheet of clear acetate with the chemiluminescence substrate mix poured onto the membrane, ensuring the surface was completely covered. An additional acetate sheet was then placed over the top of the

Table 2.2. Antibodies used for Western blotting.

Target protein	Manufacturer	Catalogue no.	Species	Dilution used
TurboGFP	Origene	TA150041	Mouse	1:10,000 (5% milk/TBST)
Phospho-histone H2A.X (S139)	Cell Signaling	9718S	Rabbit	1:1000 (5% BSA/TBST)
ATP7A	Santa Cruz	SC-376467	Mouse	1:1000 (5% milk/TBST)
ATP7B	Santa Cruz	SC-373964	Mouse	1:1000 (5% milk/TBST)
LAMP-1	Santa Cruz	SC-17768	Mouse	1:1000 (5% milk/TBST)
ABCB9	Assay Genie	PACO49334	Rabbit	1:5000 (5% milk/TBST)
CTR1	Santa Crus	SC-66847	Rabbit	1:1000 (5% milk/TBST)
GPx8	Assay Genie	CAB20390	Rabbit	1:2000 (5% milk/TBST)
ATOX1	Assay Genie	CAB19925	Rabbit	1:500 (5% milk/TBST)
Beta-actin	Santa Cruz	SC-69879	Mouse	1:10,000 (5% milk/TBST)
Anti-mouse (2°)	Cell Signaling	7076S	Rabbit	1:2000 (5% milk/TBST)
Anti-rabbit (2°)	Cell Signaling	7074P2	Goat	1:2000 (5% milk/TBST)

membrane. All air bubbles were rolled out. Membranes were inputted into the imaging system and occasionally imaged over 5 minutes.

To complete densitometry analysis, western blots developed on film were imaged using white light on the imaging system. The volume tool drew boxes around bands of interest, displaying volume intensity. The volume intensity of a band was normalized to the volume density of the loading control by dividing the band of interest by the β -actin band. Where appropriate, band volume densities were normalized to a control sample's densities.

2.5.6 ATOX1 ELISA

The Human ATOX1 enzyme-linked immunosorbent assay (ELISA) (HUF108736), purchased from Assay Genie [Dublin, Ireland], was used to assess ATOX1 levels in PDAC cell lines. ELISA was performed as per the manufacturer's recommendations. Briefly, each standard and sample was measured in duplicate, and all wells were washed with washing buffer beforehand. Protein was collected as previously described in section 2.5.2. Standards and samples were added (0.1 mL) per well where the plate was sealed and incubated at 37 °C for 90 min. The seal was removed, the plate content was discarded, followed by two washes. Next, Biotin- detection antibody working solution (0.1 mL) was added to the wells, where the plate was sealed and incubated at 37°C for 60 min. Following incubation, the cover was removed, and the plate was washed three times. Next, HRP-Streptavidin Conjugate (SABC) was added (0.1 mL) into each well, where the plate was covered and incubated at 37°C for 30 min. Again, the cover was removed, and the plate was washed five times. Then 90 μ l of TMB substrate was added to each well and incubated at 37°C in the dark for 10-20 min. Following incubation, 50 μ l of Stop solution was added to each well and mixed thoroughly. The O.D. absorbance at 450 was measured using the microplate reader.

2.6 Fluorescent Microscopy

2.6.1 Lysosomal mass/pH

A multiparametric cytotoxicity assay was performed using Cellomics® HCS reagent HitKit™ as per the manufacturer's instructions [Thermo Fisher Scientific, USA]. This kit has lysosomal pH, which is a toxicity-attributed phenomenon. Some toxins can interfere with the cell's functionality by affecting the pH of organelles such as lysosomes and endosomes or by causing an increase in the number of lysosomes. The dye used in the chosen cytotoxicity assay is a weak base that accumulates in acidic organelles, such as lysosomes and endosomes, which allows changes in lysosomal physiology to be determined. For instance, an increase or decrease in the pH of acidic organelles and the changes in lysosome numbers by compound toxicity results in a reduction or an increase in fluorescence intensity, respectively. High-resolution intracellular accumulation of fluorescently labelled nanoparticles was visualized by confocal laser scanning microscopy [Carl Zeiss, Axiovert, Germany]. All these were scanned and acquired in a stereology configuration of 5 randomly selected fields. Images were acquired at 10 X magnification using three detection channels with different excitation filters. A TRITC filter (channel 3) detected lysosomal mass and pH changes with red fluorescence at 599 nm. The fluorescent staining intensities reflecting cell lysosomal mass/pH changes (TRITC filter) were quantified for each cell in the microscopic fields by IN Cell Investigator and software (GE Healthcare, UK).

2.7 Compartment Isolation

2.7.1 Cytoplasmic and Nuclear Separation

The separated cytoplasmic and nuclear compartments were completed using the Nuclear Extraction Kit [Active Motif, UK]. In short, cells were harvested and washed in ice-cold PBS and pelleted at 200 X g for 5 min in a microcentrifuge pre-cooled at 4 °C. The cell pellet was gently

resuspended in 500 μ L Hypotonic Buffer and left on ice for 15 min. Then, 25 μ L Detergent was added and vortexed at the highest setting for 10 seconds. Ensure all cells have been efficiently lysed and the nuclei released under the microscope. Suspensions are centrifuged for 30 seconds at 14,000 X g in a microcentrifuge pre-cooled at 4 °C—transfer supernatant (cytoplasmic fraction) into a pre-chilled microcentrifuge tube. Store the supernatant at –80°C until ready to use. Use the pellet for nuclear fraction collection. OPTIONAL: A fraction of supernatant (cytoplasmic fraction) was saved to determine fractionation efficiency by Western blot. Resuspend nuclear pellet in 50 μ L Complete Lysis Buffer. OPTIONAL: 2.5 μ L Detergent can be added to help solubilise membrane-associated nuclear proteins. A very viscous pellet may form and not completely resuspend—vortex 10 seconds at the highest setting. Incubate suspension for 30 minutes on ice on a rocking platform set at 150 rpm. 3. Vortex 30 seconds at the highest setting. Centrifuge for 10 min at 14,000 X g in a microcentrifuge pre-cooled at 4 °C. Transfer supernatant (nuclear fraction) into a pre-chilled microcentrifuge tube. OPTIONAL: A fraction of supernatant (nuclear fraction) was saved to determine fractionation efficiency by Western blot.

2.7.2 Lysosomal Isolation

The lysosomal compartment isolation was completed using the Minute™ Lysosome Isolation Kit for Mammalian Cells [Invent Biotechnologies inc., USA] and followed the manufacturer's instructions. Briefly, cells were harvested and washed with ice-cold PBS. The cell suspension was centrifuged for 5 min at 500 X g. The supernatant was discarded, the cell pellet was resuspended in 500 μ L Buffer A then incubated on ice for 5-10 min, and then vortexed vigorously for 10-30 seconds. The cell suspension is immediately transferred to the filter cartridge. Cap the filter cartridge, and the sample was inverted a few times and centrifuged at 16,000 X g for 30 seconds. The filter was discarded, and the cell pellet was resuspended by vigorous vortexing for 10 seconds. The suspension was

then centrifuged at 2000 X g for 3 min, and the supernatant was transferred to a fresh 1.5 mL microfuge tube and centrifuged at 4 °C for 15 min at 11,000 X g. The pellet contains mainly mitochondria and cell debris. After centrifugation, carefully transfer 400 µL supernatant to a fresh 1.5 mL tube and spin at 16,000 X g at 4 °C for 30 min. The supernatant was removed. The pellet is resuspended in 200 µL cold Buffer A by pipetting up and down 60-100 times and vortex vigorously for 20 seconds. Centrifuge at 2000 X g for 4 min. The supernatant was carefully transferred to a fresh 1.5 mL tube. Then 100 µL Buffer B was added to the tube and vortexed briefly to mix well (the supernatant to buffer B ratio is 2:1). Samples were incubated in the tube on ice for 30 min and centrifuge at 11,000 X g for 10 min. The supernatant was removed and then spun at 11,000 X g for a few seconds to bring down the residual reagent and remove it altogether. Resuspend the pellet in 50-150 µL PBS or other buffers. This is a highly enriched lysosome fraction.

2.8 Flow Cytometry

2.8.1 Cell cycle analysis

Cell cycle analysis was performed by Propidium iodide (PI) [BioLegend, USA] staining and using the BD LSR Fortessa flow cytometer [BD Biosciences, USA]. The intensity of the PI signal is proportional to DNA content. Cells in the exponential growth phase were harvested by trypsinization as previously described. Cells were seeded at a density of 1×10^5 cells in T25 flasks and allowed to adhere overnight at 37°C in 5% CO₂/95% humidified air. Cells were then treated with cisplatin for 24 h. At 24 h, 48 h, and 72 h, cells were collected by trypsinization and collected in 15 ml tubes. Cells were centrifuged at 300 x g for 3 min, and the supernatant was decanted. The cell pellet was washed with PBS and centrifuged, and the waste was discarded. Cells were fixed and permeabilized by dropwise addition of 4.5 mL ice-cold ethanol (70% v/v in dH₂O) while vortexing to avoid the formation of aggregates. Cells were stored in the fixative overnight at 4°C. Fixed cells were centrifuged at 300

X g for 3 min, and the supernatant was decanted. Cells were then washed with 1 mL PBS and centrifuged as before. Each sample was resuspended in 0.5 mL Triton X-100 (0.1% v/v in dH₂O) [Sigma-Aldrich, Ireland], containing PI (0.02 mg/mL) and RNase A (0.2 mg/mL) [Sigma-Aldrich, Ireland], except for appropriate controls. The addition of RNase A ensures that any RNA is digested, preventing any interference with the DNA signal. Samples were incubated at 37°C for 30 min and then at RT°C for 1.5 h in the staining solution. Cells were subjected to the flow cytometer, and data was analysed using FlowJo v10 software [TreeStar Inc., Oregon, USA].

2.9 Cell-based assays

2.9.1 Clonogenic assay

The sensitivity of cells to chemotherapeutics and radiation treatment was measured by clonogenic assay, which is the standard method for measuring cytotoxicity. Cell seeding densities were optimized to ensure at least 200 viable colonies were present in a 6-well plate at the end of the clonogenic incubation (**Appendix T1**). Cell seeding densities were adjusted according to treatments, with a greater cell density where treatment would indicate lower countable colonies.

Cell number with 2 mL complete medium were added into 6-well plates and allowed to adhere overnight in a 37°C, 5% CO₂ humidified incubator. Spent media was carefully aspirated to ensure the adhered single cells were not disturbed. Cells were then subjected to chemotherapy treatment using established IC₅₀ doses for 24 h, following which treatment was carefully aspirated and 2 mL fresh complete medium applied to each well. For radiation-treated clonogenics, plates were irradiated and exposed to doses from 2 Gy to 8. After exposure, plates were returned to the incubator. Plates were incubated for 8-10 days post-seeding.

Colonies were fixed by aspirating and discarding media, then with a gentle wash with PBS to each well. A crystal violet solution (0.1% w/v crystal violet, 60% v/v MeOH, 40% v/v H₂O) was then used to stain colonies. The fixative was left at room temperature for 1 h, after which it was carefully removed. Sodium hydroxide was added to waste to inactivate crystal violet. Wells were washed carefully with water until colonies were distinct and crystal violet sediment no longer remained. Plates were left overnight to dry at room temperature. Colonies were counted using the GelCount instrument [Oxford Optronix, UK], using optimized CHARM (compact Hough and radial map) image processing algorithms for each cell line. The CHARM algorithm was optimized to distinguish and detect individual colonies. The CHARM algorithm distinguished between colonies using various functional features, including colony diameter and colony density.

Plating efficiency (PE) was calculated as the colony count divided by the number of cells seeded. Surviving fraction (SF) was therefore calculated as the colony count, divided by the control's PE, multiplied by the number of cells seeded.

Plating Efficiency (PE) = control colony count/number of control cells seeded

Surviving Fraction (SF) = colony count/(PE × number of cells seeded)

2.9.2 Proliferative Capacity Assay

A basic cumulative cell count assay was employed to detect subtle changes in proliferative capacity; 3×10^5 cells were seeded into 100 mm tissue culture dishes and allowed to adhere overnight. Subsequently, spent media was discarded to waste, and chemotherapy treatment was applied for 24 h. Treatment was aspirated, and 10 mL fresh complete medium was added to each culture dish. For radiotherapy, cells were

treated 24 h post-seeding. Cells were re-seeded at 3×10^5 every 3 days for 9 days, and a cumulative cell count was taken at each time of re-seeding.

2.9.3 MTS Assay

According to the manufacturer's instructions, cellular viability in response to chemotherapeutic/radiation treatment via CellTiter 96® Aqueous One Solution Cell Proliferation Assay [Promega, Hampshire, UK]. Briefly, Aqueous One Solution is a colorimetric-based assay that contains the tetrazolium compound MTS, which is reduced by metabolically active cells, producing a coloured formazan product which can then be measured at an absorbance of 495 nm on the VersaMax microplate reader.

Cells were seeded at a density of 5×10^3 cells per well in 100 μ L complete media within a 96-well transparent bottomed plate, with each experimental condition plated in triplicate. Cells were incubated overnight, after which the spent media was removed from each well and a suitable treatment medium added (including at least one media well on each plate). A volume of 20 μ L of MTS reagent was added to each well 72 h after treatment. After adding the MTS reagent, plates were measured at 495 nm on a plate reader. The triplicate absorbance readings were averaged, and the media-only reference was subtracted from the readings.

2.9.4 Measurement of Intracellular ROS

H₂O₂ was measured using the Fluorometric-Near Infrared ROS assay kit (Abcam, Cambridge, UK). Cells were seeded at a concentration of 1×10^4 cells/well into an opaque 96-well plate and allowed to adhere overnight at 37°C in 5% CO₂/95% humidified air. Cells were treated and left in the incubator for the appropriate time interval. Briefly, the H₂O₂ reaction mixture was prepared according to the manufacturer's instructions. A volume of 50 μ L of the H₂O₂ reaction mixture was added to each well and incubated at room temperature for 0-30 min, protected by light.

Fluorescence was measured at Ex/EM = 640/680 nm using the GloMax microplate reader [Promega, UK].

2.9.5 Measurement of Intracellular GSH Levels

Levels of reduced glutathione (GSH) were measured using the luminescence-based GSH/GSSG-Glo™ assay [Promega, UK]. Cells were seeded at a concentration of 5×10^3 cells/well into a white-bottomed 96-well plate and allowed to adhere overnight at 37°C in 5% CO₂/95% humidified air. Cells were treated with appropriate treatment for 24 h. After treatment, spent media was discarded to waste, 50 µL total GSH was applied to each well, then shaken using an orbital shaker [Medical Supply Co., Ireland] for 5 min. A volume of 50 µL of luciferase generation reagent was added to all wells, and plates were incubated for 30 min. A volume of 100 µL of luciferase detection reagent was added to all wells and incubated for 15 min at room temperature. Finally, luminescence was read with the GloMax microplate reader, with an integration time of 1000 ms.

2.9.6 Measurement of caspase 3/7 levels

Caspase 3/7 activity was measured using the ApoTox-Glo™ assay [Promega, UK]. Cells were seeded at a concentration of 1×10^4 cells/well into a white-bottomed 96-well plate and allowed to adhere overnight at 37°C in 5% CO₂/95% humidified air. Cells were treated with radiation and left for appropriate time intervals. A volume of 100 µL of caspase 3/7 substrate dissolved in caspase 3/7 buffer was added to each well. The plates were placed on the orbital-shaker for 30 seconds, left to incubate for 20 min at room temperature, and finally measured for luminescence signal using the GloMax microplate reader with 1000 ms integration time.

2.9.7 Crystal violet assay

Cells were fixed in 1% glutaraldehyde in PBS for 15 min at room temperature. Fixative was removed, and cells were washed twice with PBS and stained with 0.1% crystal violet solution for 30 min at room

temperature. The stain was removed, and cells were washed twice with H₂O and allowed to air-dry overnight. Cells were incubated with 1% Triton X-100 in PBS on a plate shaker for 2 h. Absorbance was read at 595 nm on the VersaMax microplate reader.

2.9.8 Inductively Coupled Plasma Mass Spectroscopy (ICP-MS)

Inductively Coupled Plasma Mass Spectroscopy, or ICP-MS, measures the trace elements within a given sample. Here, we adopted ICP-MS to analyse platinum (the main component of cisplatin) at the most abundant isotope, Pt₁₉₅. Cells were treated with 50 µM cisplatin for 24 h, to ensure enough platinum was quantifiable by ICP-MS, after which cells were harvested and counted, fractionated, or pulled down to isolate organelle areas of interest. Cells (2x10⁶) or fractions were incubated in HNO₃ overnight at 70°C. Following incubation, samples were sent to Essen, Germany, where ICP-MS measured platinum content. Standard curves were generated using aqueous serial dilutions of known standards. Each measurement taken was representative of 3 technical replicates from an individual sample.

2.10 Clinical Data

2.10.1 MiR-31 Kaplan Meier

The Cancer Genome Atlas (TCGA) Pancreatic Adenocarcinoma (PAAD) data RSEM RNASeq V2 normalized counts were downloaded from OncoLnc and accompanying clinical data (including overall survival) from the Broad firehose. All tissue is collected post-surgery, with all patients receiving no prior treatment. RNA was then extracted from fresh-frozen tissue and sequenced. The type of chemotherapy chosen was unknown.

For miRNA-31-5p and miRNA-31-3p, 184 patients had useable expression data. Of these patients, 117 later went on to receive chemotherapy. For use with Kaplan-Meier curves, expression was discretized into a low and high expression using the MaxStat R package, whereby an unbiased split point is selected based on the maximum Log-Rank statistic. Kaplan-Meier

operator curves were plotted using the survminer R package and visualized using ggplot2.

2.10.2 ATOX1 Kaplan Meier

The Kaplan-Meier plotter (PAN-cancer) was used to examine the effect of low and high ATOX1 expression on the overall survival of patients with PDAC ($n = 177$). To select the expression cut-off between the groups, all possible cut-off values between the lower and upper quartiles were computed, with the best-performing cut-off being selected. Cox proportional hazards regression analysis was performed to assess the relationship between ATOX1 expression levels and survival. The type of chemotherapy used was unknown. All data was taken from the TCGA database.

2.11 Statistical analysis

GraphPad Prism 10 software was used to perform statistical analysis. Experiments were repeated at least three times, and the results were displayed as mean $\pm$ SEM. The statistical significance of the results was determined by a two-tailed paired t-test, a one-sample t-test, and a one/two-way ANOVA; * $p < 0.05$ was considered a statistically significant difference.

Chapter Three

The role of miR-31 in modulating PDAC sensitivity to chemotherapy and radiotherapy

This chapter has been published in part in **McGrath, J.**, Kane, L. E., & Maher, S. G. (2022). The Influence of MicroRNA-31 on Oxidative Stress and Radiosensitivity in Pancreatic Ductal Adenocarcinoma. *Cells*, 11(15), 2294. <https://doi.org/10.3390/cells11152294>

3.1 Introduction

Pancreatic cancer represents a significant and growing health problem. Despite being the twelfth most common cancer globally, pancreatic cancer has one of the lowest (9%) 5-year survival rates among all cancer types [1, 266]. According to GLOBOCAN 2020, 495,773 new cases and 466,003 deaths were attributed to pancreatic cancer worldwide in the year 2020 [2]. Pancreatic cancer is considered the fourth cancer-related cause of death in the United States. It is projected to rise to second place in cancer mortality in Northern America by 2030, a trend reflected in Europe [1, 4, 5]. Histologically, pancreatic ductal adenocarcinoma (PDAC) is responsible for more than 90% of pancreatic cancer cases. The high mortality rate of PDAC is primarily due to late diagnosis and tumour resistance to treatment [5, 8]. Surgery is the only curative treatment option for PDAC; however, less than 15% of patients are eligible for this procedure [57]. Resistance to chemotherapy and radiotherapy has become a critical challenge in treating PDAC, with most patients displaying resistance patterns and succumbing to their disease [69-71]. The elucidation of markers and mechanisms of resistance would therefore be a significant clinical benefit and is critical for improving therapeutic efficacy for patients with PDAC.

MiRNAs are a group of small non-coding RNA molecules that regulate gene expression at the posttranscriptional level. Generally, miRNAs interact with the 3'UTR of target mRNAs to suppress expression. However, interactions of miRNAs with other regions, including the 5'UTR, coding sequence, and gene promoters, have been reported [161-163]. The sequence complementarity between nucleotides 2-8, also known as the 'seed region,' is vital for target sequence recognition. However, perfect complementarity is not essential for regulation. It has been well established that a single miRNA has the potential to target and regulate multiple genes, and a single gene can be regulated by various miRNAs [171-173]. Interestingly, it is estimated that approximately 50% of

miRNAs are encoded on fragile sites within the genome [267], hence the current interest in miRNA as modulators of cancer biology. MiRNA-31 (miR-31) is among the most frequently altered miRNA in cancer, where altered expression has been detected in various cancer types and has been thoroughly reviewed by Laurila and Kallioniemi [268].

One of the key genetic events in PDAC development is the inactivation of the p16 tumour suppressor gene [269]. The p16 gene is encoded on chromosome 9p21.3, a recognized fragile site in the human genome [270]. Interestingly, miR-31 is encoded in the exact location downstream of p16, and it is reasonable to believe that because of their proximity, they are frequently co-deleted or co-disrupted together [271]. Additionally, epigenetic modifications such as the hyper-methylation caused by EZH2 are responsible for the low expression of miR-31 [272]. However, in contrast, miR-31 is moderate to highly expressed in 16 from 23 pancreatic and colorectal cell lines when compared to HPNE “normal” cells which was developed from the human pancreatic duct [273]. The BxPC-3 and Colo205 (developed from ascitic fluid derived from a male with colon cancer) cell lines were the only cells that displayed no miR-31 expression [273].

We have previously demonstrated that miR-31 is a valuable therapeutic target regulating chemotherapy and radiotherapy sensitivity by altering drug transportation and DNA damage repair genes in other cancer types [274, 275]. However, its role in modulating chemosensitivity and radiosensitivity in PDAC remains to be explored. Therefore, the investigation of miR-31 and the elucidation as to whether it modulates chemosensitivity and radiosensitivity in PDAC cell lines is to be elucidated within this chapter.

3.2 Rationale, Aims, and Objectives

Patients diagnosed with PDAC display an extremely poor prognosis, and responding to treatment, including chemotherapy and radiotherapy, remains a challenge due to tumour resistance. Emerging evidence has revealed miRNAs as potential biomarkers and can be targeted to modify the response to treatment in PDAC and other cancers. With previously established results indicating miR-31 manipulation leads to modulating cellular sensitivity to anticancer therapies, we hypothesise that miR-31 manipulation modulates chemosensitivity and radiosensitivity in PDAC.

The objectives of this chapter were to (1) determine whether the dysregulation of miR-31 is associated with PDAC chemoresistance and radioresistance, to identify whether miR-31 may present a therapeutic target or predictive biomarker in PDAC (2) to explore the effect of miR-31 manipulation on biological endpoints relating to chemoresistance and radioresistance to characterise any alterations observed and to elucidate further and clarify the role of miR-31 in PDAC cell lines in response to therapy.

3.3 Experimental design

Assessing miRNA expression in PDAC cell lines

To analyse the status of miR-31 within a range of PDAC cell lines to establish and verify which lines would be suitable for miR-31 overexpression and suppression, the most prevalent epithelioid subtype BxPC-3 and Panc-1 cell lines were chosen, and a brief characterisation of which is noted in **Table 3.1**.

Stable expression of miR-31 in BxPC-3 and Panc-1

To establish the cell line models using BxPC-3 as a model of miR-31 overexpression and Panc-1 as a model of miR-31 suppression, cell lines were transfected using lipofectamine with either miR-31-overexpressing or miR-31-suppressing plasmids, respectively. Transfections were confirmed by western blot with GFP as a marker; however, this did not directly confirm the manipulation of miR-31 within the system, but it supported the plasmid integration and certified RT-qPCR measured support of miR-31 manipulation.

Analysis of the effect of miR-31 manipulation on anti-cancer therapies

The clonogenic assay, MTS assay, and a cumulative cell count were performed to assess PDAC cell response to chemotherapy and radiotherapy with altered miR-31 expression. The clonogenic assay is the gold standard for determining response to agents as it covers all forms of cell death, both early and late events.

3.4 Results

3.4.1 Confirmation of miR-31 status in PDAC cell lines

Resistance to chemoradiotherapy has been previously associated with miR-31 in multiple cancer types; therefore, manipulating miR-31 expression in PDAC cell lines was assessed to reveal any alterations to chemotherapy or radiation therapy. The relative expression of miR-31 was substantially reduced (** $p = 0.0038$) in the BxPC-3 parental cell line compared to the Panc-1 parental cell line (**Figure 3.1**), confirming the results of other groups. This allowed us to create an antagonistic modified miR-31 expression system within the same subtype, whereby the BxPC-3 cell line had miR-31 overexpressed, and the Panc-1 cell line had miR-31 suppressed.

3.4.2 Establishing a miR-31 manipulated stable model

To study the effect of miR-31 on cellular sensitivity to chemotherapy or radiotherapy in PDAC cell lines, a model of stable miR-31 overexpression and suppression was established. The miR-31 overexpressing plasmid encoded the miR-31 precursor sequence and produced both mature miR-31-3p and miR-31-5p. Thus, overexpression refers to general miR-31 overexpression, as it is not possible to differentiate between the contributions of miR-31-3p and miR-31-5p in this model.

Following transfection of BxPC-3 cells with either miR-VC or miR-31 expressing plasmids and transfection of Panc-1 cells with suppression plasmids Zip-miR-VC or Zip-miR-31, confirmation of miR-31 overexpression or suppression in stable expressing models were measured by RT-qPCR (**Figure 3.2 & 3.3**). The BxPC-3 miR-31 cell line showed a successful transfection by presenting a greater RQ of miR-31 than its vector control equivalent. Similarly, The Panc-1 Zip-miR-31 cell line displayed a successful transfection by presenting a reduced RQ of miR-31 compared to its vector control equivalent. However, the suppression of miR-31 in Panc-1 cells was modest and may have had a

bearing on future results. Additionally, to confirm the expression of the miR-VC or Zip-miR-VC within cells, the GFP reporter was analysed via western blot (**Figure 3.4**).

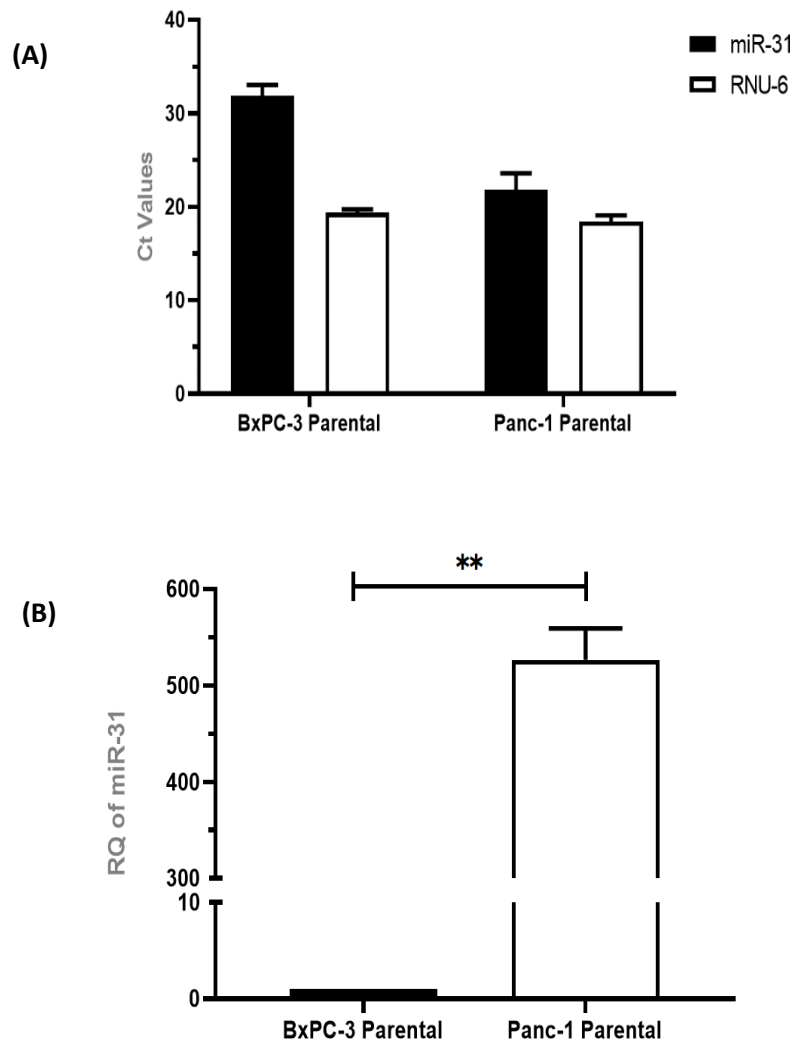


Figure 3.1 MiR-31 status in PDAC cell lines. (A) C_t values of PDAC cell lines comparing miR-31 expression. BxPC-3 parental cells have a greater mean C_t value compared to Panc-1 parental cells. **(B)** RT-qPCR displaying the relative level of expression of endogenous miR-31 between BxPC-3 parental and Panc-1 parental cells (** $p = 0.0038$). All RT-qPCR runs were loaded with 100 ng of cDNA. Relative values were normalised to the endogenous control RNU-6. A one sample t.test was applied for statistical analysis ($n = 3$).

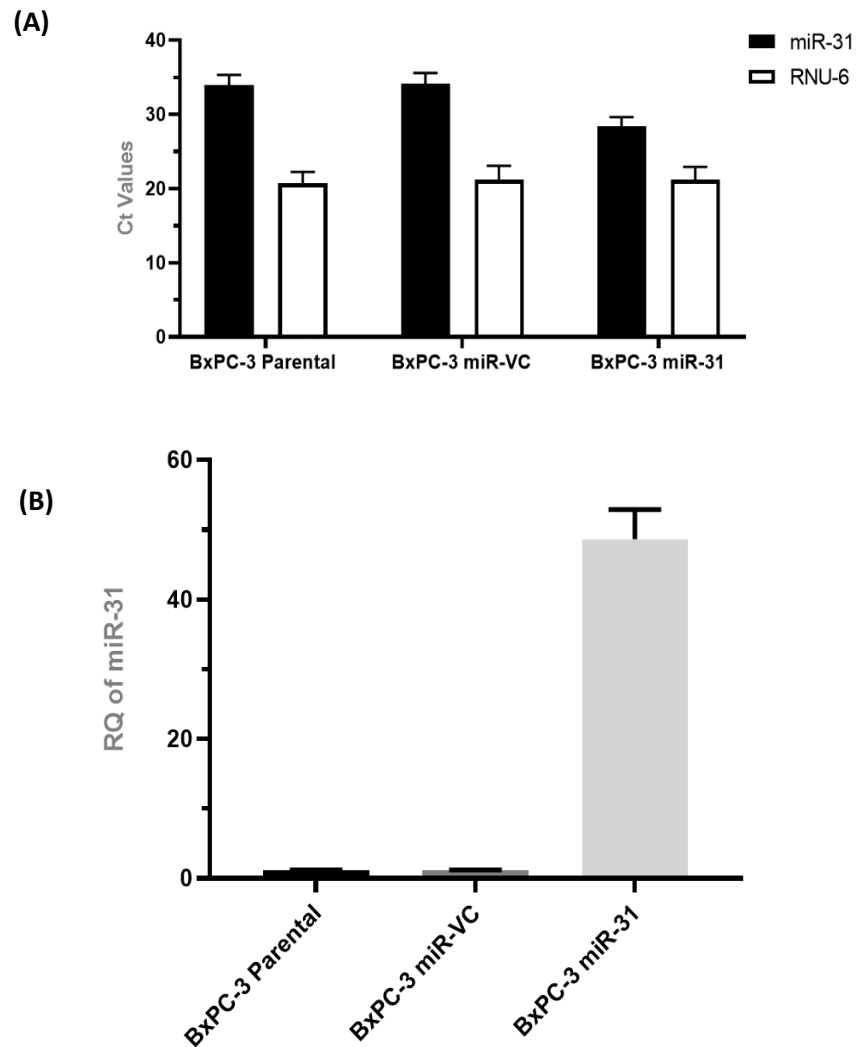


Figure 3.2 Confirmation of overexpressing miR-31 in BxPC-3 cells. (A) C_t values of BxPC-3 cell lines comparing miR-31 expression. BxPC-3 miR-31 cells have a lower mean C_t value compared to BxPC-3 miR-VC cells. **(B)** RT-qPCR displaying the relative level of expression of miR-31 between BxPC-3 miR-31 cells and BxPC-3 miR-VC cells ($n = 3$). All RT-qPCR runs were loaded with 100 ng of cDNA. Relative values were normalised to the endogenous control RNU-6.

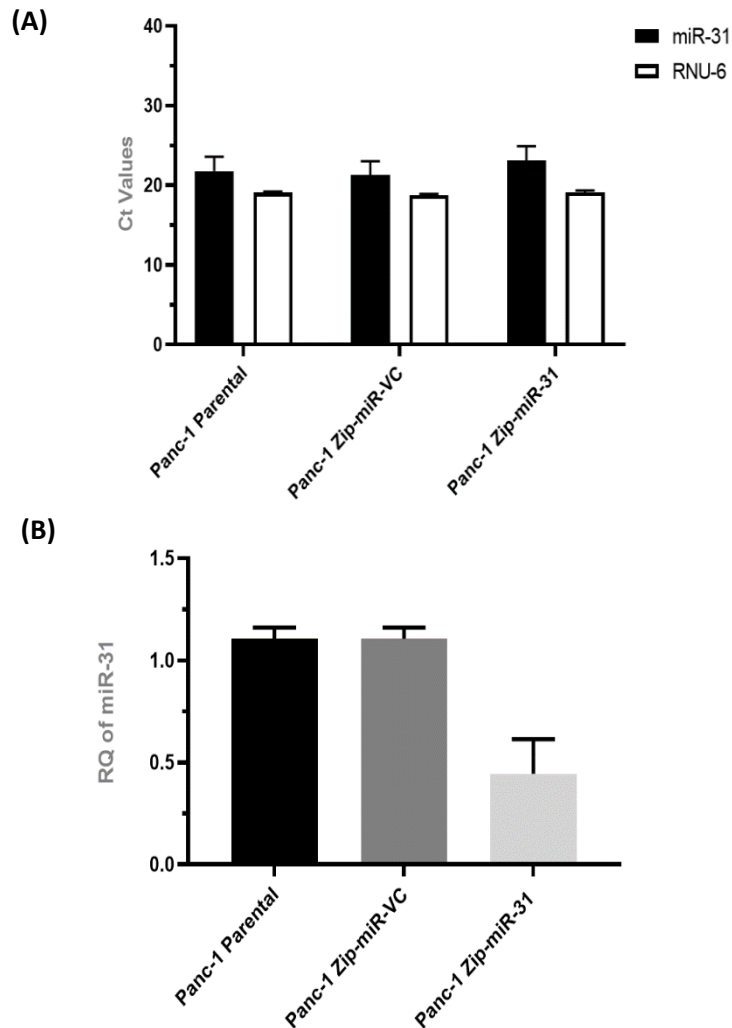


Figure 3.3 Confirmation of suppressing miR-31 in Panc-1 cells. (A) C_t values of Panc-1 cell lines comparing miR-31 expression. Panc-1 Zip-miR-31 cells have a greater mean C_t value compared to Panc-1 Zip-miR-VC cells. **(B)** RT-qPCR displaying the relative level of expression of miR-31 between Panc-1 Zip-miR-31 cells and Panc-1 Zip-miR-VC cells ($n = 3$). All RT-qPCR runs were loaded with 100 ng of cDNA. Relative values were normalised to the endogenous control RNU-6.

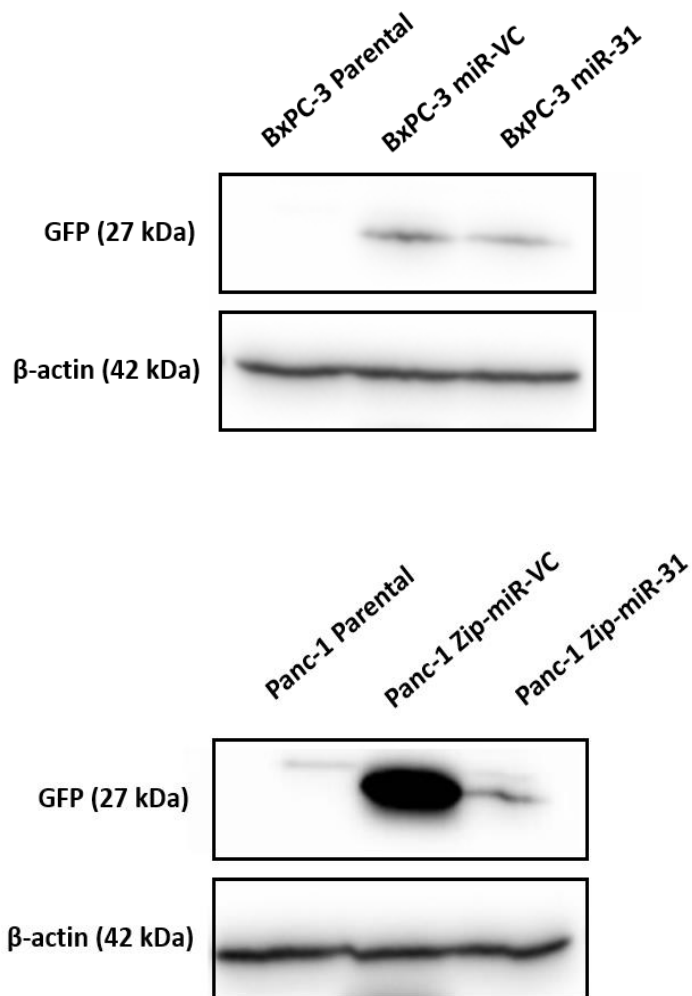


Figure 3.4 Confirmation of stable transfection in PDAC cell lines. (A) Representative western blot displaying GFP expression in transfected BxPC-3 cell lines **(B)** Representative western blot displaying GFP expression in transfected Panc-1 cell lines. β-actin was used as the loading control. The data demonstrate the successful transfection of plasmids in both cell lines. Western blots detailed are representative of

3.4.3 Mycoplasma screening of PDAC cell lines

Because mycoplasma infection can affect the changes in metabolism and cell proliferation, all cell lines used were regularly tested for mycoplasma contamination, as described in *section 2.2.5*. No mycoplasma contamination was detected in either parental or stable cell lines (**Figure 3.5**).

3.4.4 Establishing the IC₅₀ doses of chemotherapeutics in PDAC cell lines.

Before investigating if miR-31 modulates chemosensitivity in the miR-31 manipulated PDAC models, PDAC parental cells were treated with a range of a chemotherapeutic agent to establish an IC₅₀ dose, indicating how much the drug is needed to inhibit a biological process by half, and a dose-response kill curve determined this by clonogenic assay. The IC₅₀ doses of alkylating agents are shown in **Figure 3.6**, and anti-metabolite agents are shown in **Figure 3.15**.

3.4.5 MiR-31 modulates cellular sensitivity to alkylating agents in PDAC cell lines.

To investigate if miR-31 modulates cellular sensitivity to alkylating agents in miR-31 manipulated PDAC models, cells were treated with cisplatin (24 h), carboplatin (48 h), or oxaliplatin (24 h) alone. It was established that overexpressing miR-31 in BxPC-3 cells significantly promoted chemoresistance to cisplatin (**Figure 3.7**) and oxaliplatin (**Figure 3.9**) but not with carboplatin (**Figure 3.8**) when compared to its miR-VC equivalent. Furthermore, it was also established that suppressing miR-31 in Panc-1 cells significantly enhanced chemosensitivity to cisplatin treatment (**Figure 3.10**), but not with carboplatin (**Figure 3.11**) and oxaliplatin (**Figure 3.12**) when compared to its Zip-miR-VC equivalent.

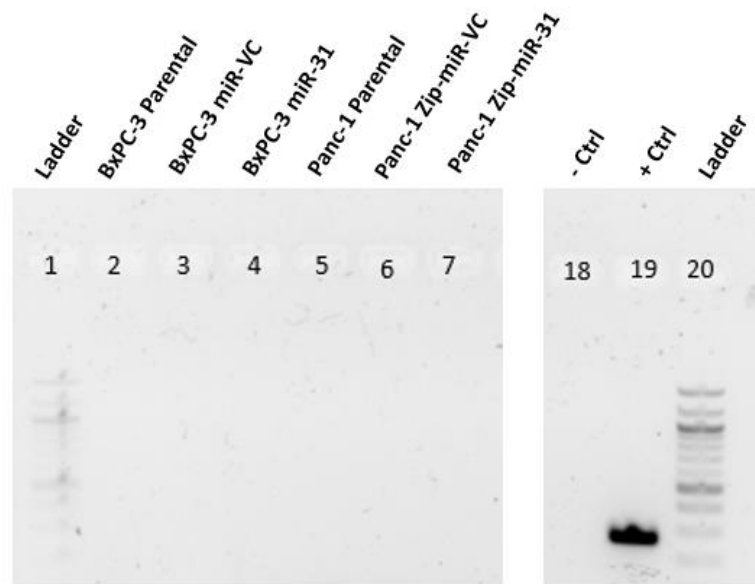


Figure 3.5 Mycoplasma testing of PDAC cell lines. Cell culture supernatants were collected from PDAC cell lines used in this study on a regular basis and tested for mycoplasma contamination. No traces of mycoplasma were observed in any cell lines when compared to the positive control (lane 19).

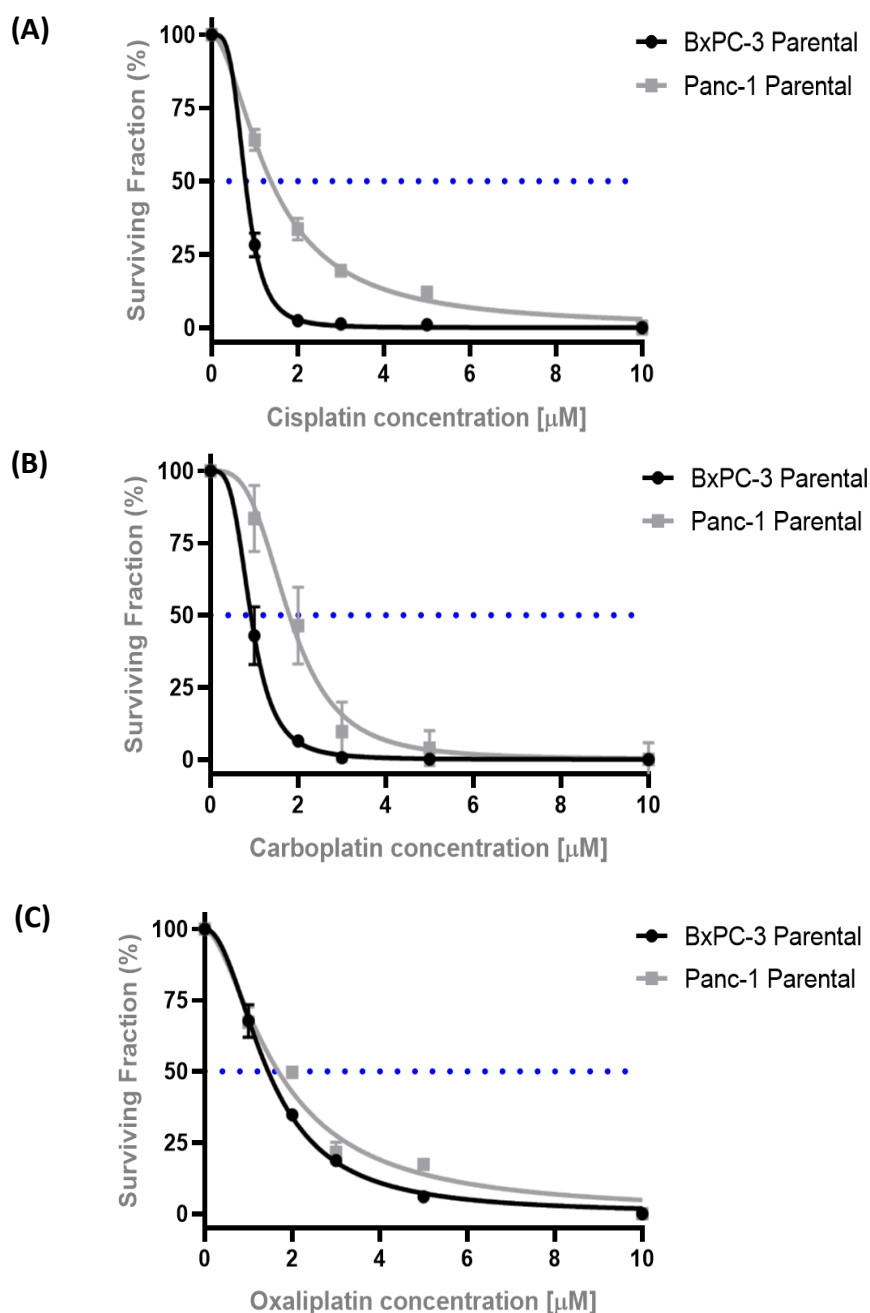


Figure 3.6 IC_{50} doses of alkylating chemotherapeutic agents in PDAC cell lines. (A) Dose-response for cisplatin treatment for 24 h in the BxPC-3 parental cell line ($\text{IC}_{50} = 0.784 \mu\text{M}$) and Panc-1 parental cell line ($\text{IC}_{50} = 1.38 \mu\text{M}$). (B) Dose-response for carboplatin treatment for 48 h in the BxPC-3 parental cell line ($\text{IC}_{50} = 0.923 \mu\text{M}$) and Panc-1 parental cell line ($\text{IC}_{50} = 1.80 \mu\text{M}$). (C) Dose-response for oxaliplatin treatment for 24 h in the BxPC-3 parental cell line ($\text{IC}_{50} = 1.45 \mu\text{M}$) and Panc-1 parental cell line ($\text{IC}_{50} = 1.68 \mu\text{M}$). All clonogenic assays were vehicle-controlled with the analysis of PBS treated controls considered when calculating the surviving fraction. IC_{50} doses were established using GraphPad Prism.

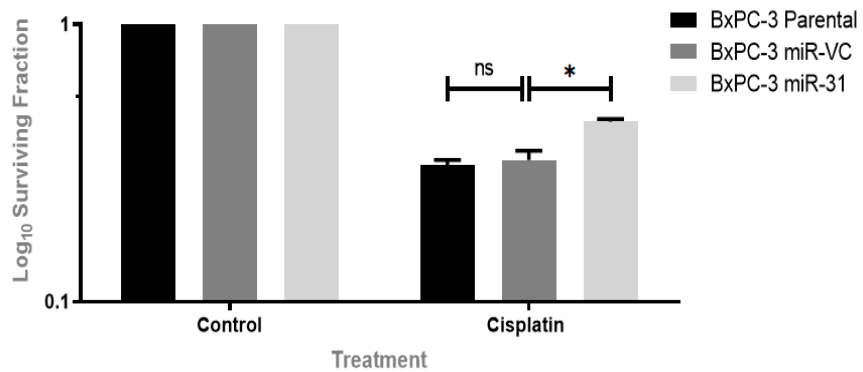


Figure 3.7 Overexpressing miR-31 in BxPC-3 cells promotes cisplatin resistance. Clonogenic analysis revealed that overexpressing miR-31 in BxPC-3 cells displayed a significant increase in the surviving fraction when compared to its miR-VC equivalent (* $p = 0.0456$). Cells were treated with the IC_{50} dose of $0.784 \mu M$ cisplatin for 24 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with PBS treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

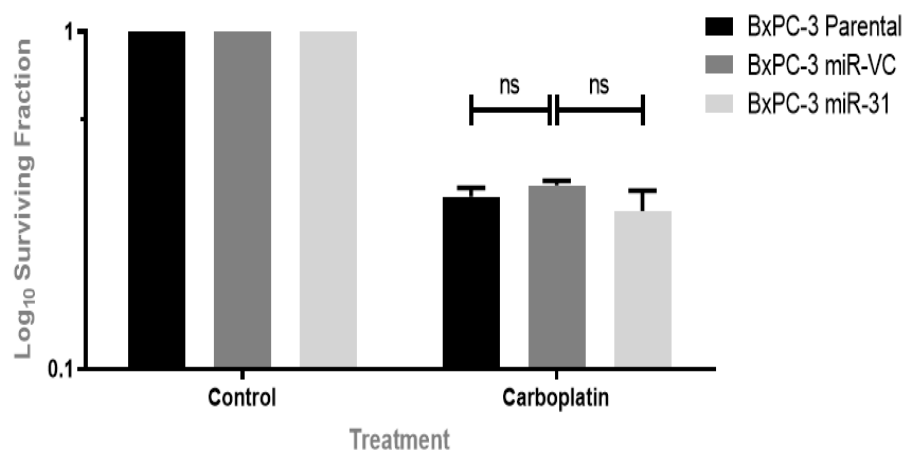


Figure 3.8 Overexpressing miR-31 in BxPC-3 cells does not modulate carboplatin resistance. Clonogenic analysis revealed that overexpressing miR-31 in BxPC-3 cells did not significantly alter the surviving fraction when compared to its miR-VC equivalent ($p = 0.390$). Cells were treated with the IC_{50} dose of $0.923 \mu M$ carboplatin for 48 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with PBS treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

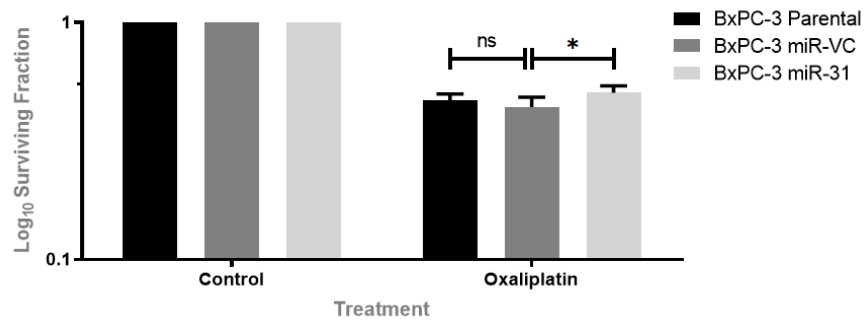


Figure 3.9 Overexpressing miR-31 in BxPC-3 cells promotes oxaliplatin resistance. Clonogenic analysis revealed that overexpressing miR-31 in BxPC-3 cells significantly increased the surviving fraction when compared to its miR-VC equivalent (* $p = 0.0165$). Cells were treated with the IC₅₀ dose of 1.45 μ M oxaliplatin for 24 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with PBS treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

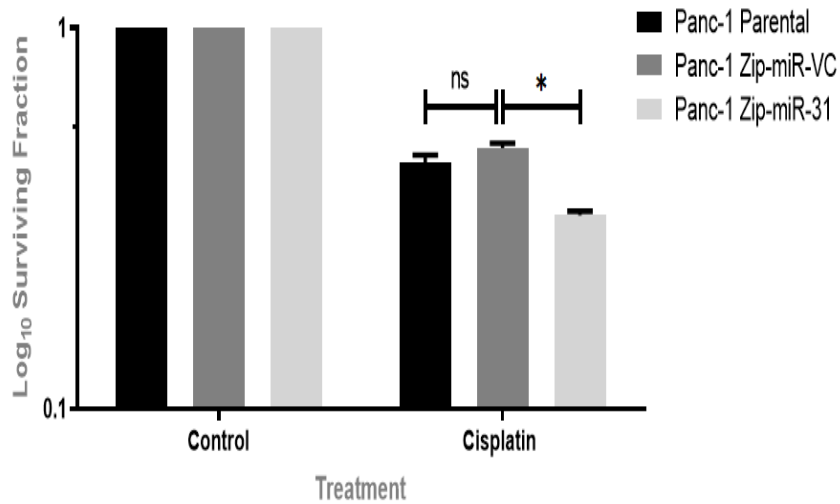


Figure 3.10 Suppressing miR-31 in Panc-1 cells enhances cisplatin sensitivity. Clonogenic analysis revealed that suppressing miR-31 in Panc-1 cells displayed a significant reduction in the surviving fraction when compared to its Zip-miR-VC equivalent (* $p = 0.0102$). Cells were treated with the IC_{50} dose of $1.38 \mu M$ cisplatin for 24 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with PBS treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

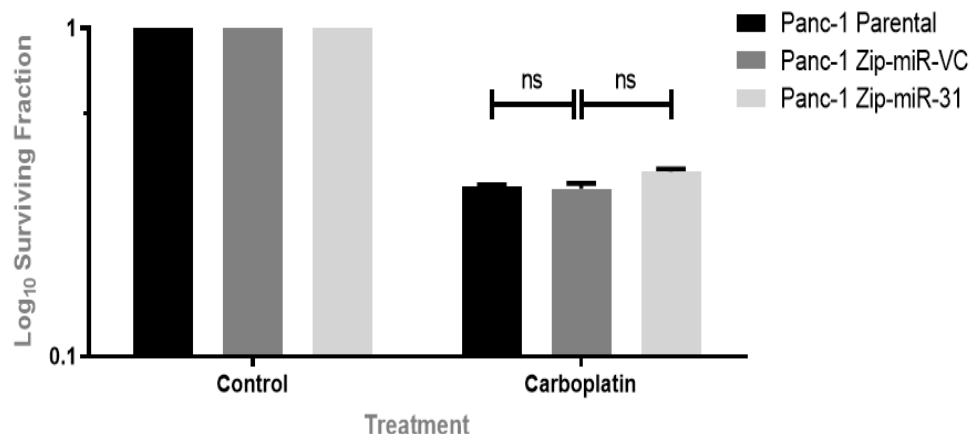


Figure 3.11 Suppressing miR-31 in Panc-1 cells does not modulate carboplatin resistance. Clonogenic analysis revealed that suppressing miR-31 in Panc-1 cells did not alter the surviving fraction when compared to its Zip-miR-VC equivalent ($p = 0.105$). Cells were treated with the IC_{50} dose of $1.80 \mu M$ carboplatin for 48 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with PBS treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

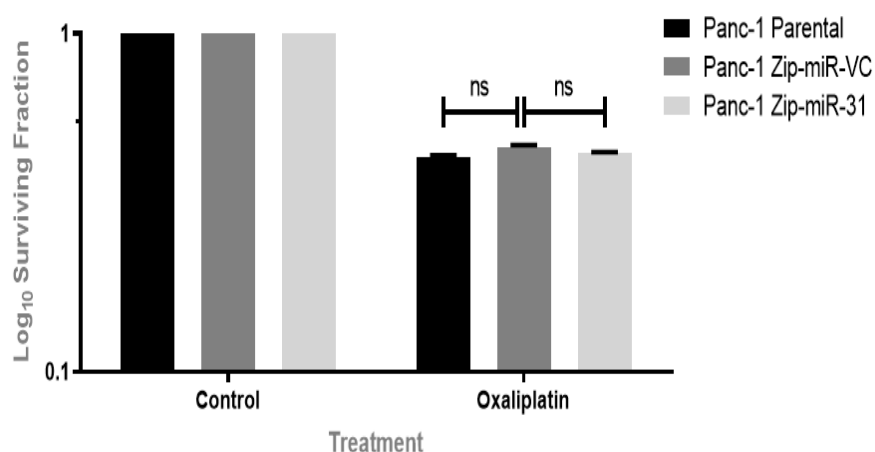


Figure 3.12 Suppressing miR-31 in Panc-1 cells does not modulate oxaliplatin resistance. Clonogenic analysis revealed that suppressing miR-31 in Panc-1 cells did not alter the surviving fraction when compared to its Zip-miR-VC equivalent ($p = 0.146$). Cells were treated with the IC_{50} dose of $1.68 \mu\text{M}$ oxaliplatin for 24 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with PBS treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

3.4.6 MiR-31 modulates cellular sensitivity to anti-metabolite agents in PDAC cell lines.

To further investigate if miR-31 modulates cellular sensitivity to anti-metabolite agents in miR-31 manipulated PDAC models, cells were treated with gemcitabine (24 h), 5-FU (24 h), or leucovorin (48 h) with 5-FU (final 24 h). It was established that overexpressing miR-31 in BxPC-3 cells significantly enhanced chemosensitivity to gemcitabine (**Figure 3.16**), 5-FU (**Figure 3.17**), and leucovorin with 5-FU (**Figure 3.18**) when compared to its miR-VC equivalent. Furthermore, it was also established that suppressing miR-31 in Panc-1 cells significantly promoted chemoresistance to 5-FU (**Figure 3.20**) and leucovorin with 5-FU (**Figure 3.21**), but not with gemcitabine (**Figure 3.19**) when compared to its Zip-miR-VC equivalent.

3.4.7 MiR-31 alters proliferation in PDAC cell lines post-cisplatin treatment.

After overexpressing miR-31 increased clonogenic survival in BxPC-3 and suppressing miR-31 decreased survival in Panc-1 cells, a cumulative cell count was undertaken first to determine whether miR-31 alone, without the influence of cisplatin, would affect proliferation. It is well established that proliferation rates can influence chemo-resistance, where cells with a slower proliferation rate are more chemo-resistant than fast-proliferating cells, so monitoring the proliferation rate indicates whether proliferation plays a role in regulating cisplatin resistance. It was found that miR-31 manipulation without the influence of cisplatin produced no significant change in the proliferation rate (**Figure 3.22**). A significant change in the proliferation of BxPC-3 miR-31 cells occurred only on day 9 (* $p = 0.0157$) post cisplatin treatment, whereas the BxPC-3 miR-VC cells were significantly affected on day 3 (* $p = 0.0268$) post cisplatin treatment. Assaying cumulative proliferation with cisplatin treatment revealed a significant reduction in proliferation only on day 9 (* $p =$

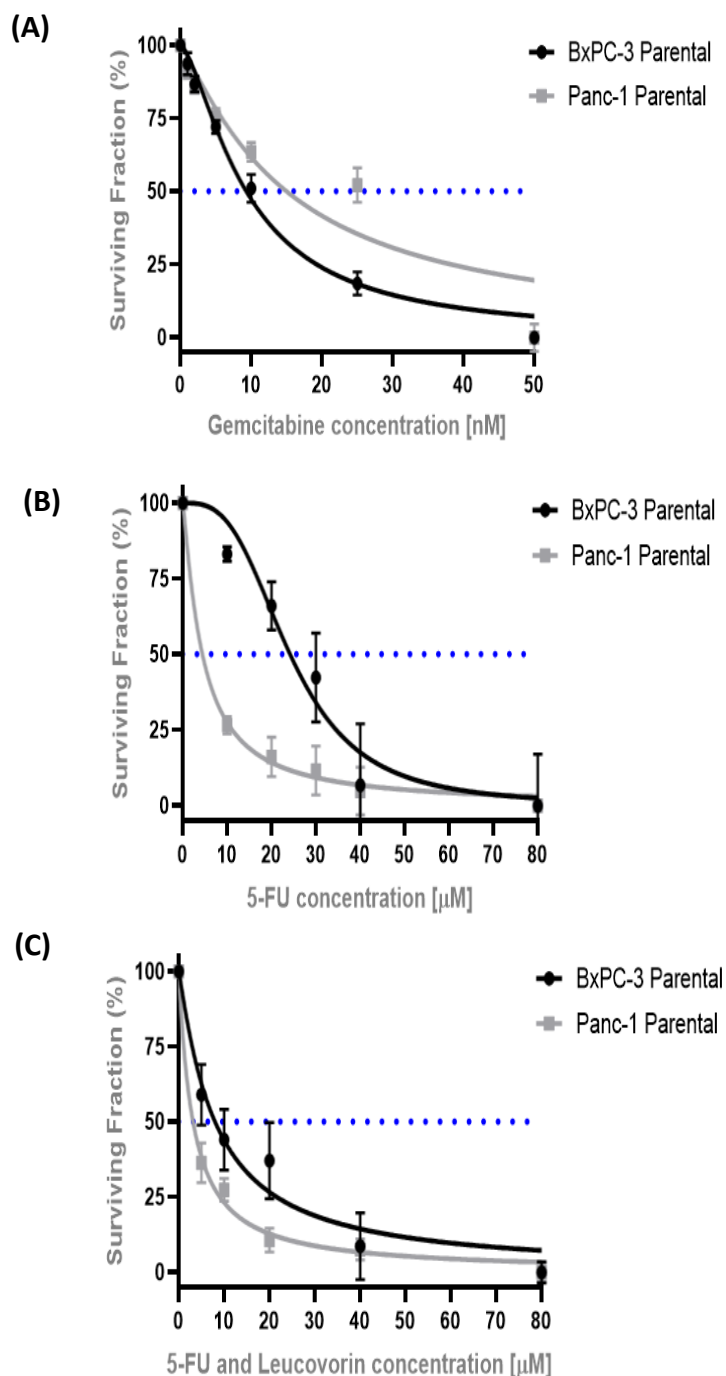


Figure 3.15 IC_{50} doses of anti-metabolite chemotherapeutic agents in PDAC cell lines. (A) Dose-response for gemcitabine treatment for 24 h in the BxPC-3 parental cell line ($IC_{50} = 9.33$ nM) and Panc-1 parental cell line ($IC_{50} = 15.0$ nM). (B) Dose-response for 5-FU treatment for 24 h in the BxPC-3 parental cell line ($IC_{50} = 24.2$ μM) and Panc-1 parental cell line ($IC_{50} = 4.44$ μM). (C) Dose-response for 5-FU treatment with 1 μM leucovorin for 24 h in the BxPC-3 parental cell line ($IC_{50} = 8.02$ μM) and Panc-1 parental cell line ($IC_{50} = 3.15$ μM). All clonogenic assays were vehicle-controlled with the analysis of PBS/DMSO treated controls considered when calculating the surviving fraction. IC_{50} doses were

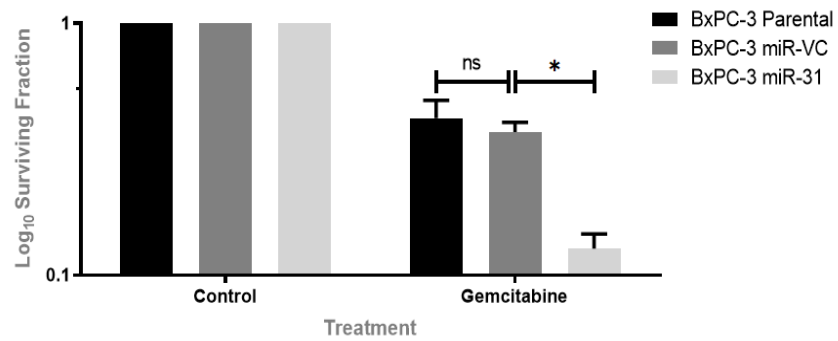


Figure 3.16 Overexpressing miR-31 in BxPC-3 cells enhances gemcitabine sensitivity. Clonogenic analysis revealed that overexpressing miR-31 in BxPC-3 cells displayed a significant reduction in the surviving fraction when compared to its miR-VC equivalent (* $p = 0.0187$). Cells were treated with the IC_{50} dose of 9.33 nM gemcitabine for 24 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with PBS treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

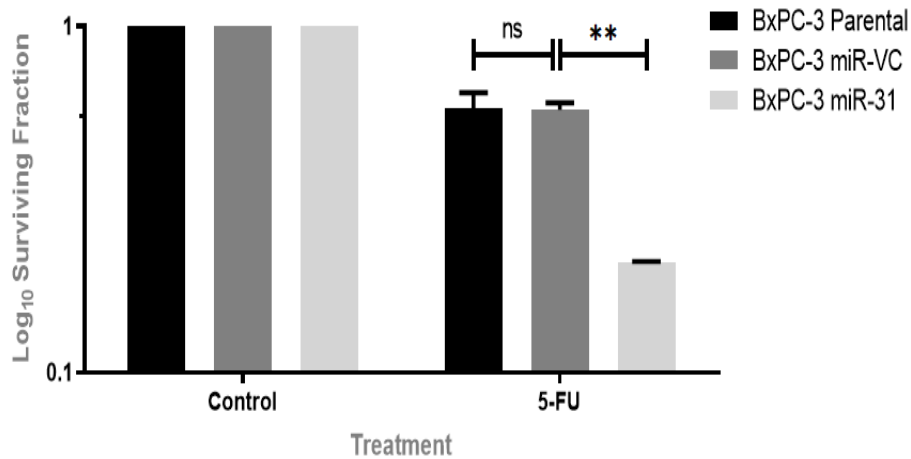


Figure 3.17 Overexpressing miR-31 in BxPC-3 cells enhances 5-FU sensitivity.

Clonogenic analysis revealed that overexpressing miR-31 in BxPC-3 cells displayed a significant reduction in the surviving fraction when compared to its miR-VC equivalent (** $p = 0.00661$). Cells were treated with the IC_{50} dose of $24.2 \mu\text{M}$ 5-FU for 24 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with DMSO treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

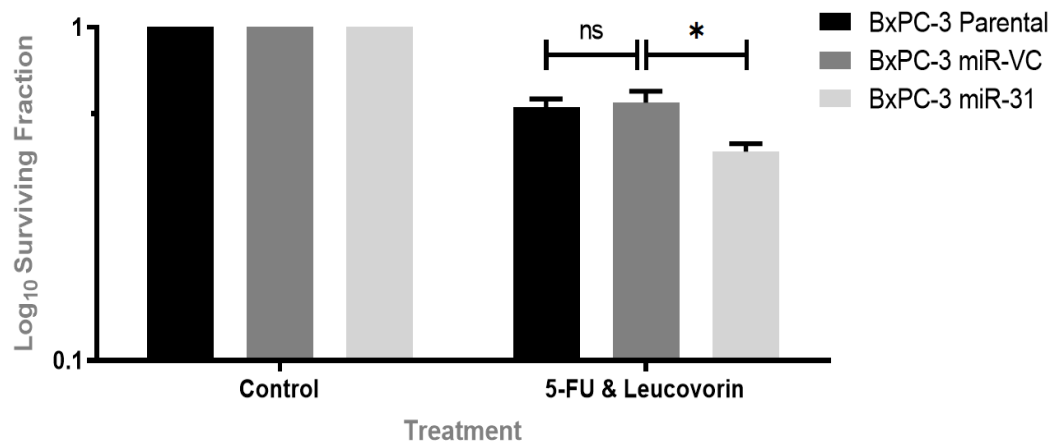


Figure 3.18 Overexpressing miR-31 in BxPC-3 cells enhances 5-FU with leucovorin sensitivity. Clonogenic analysis revealed that overexpressing miR-31 in BxPC-3 cells displayed a significant reduction in the surviving fraction when compared to its miR-VC equivalent ($*p = 0.0286$). Cells were treated 1 μ M of leucovorin in combination with the new IC_{50} dose of 8.02 μ M 5-FU for 24 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with DMSO treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

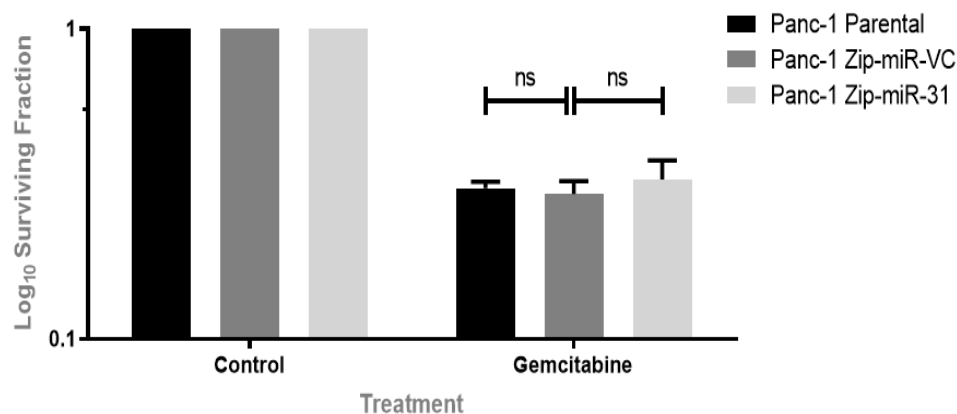


Figure 3.19 Suppressing miR-31 in Panc-1 cells does not alter gemcitabine resistance. Clonogenic analysis revealed that suppressing miR-31 in Panc-1 cells did not alter the surviving fraction when compared to its Zip-miR-VC equivalent ($p = 0.562$). Cells were treated with the IC_{50} dose of 15.0 nM gemcitabine for 24 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with PBS treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

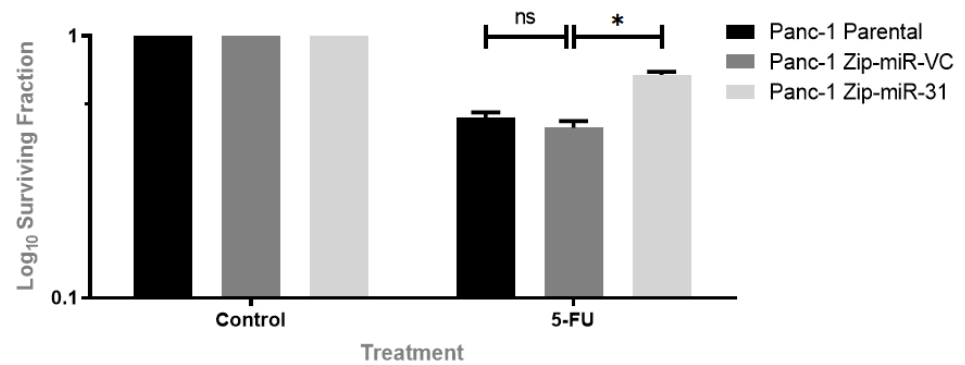


Figure 3.20 Suppressing miR-31 in Panc-1 cells promotes 5-FU resistance. Clonogenic analysis revealed that suppressing miR-31 in Panc-1 cells displayed a significant reduction in the surviving fraction when compared to its Zip-miR-VC equivalent (* $p = 0.0305$). Cells were treated with the IC_{50} dose of 4.44 μ M 5-FU for 24 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with DMSO treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

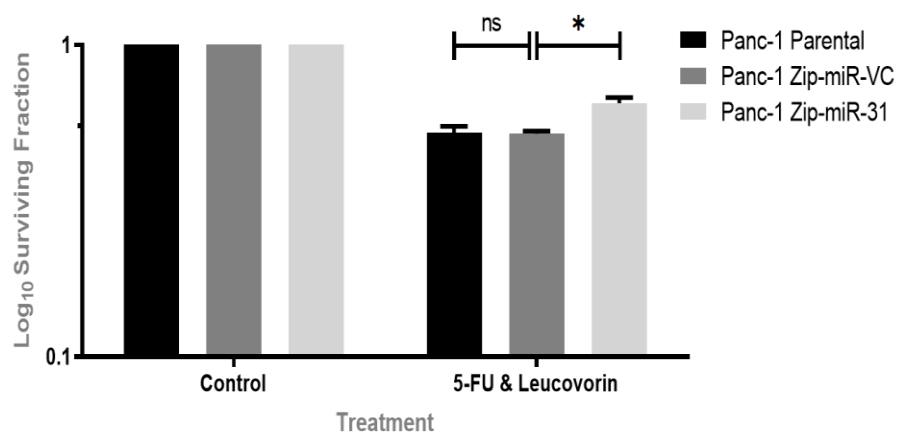


Figure 3.21 Suppressing miR-31 in Panc-1 cells promotes 5-FU with leucovorin resistance. Clonogenic analysis revealed that suppressing miR-31 in Panc-1 cells displayed a significant increase in the surviving fraction when compared to its Zip-miR-VC equivalent (* $p = 0.0456$). Cells were treated with 1 μ M of leucovorin in combination with the new IC_{50} dose of 3.15 μ M 5-FU for 24 h. All clonogenic assays were vehicle-controlled; as such, no error is associated with DMSO treatment (set to 1), with the analysis of controls considered in calculating the surviving fraction. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

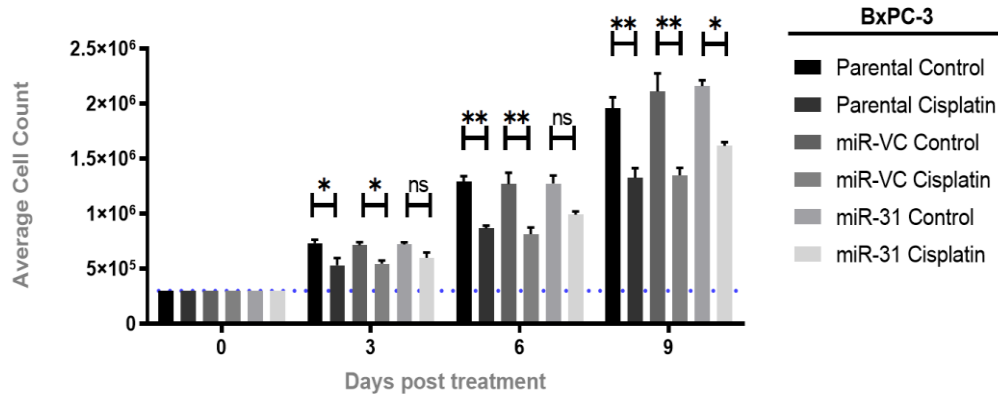


Figure 3.22 Overexpressing miR-31 in BxPC-3 cells conveys a delay in sensitivity to cisplatin. Assaying cumulative proliferation with cisplatin treatment displayed a significant reduction in cell count at all time points for parental and miR-VC cells, whereas miR-31 cells appear less sensitive to cisplatin until day 9 days post-treatment. All cells were treated with 7.84 μ M cisplatin for 24 h, and 3×10^5 cells were reseeded every third day for nine-days. At day 3 post-treatment only BxPC-3 parental cells (* $p = 0.0251$) and miR-VC cells (* $p = 0.0268$) displayed a significant reduction in cell count sooner compared to its untreated equivalent. At day 6 post-treatment, parental cells (** $p = 0.0042$) and miR-VC cells (** $p = 0.0026$). At day 9 post-treatment, parental cells (** $p = 0.0056$), miR-VC cells (** $p = 0.0012$), and miR-31 cells (* $p = 0.0157$). Day 0 results are all seeded with 3×10^5 cells, as such no error is associated. Data are presented as the mean $\pm$ SEM. One-way ANOVA with Tukey's post-hoc test was applied for statistical analysis ($n = 3$).

0.0207) in Panc-1 Zip-miR-VC cells, whereas Zip-miR-31 cells appeared more sensitive to the cisplatin treatment on day 6 (** $p = 0.0015$) (**Figure 3.23**). As assessed by a cumulative proliferation assay, miR-31 alone does not alter cell proliferation. Although, post cisplatin treatment, loss of miR-31 encouraged cell death, displayed by a reduced cell count, which can explain the differences in clonogenicity.

3.4.8 Loss of miR-31 is associated with better overall survival in patients with PDAC.

To determine whether miR-31-5p or miR-31-3p expression was correlated with predictive outcomes for patients receiving chemotherapy, expression values were related to overall survival following surgery using the Kaplan-Meier method. For both miR-31-5p (**Figure 3.24A**) and miR-31-3p (**Figure 3.24B**), higher expression displayed poor overall survival, while lower expression resulted in an improved prognostic outcome. Cox regression also confirmed a significant association with overall survival for the ascertained split-point. Patients with higher expression of miR-31-5p were around 1.82 times more likely to die than those with low expression (* $p = 0.0367$). Patients with high miR-31-3p expression were around 1.66 times more likely to die ($p = 0.095$) within the follow-up period, although no statistical significance was found.

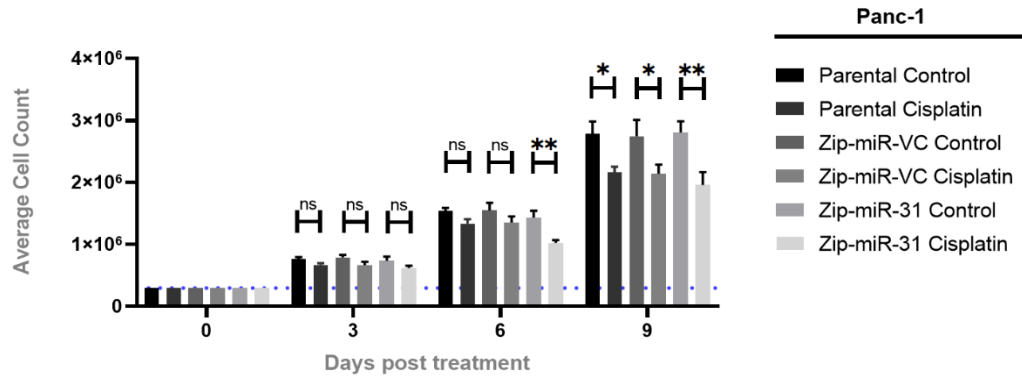


Figure 3.23 Suppressing miR-31 in Panc-1 cells reduces cell count post cisplatin treatment. Assaying cumulative proliferation with cisplatin treatment displayed a significant reduction in cell count sooner for Zip-miR-31 cells, whereas Parental and Zip-miR-VC cells appear less sensitive to cisplatin until day 9 days post-treatment. All cells were treated with 13.8 μ M cisplatin for 24 h and 3×10^5 cells were reseeded every third day for nine days. On day 6 post-treatment, there was a significant reduction in cell count for cisplatin-treated Zip-miR-31 cells only when compared to its untreated cells (** $p = 0.0015$). At day 9 post-treatment, parental cells (* $p = 0.0168$), Zip-miR-VC cells (* $p = 0.0207$), and Zip-miR-31 cells (** $p = 0.0015$). Day 0 results are all seeded with 3×10^5 cells, as such no error is associated. Data are presented as the mean $\pm$ SEM. One-way ANOVA with Tukey's post-hoc test was applied for statistical analysis ($n = 3$).

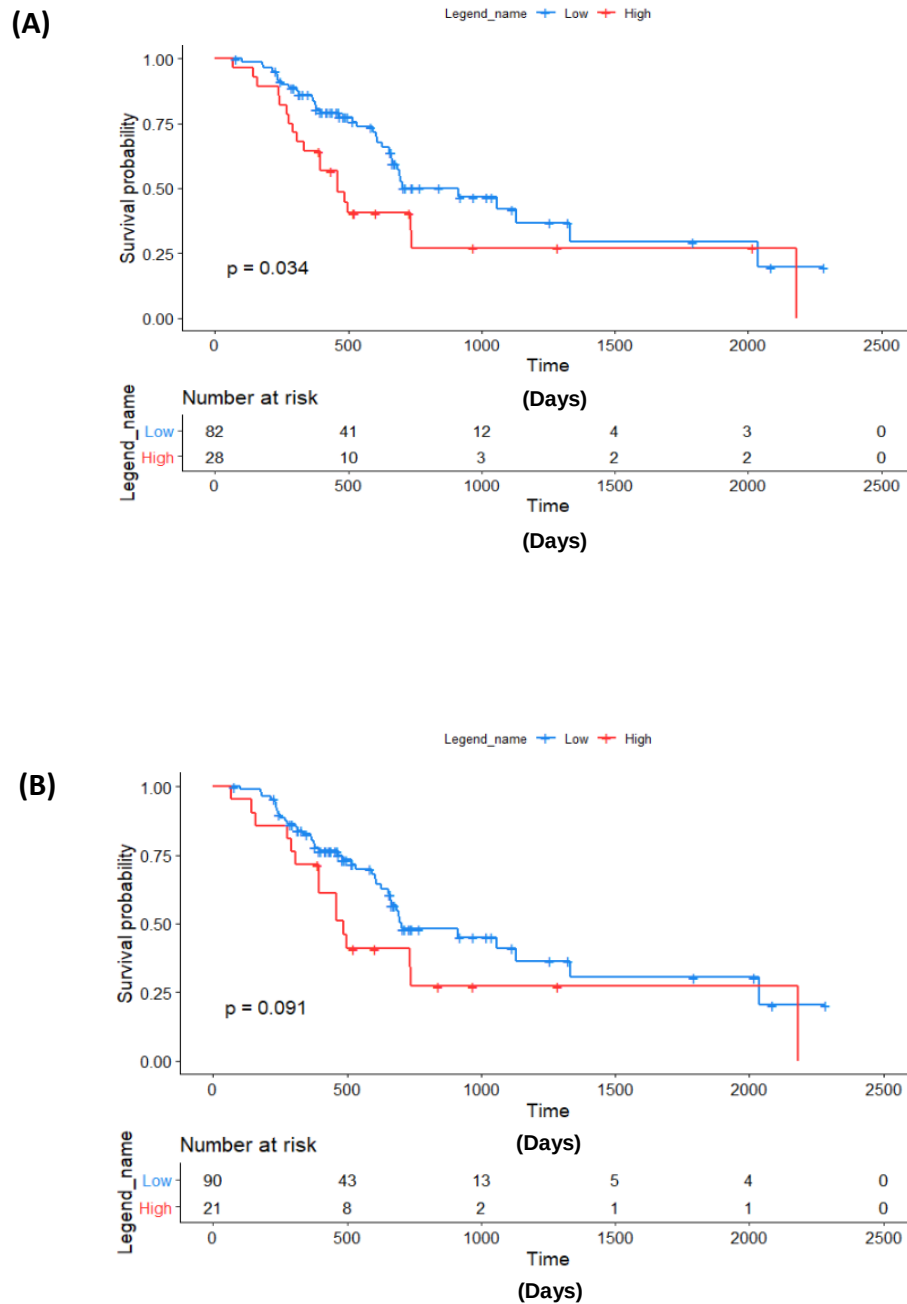


Figure 3.24 The effect of miR-31 expression on survival for patients who received chemotherapy. Kaplan Meier curves demonstrating the effect of **(A)** miR-31-5p and **(B)** miR-31-3p on survival. MiR-31 was separated into two groups: low expression vs high expression. Survival is measured in days post-surgery. Log-rank (Mantel-Cox) test was applied for statistical analysis.

3.4.9 MiR-31 modulates sensitivity to radiation treatment in PDAC cell lines.

To investigate if miR-31 modulates sensitivity to radiation treatment in PDAC cell lines, the clonogenic assay was applied with clinically relevant treatment doses, from 2 Gy to 8 Gy. Seeding densities were optimized to ensure adequate statistical power. BxPC-3 parental cells displayed a more radioresistant phenotype when compared with Panc-1 parental cells across all radiation doses, but only statistically significant at 2 Gy and 4 Gy (**Figure 3.25**). For future experiments, all cell lines were treated with 4 Gy radiation. This represented an approximate IC₅₀ dose, so it provided a representable margin above and below to determine the effect of miR-31 on cell survival. Our results show that overexpressing miR-31 into BxPC-3 cells significantly enhanced sensitivity to radiation treatment, as measured via surviving fraction (**Figure 3.26**). Reciprocally, there was a modest yet statistically significant increase in surviving fraction when suppressing miR-31 in Panc-1 cells, promoting resistance to radiation treatment (**Figure 3.27**).

The cellular viability was also assessed to support our previous observations by measuring metabolism levels. Here it was shown that overexpressing miR-31 in BxPC-3 cells significantly reduced cellular viability 24 h post-radiation treatment (**Figure 3.28**). In contrast, suppressing miR-31 in Panc-1 cells increased cellular viability (**Figure 3.29**). These results indicate that miR-31 may be a functional modulator of sensitivity to radiation treatment in PDAC cell lines.

3.4.10 MiR-31 alters proliferation in PDAC cell lines post-radiation treatment.

Following the observation that overexpressing miR-31 reduced clonogenic survival and cellular viability in BxPC-3 cells and increased survival and cellular viability in the miR-31-suppressed Panc-1 cells, a cumulative cell count was undertaken first to determine whether miR-31

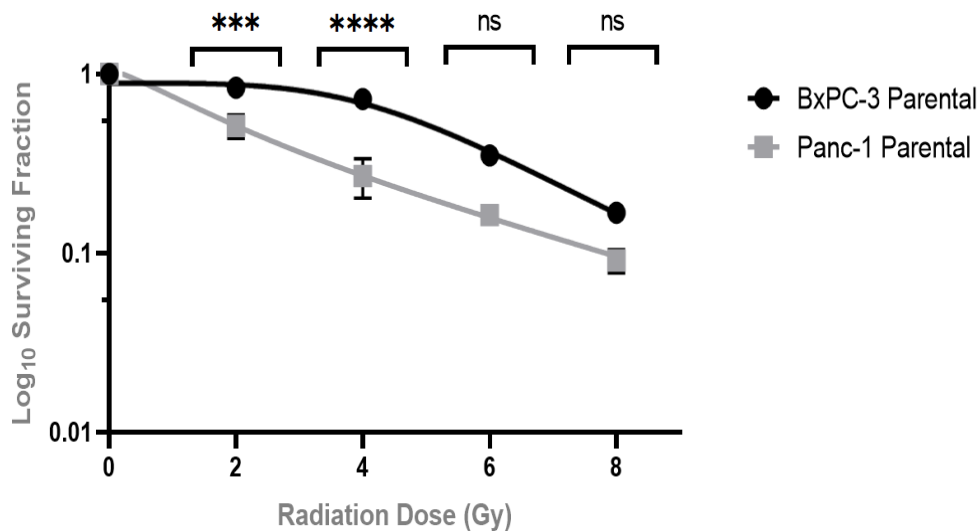


Figure 3.25 Clonogenic survival of PDAC cell lines when treated with radiation. The radiosensitivity of PDAC lines was assessed by clonogenic assay. BxPC-3 and Panc-1 parent cell lines were irradiated with 2 Gy, 4 Gy, 6 Gy, and 8 Gy. Control cells were mock-irradiated. At the end of the incubation period (8-10 days), surviving colonies were counted, and the surviving fraction was determined. The BxPC-3 cell line were more radioresistant than the Panc-1 cell line at 2 Gy and 4 Gy. Data are represented as the mean $\pm$ SEM ($n = 3$). Two-way ANOVA with Tukey's post-hoc test adopted for statistical analysis; comparing BxPC-3 cells to Panc-1 cells at 2 Gy (** $p = 0.0010$); 4 Gy (**** $p < 0.0001$); 6 Gy ($p = 0.1213$); and 8 Gy ($p = 0.9495$).

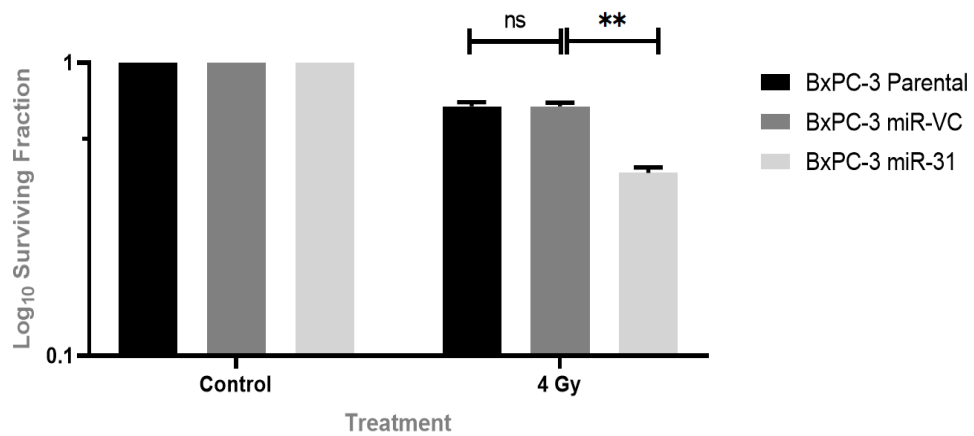


Figure 3.26 Overexpressing miR-31 in BxPC-3 cells enhances radiosensitivity.

BxPC-3 cells were stably transfected with a miR-VC or miR-31 plasmid. Cells were treated at 4 Gy at 24 h post-seeding while controls were mocked irradiated (0 Gy). Surviving colonies were counted at the end of the incubation period (8-10 days), and a clonogenic assay determined the surviving fraction. Treating at 4 Gy significantly reduced survival in miR-31 overexpressed BxPC-3 cells when compared to miR-VC it's equivalent (** $p = 0.00891$). No significant difference was observed between parental and miR-VC cells ($p = 0.913$). Data are expressed as the mean $\pm$ SEM and analysed by a two-tailed paired t-test ($n = 3$).

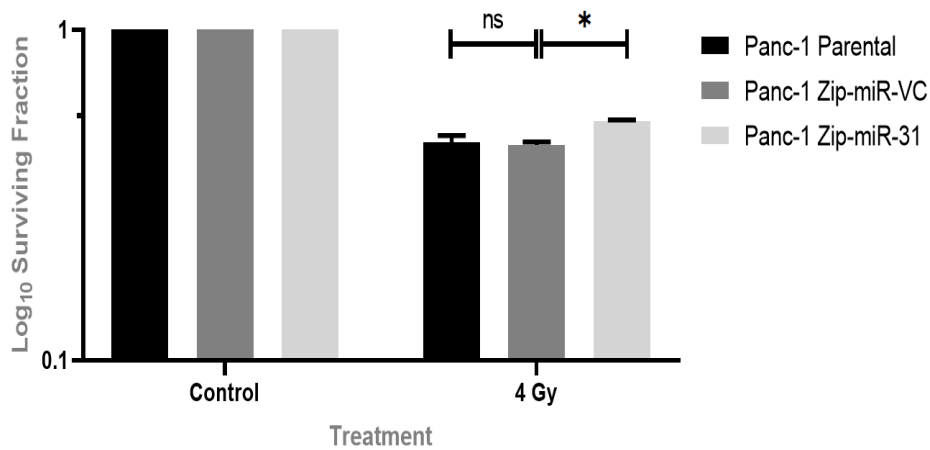


Figure 3.27 Suppressing miR-31 in Panc-1 cells promotes radioresistance. Panc-1 cells were stably transfected with either a Zip-miR-VC or Zip-miR-31 plasmid. Cells were treated at 4 Gy at 24 h post-seeding while controls were mocked irradiated (0 Gy). Surviving colonies were counted at the end of the incubation period (8-10 days), and a clonogenic assay determined the surviving fraction. Treating at 4 Gy significantly increased survival in miR-31 suppressed Panc-1 cells compared to its Zip-miR-VC equivalent (* $p = 0.0211$). No significant difference was observed between parental and Zip-miR-VC cells ($p = 0.835$). Data are expressed as the mean $\pm$ SEM and analysed by a two-tailed paired t-test ($n = 3$).

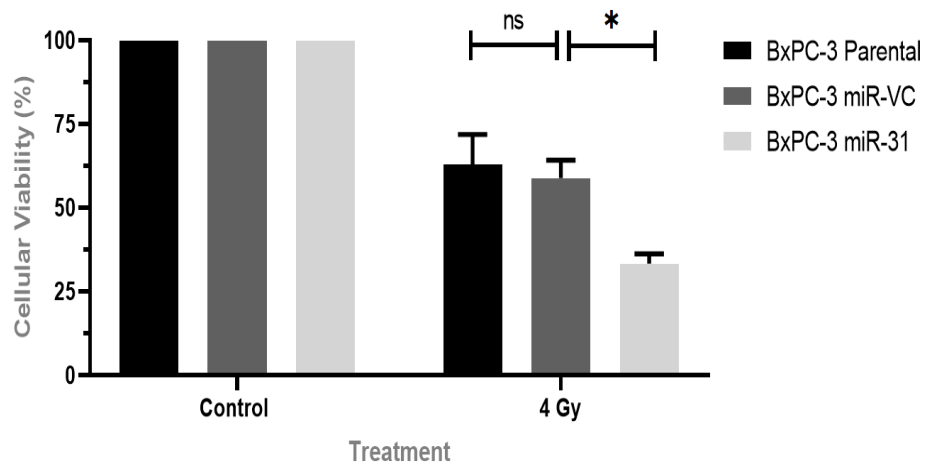


Figure 3.28 Overexpressing miR-31 in BxPC-3 cells reduces cellular viability post-radiation treatment. BxPC-3 cells were stably transfected with a miR-VC or miR-31 plasmid. Cells were treated at 4 Gy at 24 h post-seeding while controls were mocked irradiated (0 Gy). Treating at 4 Gy significantly reduced cellular viability (as measured by metabolism) in miR-31 overexpressed BxPC-3 cells compared to miR-VC equivalent (* $p = 0.0484$). No significant difference was observed between parental and miR-VC cells ($p = 0.752$). Data are expressed as the mean $\pm$ SEM and analysed by a two-tailed paired t-test ($n = 3$).

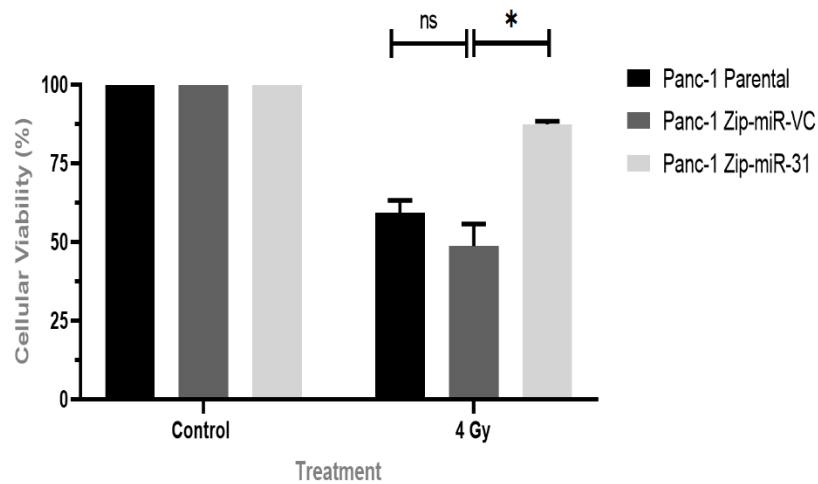


Figure 3.29 Suppressing miR-31 in Panc-1 cells increases cellular viability post-radiation treatment. Panc-1 cells were stably transfected with a miR-VC or miR-31 plasmid. Cells were treated at 4 Gy at 24 h post-seeding while controls were mocked irradiated (0 Gy). Treating at 4 Gy significantly increased cellular viability (as measured by metabolism) in miR-31 suppressed Panc-1 cells, compared to Zip-miR-VC; it's equivalent (* $p = 0.033$). No significant difference was observed between parental and Zip-miR-VC cells ($p = 0.199$). Data are expressed as the mean $\pm$ SEM and analysed by a two-tailed paired t-test ($n = 3$).

alone, without the influence of radiation, would affect proliferation. Moreover, the cumulative cell count attempted to establish when miR-31 might influence proliferation. It was demonstrated that miR-31 manipulation without the influence of radiation produced no significant change in proliferation rate; the cells only responded differently after radiation, indicating miR-31 plays an active role in the response post-radiation treatment. We observed a reduction in cell count when over-expressing miR-31 in BxPC-3 cells three days post-radiation treatment, whereas no significant difference was observed in its vector control equivalent (**Figure 3.30**). Additionally, there was an increase in cell count when suppressing miR-31 in Panc-1 cells 3- and 6-days post-radiation treatment, while no significance was found with its vector control equivalent (**Figure 3.31**).

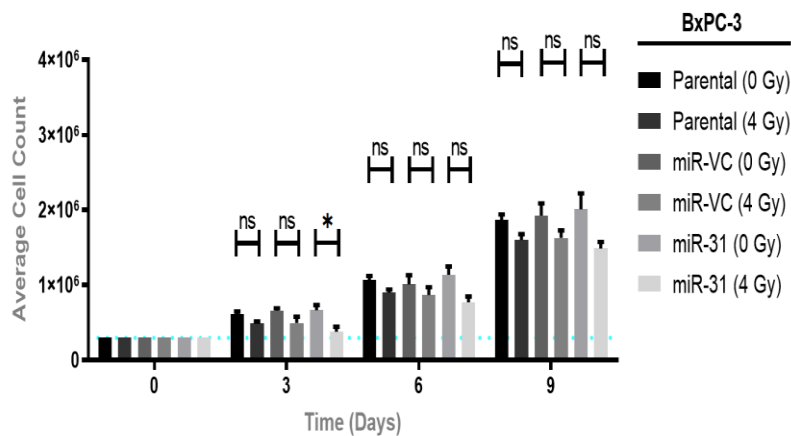


Figure 3.30 Overexpressing miR-31 alters cell proliferation post-radiation treatment. Assaying cumulative proliferation with radiation treatment revealed no significant decrease in proliferation at any time points in BxPC-3-miR-VC treated cells when compared to untreated cells. MiR-31 treated cells significantly reduced cell count at day 3 (* $p = 0.0353$) compared to untreated cells. Data are expressed as the mean $\pm$ SEM. One-way ANOVA with Tukey's post-hoc test was adopted for statistical analysis ($n = 3$).

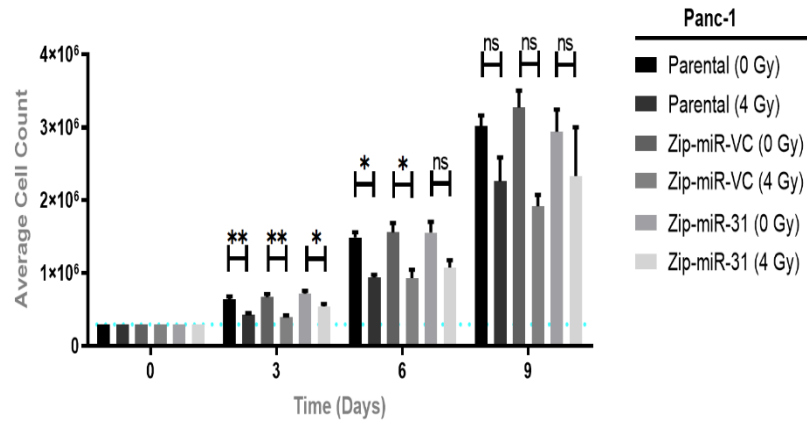


Figure 3.31 Suppressing miR-31 alters cell proliferation post-radiation treatment. Assaying cumulative proliferation with radiation treatment revealed a significant reduction in proliferation on day 3 (** $p = 0.00122$) and day 6 (* $p = 0.0120$) in Panc-1 Zip-miR-VC cells treated cells compared to untreated cells. In comparison, Zip-miR-31 cells only displayed a reduction in cell count at day three (* $p = 0.0354$) compared to untreated cells. Data are expressed as the mean $\pm$ SEM. One-way ANOVA with Tukey's post-hoc test was adopted for statistical analysis ($n = 3$).

3.5 Discussion

Research on miR-31 shows that it displays altered expression levels in various tumours. There is evidence to support oncogenic and tumour suppressive functions in cancer cells, where it has been reported that miR-31 is lost in ovarian, prostate, and oesophageal cancers [271]. In comparison, high expression of miR-31 has been found in lung cancer, colorectal cancer, and head and neck squamous cell carcinoma [271, 276]. Within PDAC, the fragile site 9p21, where miR-31 is encoded, is lost in approximately 85% of PDAC tumours [270, 271]. Consequently, it is supposed that miR-31 is lost in most PDAC tumour [277]. However, high expression of miR-31 has been reported in PDAC from multiple studies [278]. Although, in dispute with previous research, Papaconstantinou *et al.* [279] found miR-31 to be down-regulated in eighty-eight samples of PDAC. This discrepancy between miR-31 expression and PDAC tumour suggests that further research is needed to identify if loss of miR-31 in PDAC correlates to better or poor prognosis.

The data presented in this chapter showed that miR-31-expressing cells promote resistance to platinum-based chemotherapies in PDAC cell lines. However, by enhancing cellular sensitivity to treatment, miR-31-expressing cells display an opposite effect to anti-metabolic agents and radiation alone. Although, it was found that suppressing miR-31 in Panc-1 cells did not promote gemcitabine resistance. From all PDAC cell lines, Panc-1 is the most resistant to gemcitabine [280], so encouraging cells to display a more resistant phenotype when already inherently resistant presents a challenging task. Nevertheless, the differences observed in cellular sensitivity between platinum agents (e.g., cisplatin, carboplatin, or oxaliplatin) and anti-metabolites (e.g., gemcitabine or 5-fluorouracil) when manipulating miR-31 may be explained by the differences in the mechanism of action of the drugs. All platinum agents act as pro-drugs, where the chloride ligands are generally removed via hydrolysis, which is necessary before they can target DNA [84]. Subsequently, this binding

between DNA and platinum agents induces apoptosis, a form of programmed cell death. Whereas anti-metabolites function by mimicking the molecules that a cell needs to survive. Gemcitabine is a broad-spectrum agent and displays different mechanisms of action depending on its phosphorylation state [281]. 5-FU, phosphorylated, can inhibit thymidylate synthase, resulting in an imbalanced pool of deoxythymidine triphosphate required for DNA synthesis [282]. Furthermore, the primary effect of radiation treatment harming cells is directly affecting DNA or indirectly by producing reactive oxygen species derived from the ionisation of the water component of the cells [283, 284].

Because miR-31 can alter hundreds of genes simultaneously and due to the differences in the mechanism of action from platinum agents, anti-metabolites, and radiation treatment, it is not surprising that opposing effects are observed in cellular sensitivity. Manipulating miR-31 in PDAC cell lines may alter gene expression of a particular pathway specific to platinum agents only and has no significance or plays antagonistic roles with anti-metabolite or radiation treatment. Interestingly, a recent study that generated an isogenic model of OE33 Cis P (cisplatin-sensitive) and OE33 Cis R (cisplatin-resistant) cells found OE33 Cis R cells to be more sensitive to 5-FU and radiation treatment when compared to OE33 P cells [285]—supporting our observations where miR-31 expressing cells promote cisplatin resistance but enhance sensitivity to 5-FU and radiation treatment. Furthermore, Hummels *et al.* [286] found two miRNAs from an 848-miRNA panel, miR-31 and miR-125a, to have altered expression in both cisplatin-resistant and 5-FU-resistance oesophageal cells. Suggesting that miR-31 expression plays a role in modulating chemotherapy in various cancer types, but it appears to be chemotherapy specific, where both a chemo-sensitive and chemo-resistant phenotype can be expected depending on the choice of drug.

There are differences between cisplatin and carboplatin pharmacology and pharmacokinetics [287, 288]. Cisplatin is a smaller compound

considered more potent than carboplatin. Additionally, carboplatin is thought to be more dependent upon passive diffusion across the cell membrane, implying that membrane transporters are not required for the cellular intake of carboplatin [287, 288]. Because no significant differences in the survival fraction were observed in the miR-31 manipulated models when treated with carboplatin, this may suggest that miR-31 alters the trafficking of cisplatin across the cell membrane or affects how cisplatin is activated and sequestered once inside the cell. Interestingly, only miR-31 overexpressed cells displayed significance in oxaliplatin sensitivity. Oxaliplatin is a larger compound than cisplatin yet smaller than carboplatin, and oxaliplatin has been demonstrated to be more potent than carboplatin but not cisplatin [289]. Like cisplatin, oxaliplatin also relies on similar modes of transport across the cell membrane [289, 290]. Furthermore, our findings show that miR-31-expressing cells display cisplatin sensitivity. However, it just takes a more extended period, indicating the potential miR-31 has on altering the availability of cisplatin to the nucleus, where it is needed to target DNA and induce cell death, rather than the need to enhance the DNA damage and repair system.

Supporting our findings, Moody *et al.* [274] revealed that overexpressing miR-31 also promoted cisplatin resistance in malignant pleural mesothelioma cells. In another study, Samuel *et al.* [291] found that overexpressing miR-31 led to increased cisplatin resistance in ovarian cancer cell lines. Although, miR-31 was displayed to weaken the cisplatin resistance in medulloblastoma cell lines [292]. Additionally, Chen *et al.* [293] demonstrated that upregulating miR-31 in hepatocellular carcinoma cell lines reduced chemoresistance to cisplatin. Nevertheless, miR-31 can modulate cisplatin sensitivity, but if a resistant or sensitive phenotype is observed seems to depend on the cancer type.

So far, no reliable target molecules exist to predict or influence the efficacy of gemcitabine in PDAC. One recent study proposed that miRNAs

play an essential role in gemcitabine resistance in PDAC cells [228]. After generating stable gemcitabine-resistant variants of PDAC cell lines, a miRNA screening revealed miR-31 (among other miRNAs) was significantly downregulated in gemcitabine-resistant cells [228]. The data here show that loss of miR-31 is also associated with gemcitabine resistance, alluding to the importance of miR-31 modulating gemcitabine sensitivity in PDAC cell lines.

5-FU signifies the chemotherapeutic backbone for numerous cancer types' neoadjuvant, adjuvant, and palliative treatment [294]. Although 5-FU is still considered inferior to gemcitabine treatment, tumour resistance remains a significant challenge [107]. To our knowledge, it was established that miR-31 enhances 5-FU sensitivity in PDAC cell lines for the first time. Comparably, Korourian *et al.* [295] demonstrated that the induction of miR-31 expression increased 5-FU sensitivity in gastric adenocarcinoma cell lines. Interestingly, like cisplatin treatment, miR-31 expression can reverse 5-FU resistance in other cancers. Nakagawa *et al.* [296] and Li *et al.* [297] revealed that the increased miR-31 expression was linked to 5-FU resistance.

Moreover, the ability to use a lower dosage of chemotherapy but still display a large amount of cell kill would be ideal for patients as it would reduce side effects. Leucovorin is a potentiator of 5-FU efficacy and depicts a synergistic relationship [298]. This is achieved by increasing the rate of thymidylate synthase inhibition. Here it was shown that pre-treating miR-31 expressing cells with leucovorin, and a lower dose of 5-FU, achieved the same amount of cell death compared to a higher dose of 5-FU only. Further investigation is needed to know how miR-31 enhances 5-FU toxicity, but our findings suggest that miR-31 encourages 5-FU sensitivity the same way, at least to some degree, as leucovorin does with 5-FU.

Approximately 10-30% of patients with PDAC will receive radiation therapy as part of their treatment plan, although radioresistance remains

a problem [65, 70]. Here it was revealed that overexpressing miR-31 enhances radiosensitivity while suppressing miR-31 promotes radioresistance in PDAC cell lines. Interestingly, Wen *et al.* [299] displayed similar results in hepatocellular carcinoma cell lines, where miR-31 knockdown desensitised cells to radiation, whereas miR-31 upregulation gave the opposite effect. In addition, Korner *et al.* [300] demonstrated that mimicking miR-31 expression in breast cancer cell lines enhanced cellular sensitivity to radiation treatment. Moreover, a recent study revealed that the ectopic re-expression of miR-31 significantly re-sensitised radioresistant oesophageal cells to radiation [275].

Platinum agents, including cisplatin and oxaliplatin, have been widely shown to enhance radiosensitivity [301]. However, from the findings here, depending on a tumour's miR-31 status, this combination therapy may offer no benefit compared to a standalone treatment. Additionally, it is well established that 5-FU and gemcitabine behave as radiosensitisers. If the miR-31 expression profile of a tumour is known, it could be beneficial to improve the efficacy of treatment. However, using conventional chemotherapies as a radio-sensitisation may lead to enhanced toxicity and adverse side effects in patients [302]. Therefore, a chemotherapeutic-free-radiosensitiser would be of significant interest, and miR-31 could present as a novel target for modulating anti-cancer therapies, especially in PDAC.

The ability to induce DNA damage is what all platinum agents, anti-metabolites, and radiation treatment have in common. Thus, assessing levels of DNA damage induction post-treatment would be helpful to underpin the mechanisms behind resistance due to miR-31 manipulation in PDAC cell lines. Suppose any discrepancies in DNA damage induction were observed between therapy-resistant cells versus therapy-sensitive cells due to miR-31 manipulation. In that case, this implies that miR-31 alters a pathway specific to platinum-based agents or vice versa. This may include the transport across the cell membrane into the cell, drug

sequestration, or the trafficking to the nucleus where it is needed to induce DNA damage and, consequently, cell death. The next chapter will explore the relationship between miR-31 and how it might modulate platinum-based chemotherapeutics.

Chapter Four

Part I

MiR-31 alters the drug trafficking of
chemotherapeutics in PDAC.

4.1 Introduction

Anticancer drug resistance in cancer cells is a complex phenomenon, and unfortunately, many patients will likely develop resistance and respond poorly to treatment [72-76]. Therefore, a better understanding of drug resistance mechanisms will likely improve drug efficacy and therapeutic strategies in oncology. MicroRNAs regulate numerous protein-coding genes, including essential genes in cancer, particularly in cancer drug resistance [188, 189]. MiR-31 has significantly regulated genes that potentiate drug resistance in various cancer types [274, 292, 297]. In the previous chapter, it was displayed that there was a correlation between miR-31 overexpression and increased therapeutic resistance to platinum-based agents. Although, miR-31 overexpression enhanced cellular sensitivity to anti-metabolites and radiation treatment independently. Pathways that respond to all platinum-based agents, anti-metabolites, and radiotherapy include nucleotide excision repair (NER) and base excision repair (BER) pathways, which promote DNA damage repair [90]. Because miR-31 displayed a paradoxical effect by promoting resistance to platinum-based agents but enhanced sensitivity to anti-metabolite agents and radiation, miR-31 is likely to be altering a pathway specific to platinum-based agents where changes to the cellular drug metabolism and drug transportation may well be contributing to the resistance observed.

Cisplatin, a generally used chemotherapeutic agent for solid tumours, is an effective single agent or can be used in combination with other drugs, including gemcitabine, to treat PDAC [304]. However, due to tumour resistance, many patients with PDAC will respond poorly to cisplatin and succumb to their disease [74]. Consequently, understanding the mechanisms associated with the chemo-resistance of PDAC cells is essential and can redefine the use of cisplatin in PDAC chemotherapy. Resistance to cisplatin has generally been attributed to altered DNA repair, altered accumulation, and drug detoxification [304]. Perhaps the

most studied resistance model involves drug transport across the plasma membrane [304, 305]. Recent evidence suggests that cisplatin enters cells by active or passive transport [306], and it is well established that the copper transporter CTR1 plays a role as the primary facilitator of cisplatin influx into the intracellular environment [307]. CTR1 has previously displayed clinical relevance since high expression was associated with an excellent therapeutic response [308]. At the same time, low levels resulted in poor therapeutic outcomes in patients with ovarian cancer [309]. Deletion of CTR1 was reported to reduce cisplatin accumulation and increase resistance *in vitro* and *in vivo* [310]. Additionally, the proteins primarily responsible for the efflux of cisplatin are the copper transporters ATP7A and ATP7B, which are found in the secretory pathway (e.g., Golgi body) and can also be located on the plasma membrane [311]. Studies have associated ATP7A and ATP7B with cisplatin resistance, where cells lacking these efflux transporters were markedly more sensitive to cisplatin chemotherapy [311, 312].

Once in the cell, cisplatin can be detoxified by the antioxidant glutathione (GSH) and has been strongly linked to cisplatin resistance [87-89]. It is well established that lysosomes play a vital role in detoxifying and sequestering drugs, including cisplatin [313]. Generally, lysosomes have a low pH and are essential for capturing and neutralizing toxins, drugs, and heavy metals [314]. The ability of lysosomes to sequester drugs has been reported in various cancer cell lines [313, 315]. Additionally, a known lysosomal-bound drug transporter, ABCB9, has been linked to modulating resistance [316].

The role of cisplatin transport in PDAC remains poorly understood. Therefore, this chapter explores the potential mechanisms of why miR-31 promotes cisplatin resistance.

4.2 Rationale, Aims, and Objectives

PDAC cells expressing miR-31 display increased resistance to platinum-based chemotherapeutic treatment. It has been previously demonstrated that miRNAs alter numerous resistance-associated pathways, including modulation of drug sequestration and transportation. We hypothesized that miR-31 might regulate how cisplatin is sequestered and transported within PDAC cell lines. Therefore, the ability of miR-31 to control drug trafficking within the cellular environment was investigated.

The objectives of this chapter were (1) to explore the effect of the miR-31 expression on the levels of cisplatin uptake in PDAC cells and (2) to identify potential molecular mechanisms underpinning any miR-31 mediated alterations in cisplatin transport in PDAC cells.

4.3 Experimental design

Assessing the concentration of platinum within PDAC cells

To analyse cisplatin uptake in PDAC cells, platinum content was evaluated and quantified using ICP-MS.

Analysis of copper transporters

To establish whether influx, efflux, and sequestration/transporter proteins were involved in the chemo-resistant phenotype demonstrated within the miR-31 manipulated PDAC models. Western blot was adopted to assess protein expression.

Analysis of lysosomal pH

Fluorescence microscopy was utilized to evaluate lysosomal acidity to determine if alterations in lysosomal pH modulated chemoresistance in PDAC cells.

Isolation of the lysosomal compartment

The investigation of the platinum content within lysosomes was approached using a spin-column-based technology that is simple, rapid, and efficient. The number of starting cells required is much smaller than that of traditional methods. This method can significantly enrich cultured-cell lysosomes without using a Dounce homogenizer and ultracentrifugation. Although fractionation has long been utilized to separate lysosomes, this technique does not delineate between lysosomes and other membrane-bound organelles, including peroxisomes and mitochondria, meaning that the analysis of the fraction is misleading.

4.4 Results

4.4.1 *Manipulating miR-31 alters the intracellular accumulation of cisplatin in PDAC cell lines.*

One of the main challenges of cisplatin therapy in cancer is the uptake of the chemotherapeutic agent across the plasma membrane into the cell. Therefore, the concentration of Pt₁₉₅ (considered the most abundant isotope of platinum) was assessed in the miR-31 manipulated BxPC-3 and Panc-1 cell lines to establish a potential mechanism underpinning miR-31-mediated cisplatin resistance. No differences in Pt₁₉₅ levels were found in the supernatants of cisplatin-treated cells (**Figure 4.1**). Interestingly, the intracellular level of Pt₁₉₅ was increased but only approached statistical significance in BxPC-3 miR-31 cells compared to its miR-VC equivalent (**Figure 4.2A**). A reduction in Pt₁₉₅ but no statistically significant difference was observed intracellularly in Panc-1 Zip-miR-31 cells compared to its Zip-miR-VC equivalent (**Figure 4.2B**). These results suggest that despite miR-31 promoting cisplatin resistance, the resistant population of cells surprisingly had a greater concentration of cisplatin within them; however, no statistical significance was observed. The assimilation of cisplatin into the cells was thought to occur by passive diffusion; however recent studies reveal the significant role of specific membrane transporters, such as the copper transporter CTR1 for the uptake and ATP7A/B for the export of platinum-based drugs, including cisplatin. Moreover, previous studies have associated the importance of these influx and efflux pumps in chemo-resistance in various cancer types. However, there were no statistically significant differences in the expression of CTR1 in either miR-31 manipulated BxPC-3 or Panc-1 cell lines (**Figure 4.3**). Additionally, no statistically significant differences were displayed in ATP7A/ATP7B expression (**Figures 4.4 & 4.5**). Although, there appeared to be a trend toward increased expression of ATP7B in BxPC-3 miR-31 cells ($p = 0.0876$) and decreased expression of ATP7B in Panc-1 Zip-miR-31 cells ($p = 0.0873$)

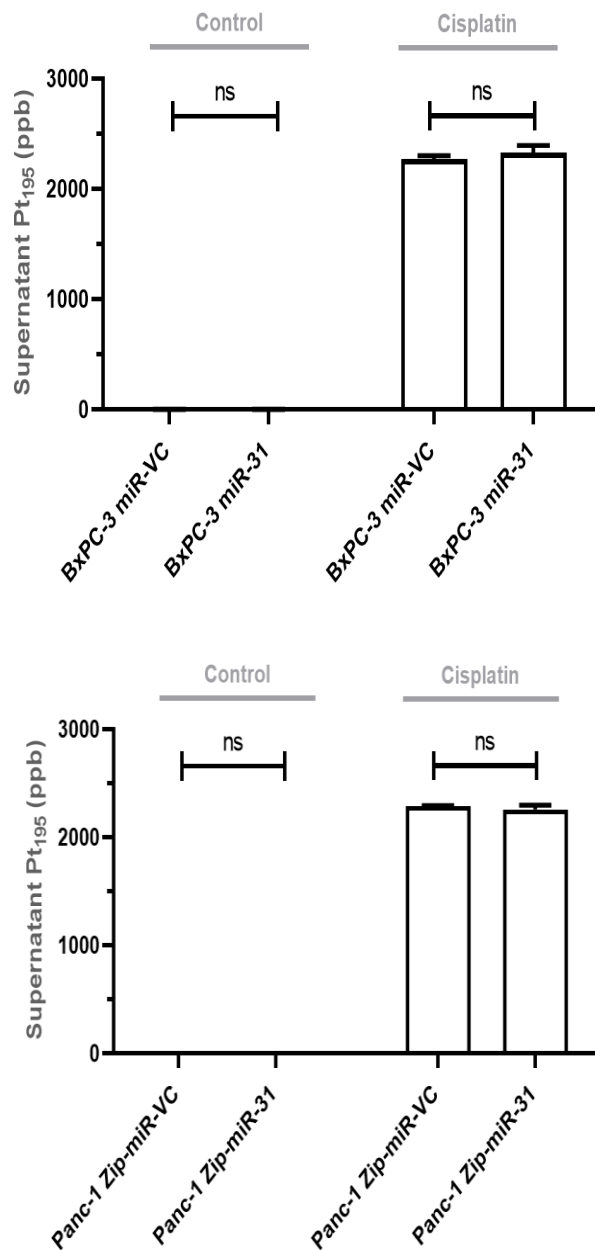


Figure 4.1 Supernatant cisplatin content is unaltered with miR-31 manipulation in PDAC cell lines. ICP-MS analysis of supernatant from cells treated with 50 μ M cisplatin for 24 h. **(A)** No significant changes in platinum (Pt₁₉₅) were observed in the supernatants of BxPC-3 miR-31 cells compared to the miR-VC equivalent. **(B)** No significant differences in platinum (Pt₁₉₅) were observed in the supernatants of Panc-1 Zip-miR-31 cells compared to the Zip-miR-VC equivalent. Data presented as the $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

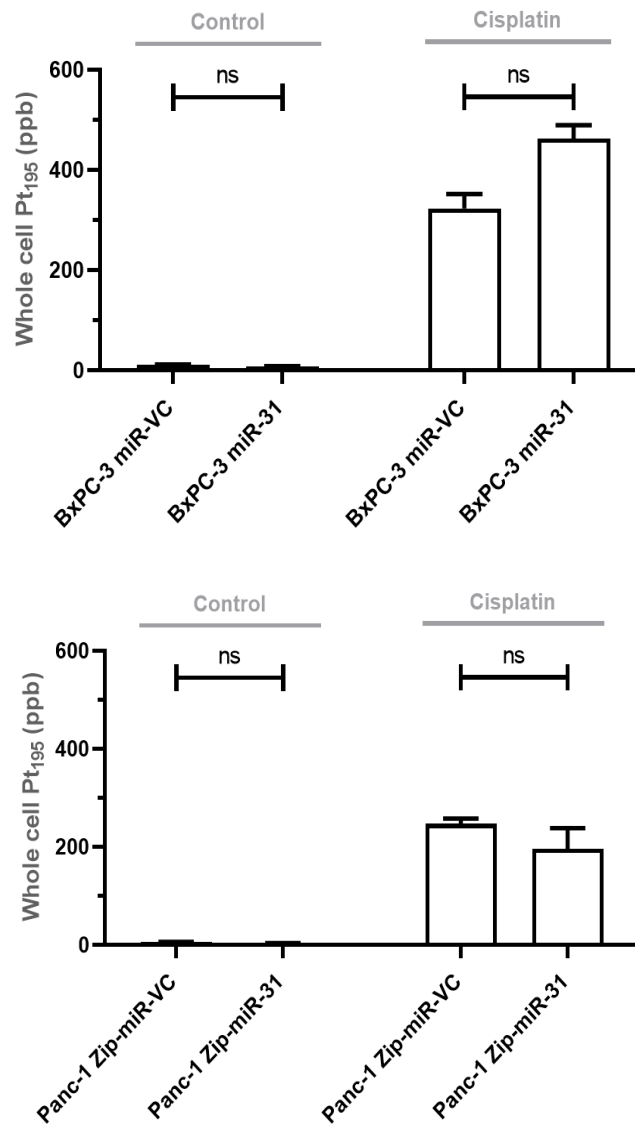


Figure 4.2 Intracellular cisplatin content is unaltered with miR-31 manipulation in PDAC cell lines. ICP-MS analysis of cells treated with 50 μ M cisplatin for 24 h. **(A)** A trend displayed increased platinum (Pt₁₉₅) levels in BxPC-3 miR-31 cells compared to the miR-VC equivalent, although no significant differences were observed. **(B)** There is a trend toward decreased levels of Pt₁₉₅ in Panc-1 Zip-miR-31 cells compared to the Zip-miR-VC equivalent, although no significant differences were observed. Data presented as the $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n=3$).

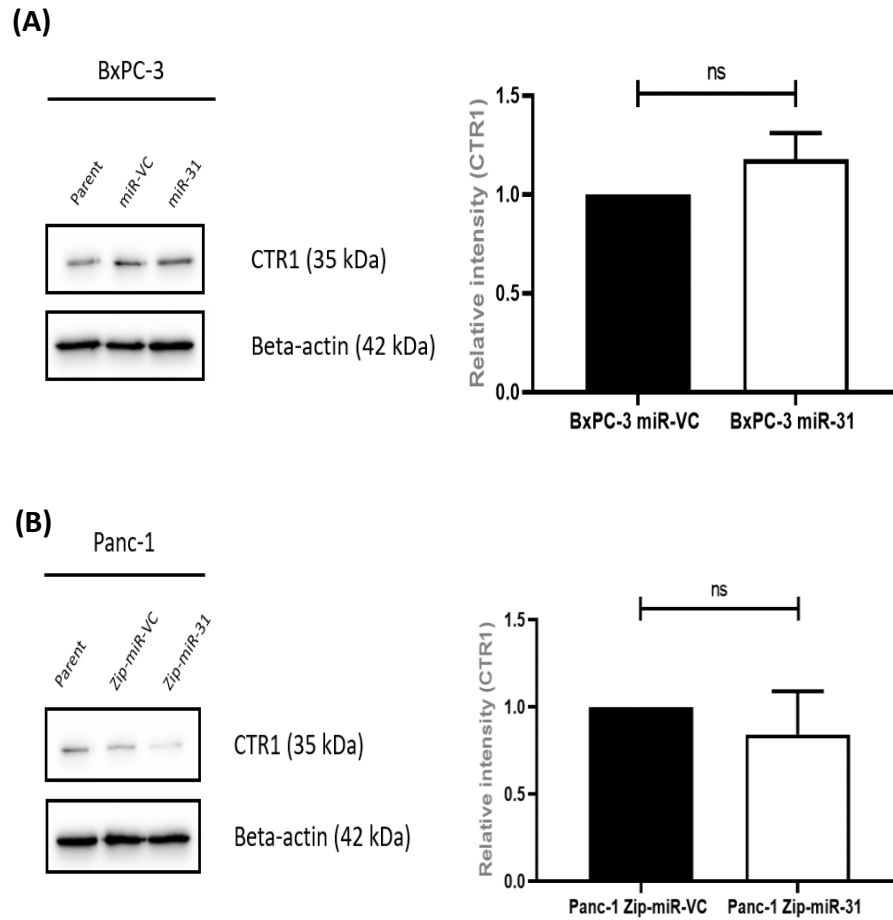


Figure 4.3 Manipulating miR-31 in PDAC cell lines does not significantly alter the expression of drug influx transporter CTR1. Representative western blot illustrates the drug influx transporter copper transporter 1 (CTR1) expression to be **(A)** not altered by miR-31 overexpression in BxPC-3 cells, and **(B)** not altered by miR-31 suppression in Panc-1 cells. Densitometry analysis was applied displaying the relative intensity of CTR1 expression. Data are presented as the mean $\pm$ SEM. BxPC-3 miR-VC/Panc-1 Zip-miR-VC is utilised as the relative control and is set to 1, as such no error is associated. A one-sampled t.test was applied for statistical analysis ($n=3$).

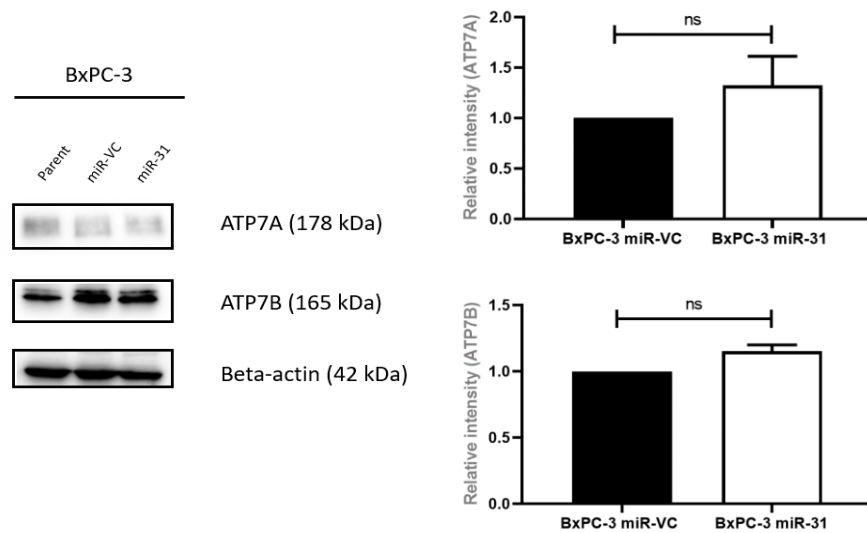


Figure 4.4 Manipulating miR-31 in BxPC-3 cells does not significantly alter the expression of drug efflux transporters ATP7A and ATP7B. Representative western blot illustrates no alterations between the drug efflux transporters ATP7A and ATP7B by miR-31 overexpression in BxPC-3 cells. Densitometry analysis was applied displaying the relative intensity of ATP7A and ATP7B expression. Data are presented as the mean $\pm$ SEM. BxPC-3 miR-VC is utilised as the relative control and is set to 1, as such no error is associated. A one-sampled t.test was applied for statistical analysis ($n = 3$).

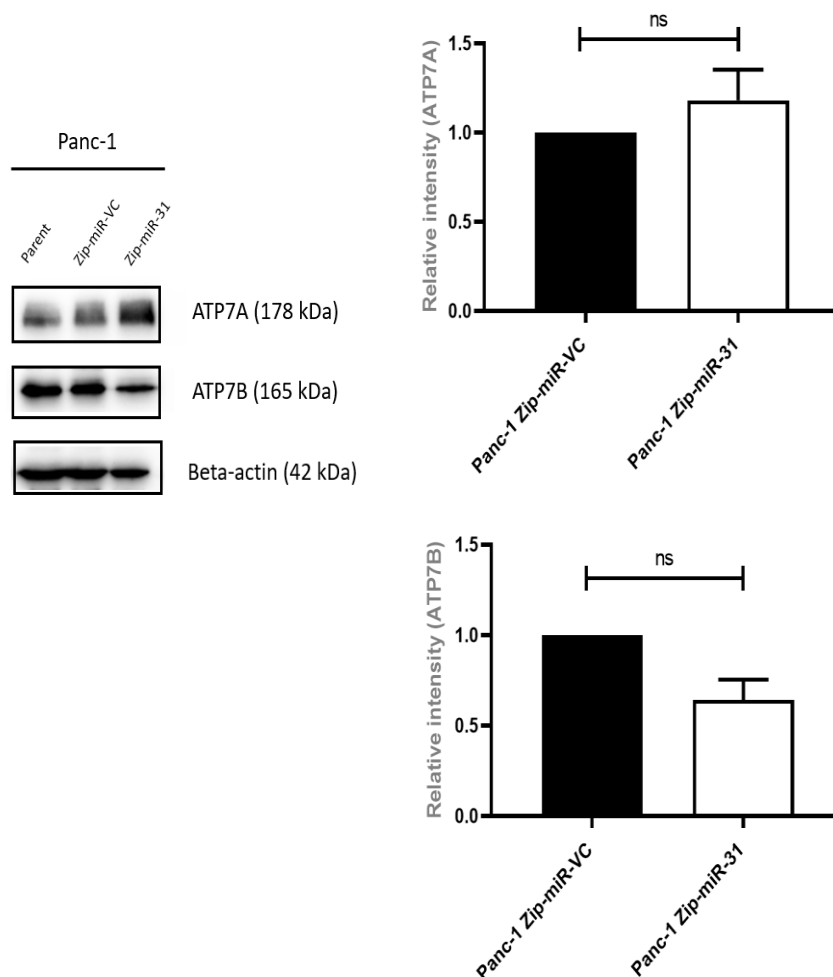


Figure 4.5 Manipulating miR-31 in Panc-1 cells does not significantly alter the expression of drug efflux transporters ATP7A and ATP7B. Representative western blot illustrates no alterations between the drug efflux transporters ATP7A and ATP7B by miR-31 suppression in Panc-1 cells. Densitometry analysis was applied displaying the relative intensity of ATP7A and ATP7B expression. Data are presented as the mean $\pm$ SEM. Panc-1 Zip-miR-VC is utilised as the relative control and is set to 1, as such no error is associated. A one-sampled t.test was applied for statistical analysis ($n = 3$).

could potentially promote cisplatin resistance in BxPC-3 cells and enhance cisplatin sensitivity in Panc-1 cells. Still, it does not explain the modest increased levels (yet not significant) of Pt₁₉₅ found in the more resistant cells. To this end, Pt₁₉₅ levels within the cytoplasmic and nuclear compartments were assessed independently post-cisplatin treatment.

4.4.2 Manipulating miR-31 alters the nuclear accumulation of cisplatin in PDAC cell lines.

Further investigation was needed to resolve how miR-31 expressing cells (BxPC-3 miR-31 & Panc-1 Zip-miR-VC cells) displayed cisplatin resistance in defiance of a modest increase (but not significant) in the levels of intracellular cisplatin as measured by Pt₁₉₅. Therefore, both the cytoplasmic and nuclear compartments were separated from cells, and ICP-MS post-cisplatin-treatment measured levels of Pt₁₉₅ to determine if there was a discrepancy in the accumulation of Pt₁₉₅ within the nuclear region, where it carries out its function by promoting cross-linkage damage. ICP-MS analysis displayed a statistically significant increase of Pt₁₉₅ within the cytoplasmic compartment in our BxPC-3 miR-31 cells **(Figure 4.6A)**. Nevertheless, a statistically significant reduction of Pt₁₉₅ found in the nuclear case in BxPC-3 miR-31 cells could potentially explain the cisplatin-resistant phenotype observed here **(Figure 4.7A)**. Conversely, the opposite trend was observed within our Panc-1 miR-31 suppressed cells, although no statistical significance was found **(Figures 4.6B & 4.7B)**. This data strongly imply that the increased cisplatin resistance observed upon miR-31 expression is likely due to the altered sequestration and trafficking of drugs to the nucleus.

4.4.3 Manipulating miR-31 alters DNA damage induction and repair post cisplatin treatment.

To confirm if miR-31 altered levels of cisplatin within the nucleus, the influence of miR-31 on DNA damage induction was measured by investigating levels of phospho-histone H2A.X.

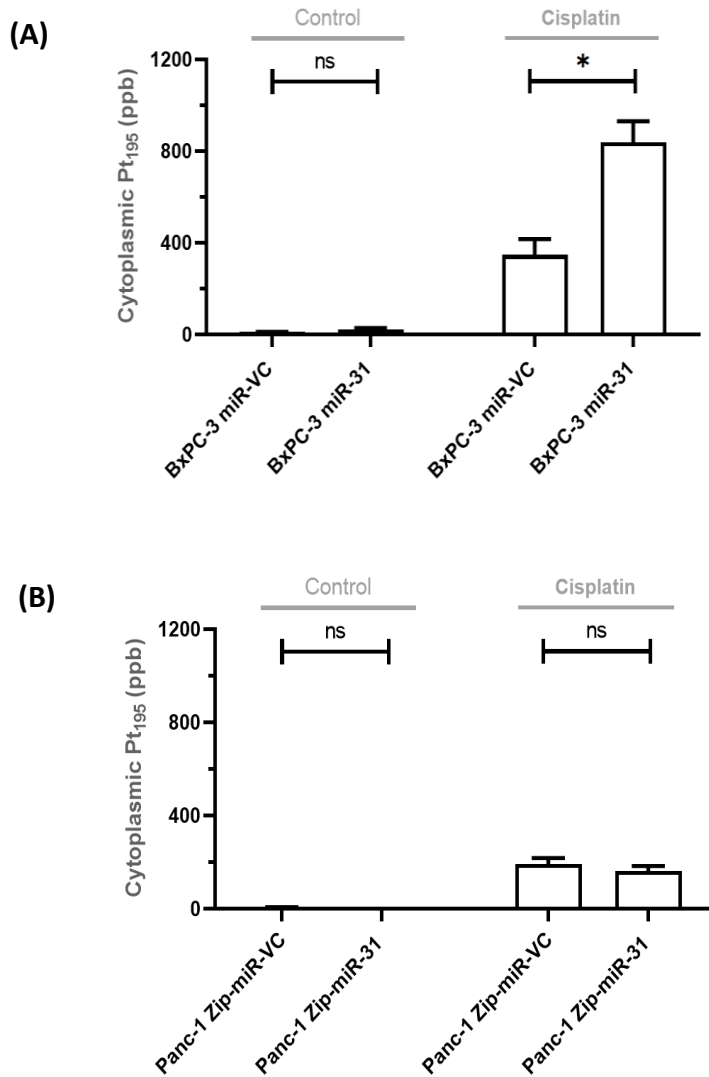


Figure 4.6 Cytoplasmic cisplatin content is altered with miR-31 manipulation in PDAC cell lines. ICP-MS analysis of the cytoplasmic compartment of cells treated with 50 μ M cisplatin for 24 h. **(A)** There is a significant (* $p = 0.0384$) increase in levels of platinum (Pt₁₉₅) in BxPC-3 miR-31 cells compared to the miR-VC equivalent. **(B)** There is a trend toward decreased levels of Pt₁₉₅ in Panc-1 Zip-miR-31 cells compared to the Zip-miR-VC equivalent. Data presented as the $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

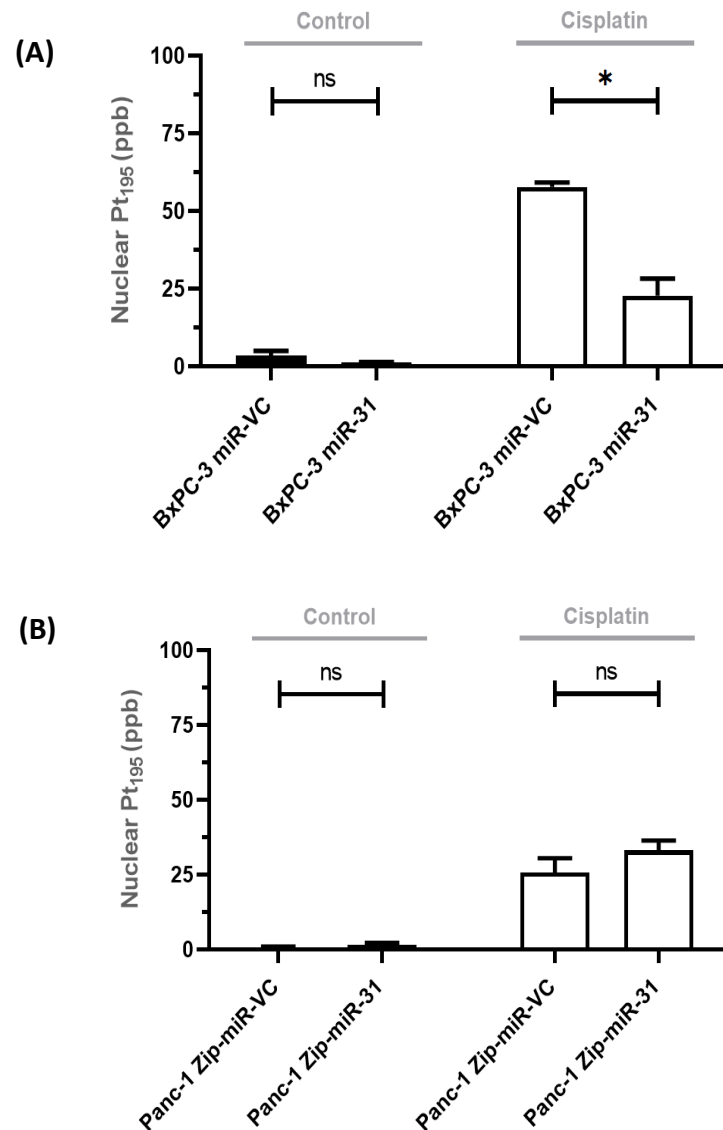


Figure 4.7 Nuclear cisplatin content is altered with miR-31 manipulation in PDAC cell lines. ICP-MS analysis of the nuclear compartment of cells treated with 50 μ M cisplatin for 24 h. **(A)** There is a significant (* $p = 0.0206$) decrease in levels of platinum (Pt₁₉₅) in BxPC-3 miR-31 cells compared to the miR-VC equivalent. **(B)** There is a trend towards increased levels of Pt₁₉₅ in Panc-1 Zip-miR-31 cells compared to the Zip-miR-VC equivalent. Data presented as the $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

(gamma-H2A.X), a marker of double-strand breaks (DSBs) and is associated with cell death. In response to cisplatin, it was found that BxPC-3 miR-31 cells reduced gamma-H2A.X levels compared to their miR-VC equivalent (**Figure 4.8**), while Panc-1 Zip-miR-31 cells increased gamma-H2A.X levels compared to their Zip-miR-VC equivalent (**Figure 4.9**). The loss of gamma-H2A.X is generally consistent with DNA repair and is a vital survival mechanism that is likely to play a role in the cisplatin resistance of miR-31 abundant cells. Although, low gamma-H2A.X levels may also indicate either alteration in cisplatin detoxification or the inadequacy of cisplatin being transported into the nucleus, thus resulting in fewer DSBs.

4.4.4 Cell cycle checkpoint operation in miR-31 manipulated PDAC models.

The effect of cisplatin on cell cycle distribution in miR-31 manipulated BxPC-3 cells was assessed by PI staining and flow cytometry to investigate any alterations in cell cycle checkpoint operation. It is well established that cell cycle analysis is associated with chemoresistance. A trend indicated that the BxPC-3 cisplatin-resistant cells accumulated in the S-phase at all time points post-cisplatin treatment (**Figure 4.10**). Because no statistical analysis was performed, further investigation into the cell cycle is required to determine if the cell cycle plays a role in either miR-31 promoting cisplatin resistance or supporting the delay of cisplatin enhancing cellular sensitivity.

4.4.5 Oxidant and antioxidant levels in miR-31 manipulated PDAC models.

It is well established that differences in oxidants and antioxidant levels have been associated with chemo-resistance and detoxification of platinum-based drugs. Therefore, to prove whether oxidant and antioxidant levels contributed to cisplatin resistance in our miR-31 manipulated models, hydrogen peroxide generation which is considered the primary type of reactive oxygen species (ROS), and total glutathione (GSH) levels were assessed. There were

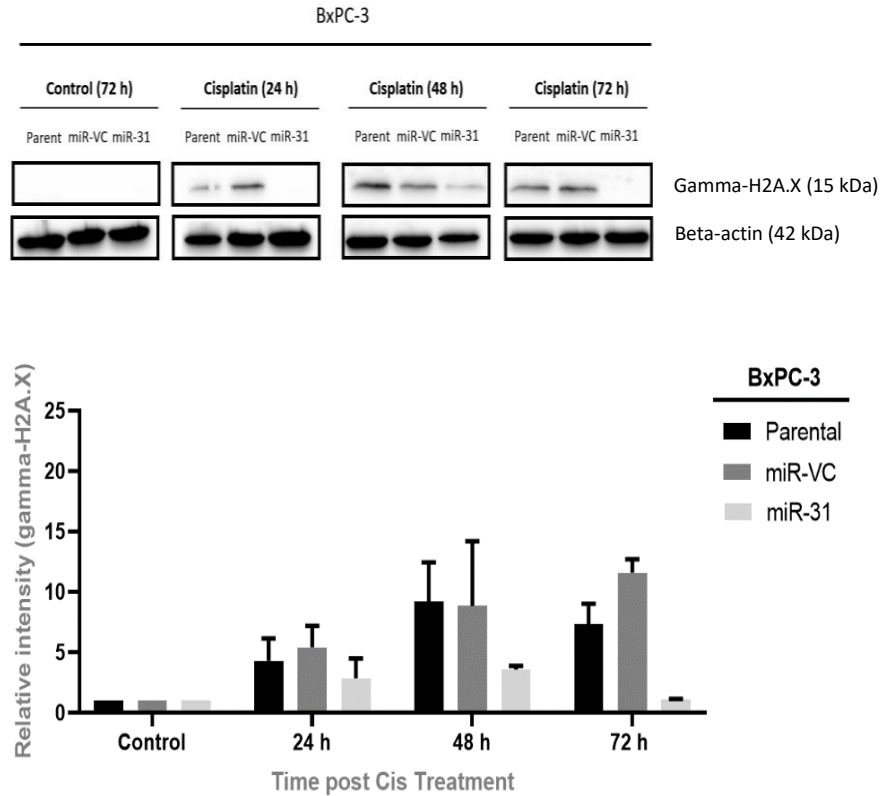


Figure 4.8 Overexpressing miR-31 in BxPC-3 cells correlates to reduced gamma-H2A.X levels. Representative western blot time course and densitometry analysis for gamma-H2A.X as a marker of DNA damage with cisplatin (Cis) treatment in BxPC-3 miR-31 cells compared to the miR-VC equivalent. Cells were treated with 7.84 μ M Cis for 24 h. Interestingly, levels of gamma-H2A.X were reduced but no significant differences were observed post-cisplatin treatment. Data are represented as the mean $\pm$ SEM. Two-way ANOVA with Tukey's post-hoc test was adopted for statistical analysis ($n = 3$).

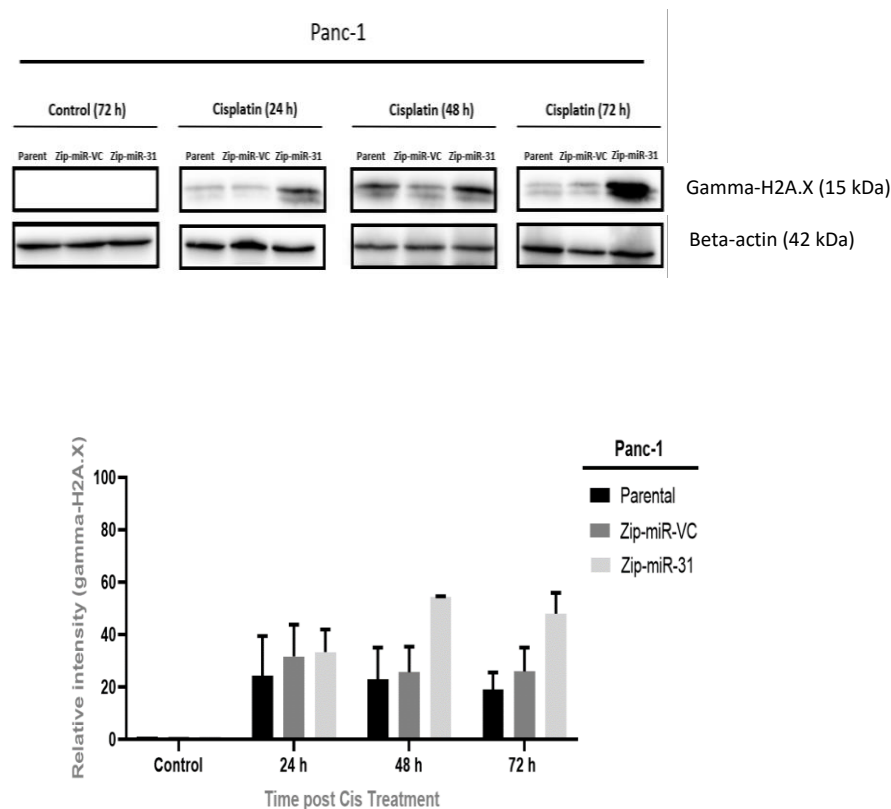


Figure 4.9 Suppressing miR-31 in Panc-1 cells correlates to increased gamma-H2A.X levels. Representative western blot time course and densitometry analysis for gamma-H2A.X as a marker of DNA damage with cisplatin (Cis) treatment in Panc-1 Zip-miR-31 cells compared to the Zip-miR-VC equivalent. Cells were treated with 13.8 μ M Cis for 24 h. Interestingly, levels of gamma-H2A.X were increased but no significant differences were observed post-cisplatin treatment. Data are represented as the mean $\pm$ SEM. Two-way ANOVA with Tukey's post-hoc test was adopted for statistical analysis ($n = 3$).

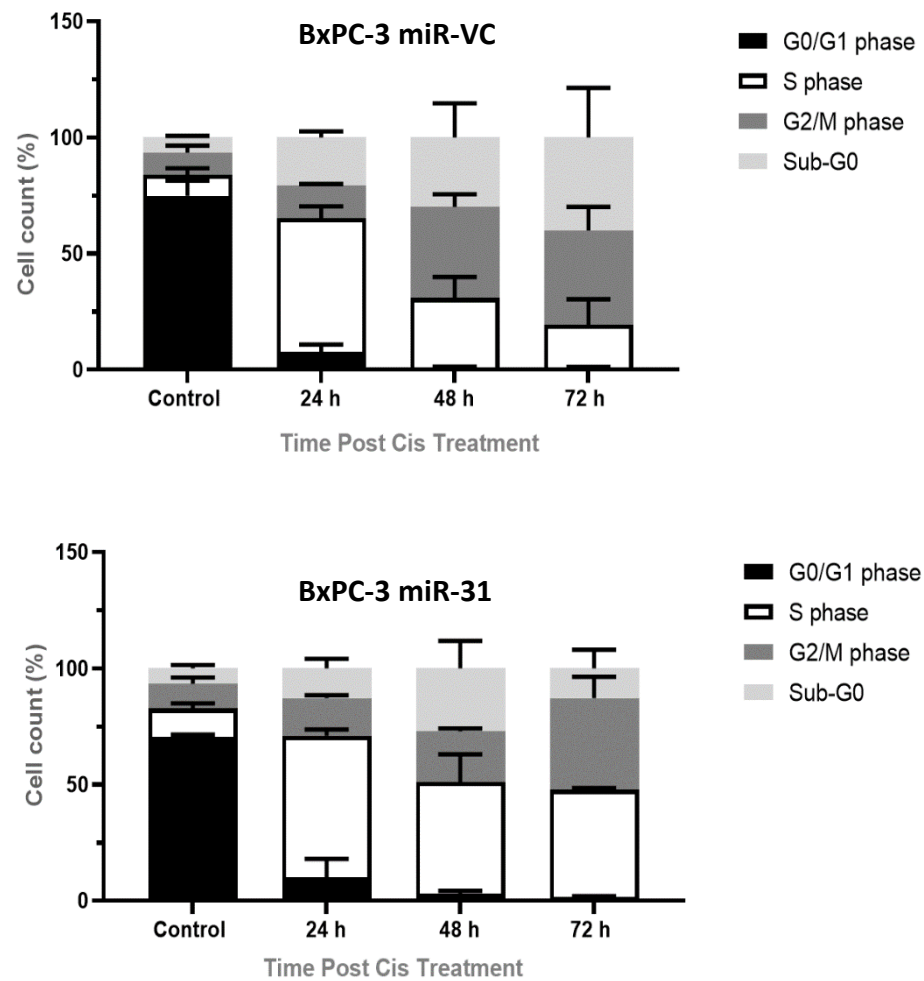


Figure 4.10 Overexpressing miR-31 may induce S-Phase arrest in BxPC-3 cells post-cisplatin treatment. The effect of cisplatin treatment on the cell cycle distribution of BxPC-3 miR-VC and miR-31 cells was investigated at 24 h, 48 h, and 72 h post cisplatin treatment. Cells were treated with 7.84 μ M cisplatin for 24 h. The analysis of cell cycle was completed $n = 2$, therefore, statistics was not applied. Data are represented as the mean $\pm$ SEM.

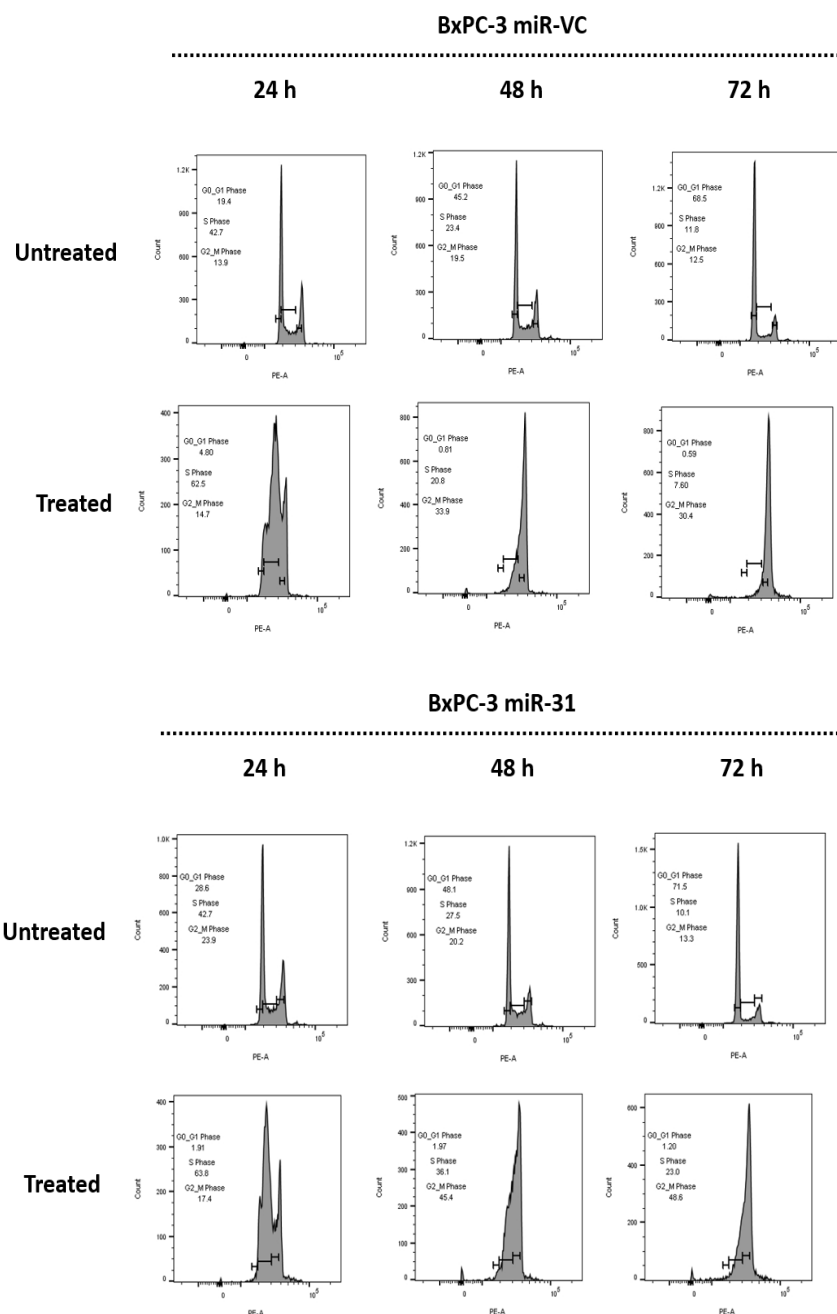


Figure 4.10 Overexpressing miR-31 may induce S-Phase arrest in BxPC-3 cells post-cisplatin treatment *continued*. The effect of cisplatin treatment on the cell cycle distribution of BxPC-3 miR-VC and miR-31 cells was investigated at 24 h, 48 h, and 72 h post cisplatin treatment. Cells were treated with 7.84 μ M cisplatin for 24 h. The analysis of cell cycle was completed $n = 2$, therefore, statistics was not applied. Data are represented as the mean $\pm$ SEM.

no statistically significant differences in ROS levels were found between cisplatin-treated cells and untreated cells in BxPC-3 cells (**Figure 4.11A**) and Panc-1 cells (**Figure 4.12A**). Similarly, there were no significant changes in total GSH levels in BxPC-3 cells (**Figure 4.11B**) and Panc-1 cells (**Figure 4.12B**). This data suggests that miR-31 promotes cisplatin resistance mainly independent of ROS and GSH biology.

4.4.6 Correlation between miR-31 and lysosomal pH for regulating cisplatin resistance.

Lysosomes are acidic organelles and generally show a pH of 4.5 - 5.5 achieved by a membrane V-ATPase pump and play many vital roles, including multidrug resistance. Interestingly, it was found that overexpressing miR-31 in BxPC-3 cells, without any treatment, significantly increased pH_{Lys} when compared to its miR-VC equivalent (**Figure 4.13A**). Additionally, a reduction in pH_{Lys} was displayed while suppressing miR-31 in Panc-1 cells, but no statistically significant differences were observed (**Figure 4.13B**). One possible explanation is that miR-31 expressing PDAC cells may present fewer V-ATPases on the lysosomal membrane, reducing lysosomal acidification.

Bafilomycin A1 (BA1) is a frequently used inhibitor of lysosomal function by blocking the V-ATPase pumps found on the lysosomal membrane. BA1 prevents lysosomal acidification and consequently increases pH_{Lys} and has been demonstrated to promote cisplatin resistance. We propose that BA1 treatment would offer protection in PDAC cell lines against cisplatin by increasing pH_{Lys} . It was found that treating with BA1 and cisplatin separately or in combination increased pH_{Lys} in BxPC-3 miR-31 cells compared to its miR-VC equivalent (**Figure 4.15A**). However, treating with BA1 increased cisplatin sensitivity rather than resistance as expected, thus displaying a synergistic relationship, especially in miR-31 expressing cells (**Figure 4.15B**). Additionally, BA1 treatment increased cisplatin sensitivity in Panc-1 Zip-miR-31 cells

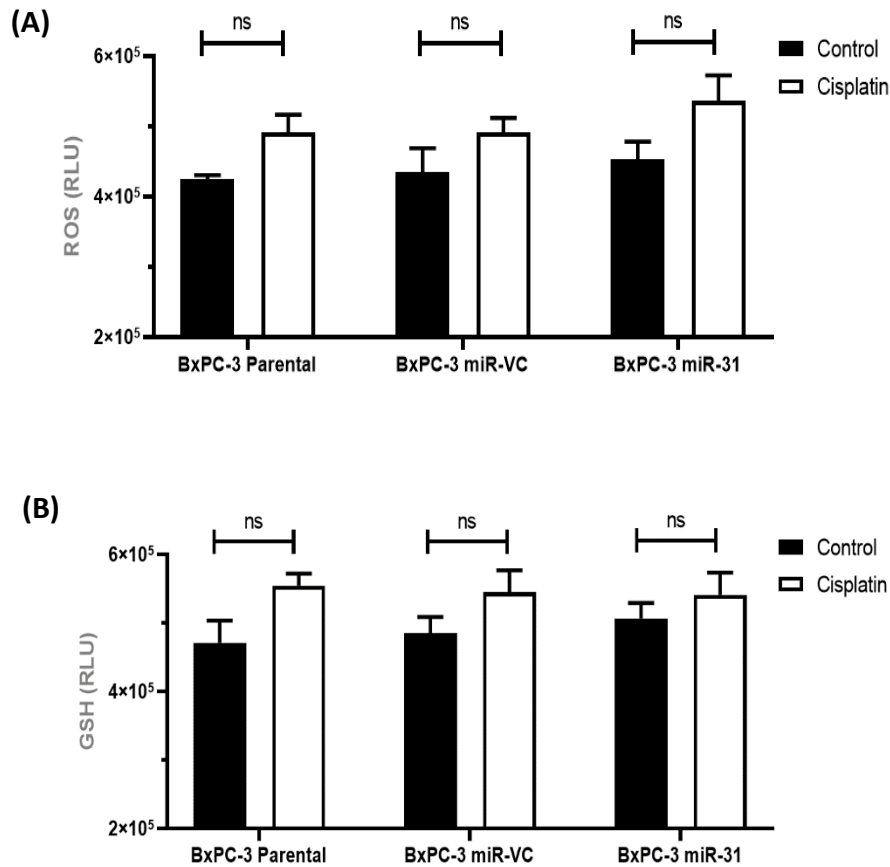
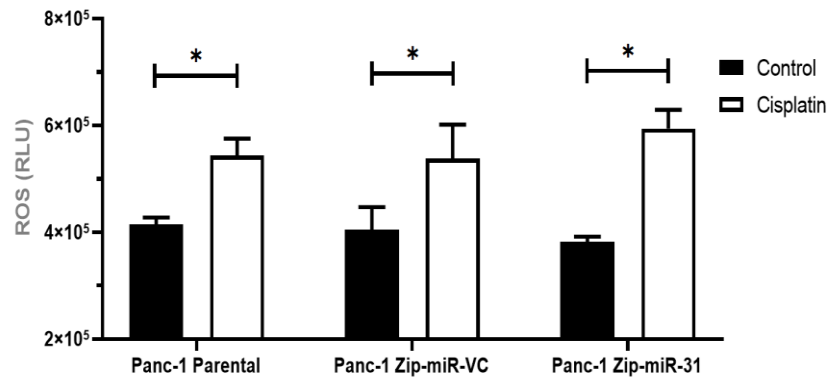


Figure 4.11 Overexpressing miR-31 in BcPC-3 cells does not alter reactive oxygen species or glutathione generation post-cisplatin treatment. BxPC-3 cells were treated with 7.84 μ M cisplatin for 24 h. **(A)** Reactive oxygen species (ROS) was assessed 24 h post cisplatin treatment. **(B)** Glutathione (GSH) was assessed 24 h post cisplatin treatment. No significant differences were observed in all cell lines, suggesting that ROS and GSH generation play no role in miR-31 modulating cisplatin resistance. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

(A)



(B)

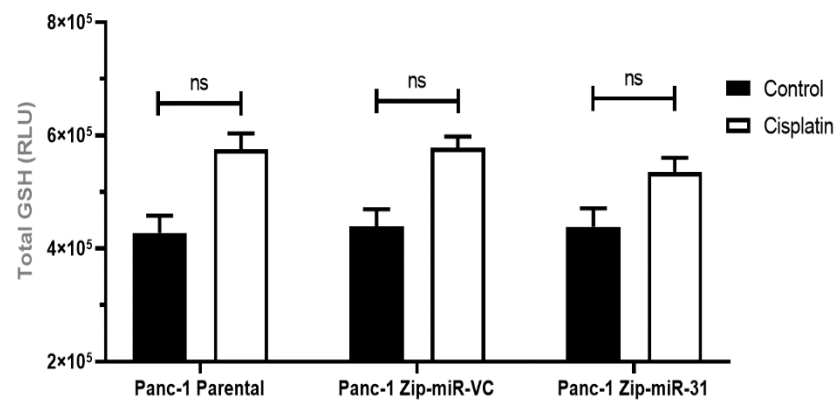


Figure 4.12 Suppressing miR-31 in Panc-1 cells does not alter reactive oxygen species or glutathione generation post-cisplatin treatment. Panc-1 cells were treated with $13.8 \mu\text{M}$ cisplatin for 24 h. **(A)** Reactive oxygen species (ROS) was assessed 24 h post cisplatin treatment. **(B)** Glutathione (GSH) was assessed 24 h post cisplatin treatment. Similar ROS and GSH levels were observed in all cell lines, suggesting that ROS and GSH generation play no role in miR-31 modulating cisplatin resistance. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

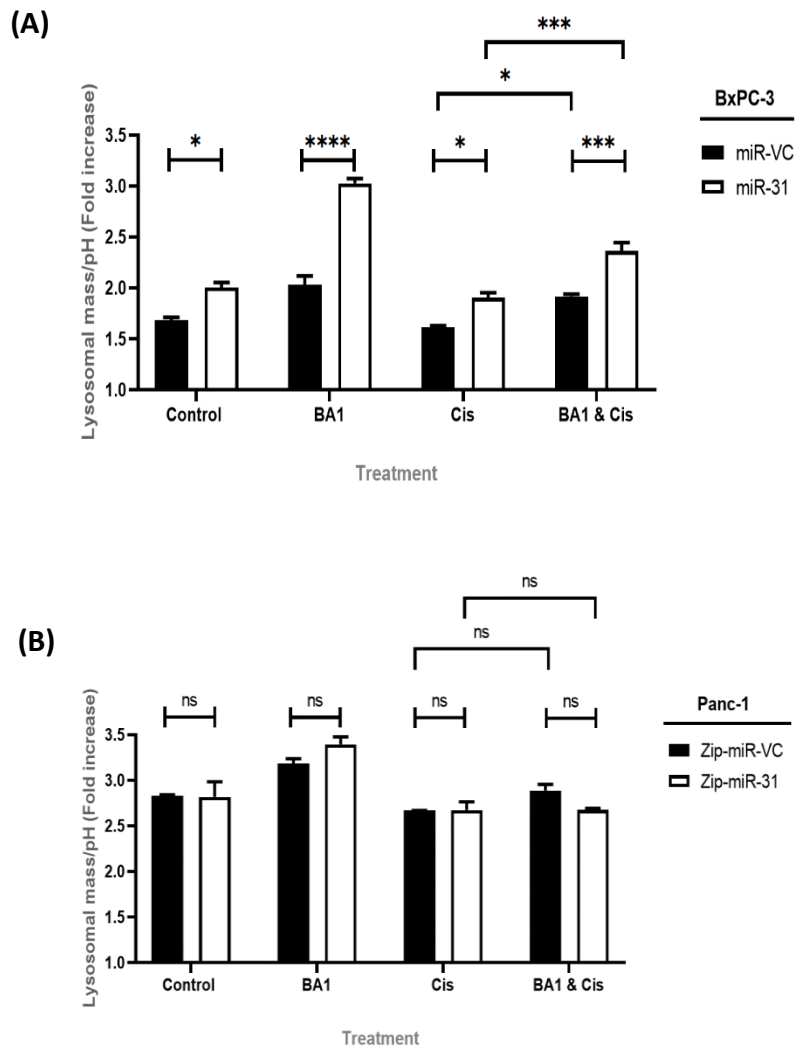


Figure 4.13 Manipulating miR-31 alters lysosomal mass/pH in PDAC cell lines. (A) Overexpressing miR-31 in BxPC-3 cells significantly increases lysosomal mass/pH in untreated cells (* $p = 0.0123$), bafilomycin A1 (BA1) treated cells (**** $p < 0.0001$), cisplatin-treated cells (* $p = 0.0282$), and BA1 with cisplatin-treated cells (*** $p = 0.0006$). **(B)** Suppressing miR-31 in Panc-1 cells displayed no significant differences in lysosomal mass/pH. Cells were pre-treated with 10 nM BA1 for 48 hours, followed by IC_{50} doses of cisplatin for the last 24 h. Data are presented as the mean $\pm$ SEM. A Two-way ANOVA was applied for statistical analysis ($n = 3$).

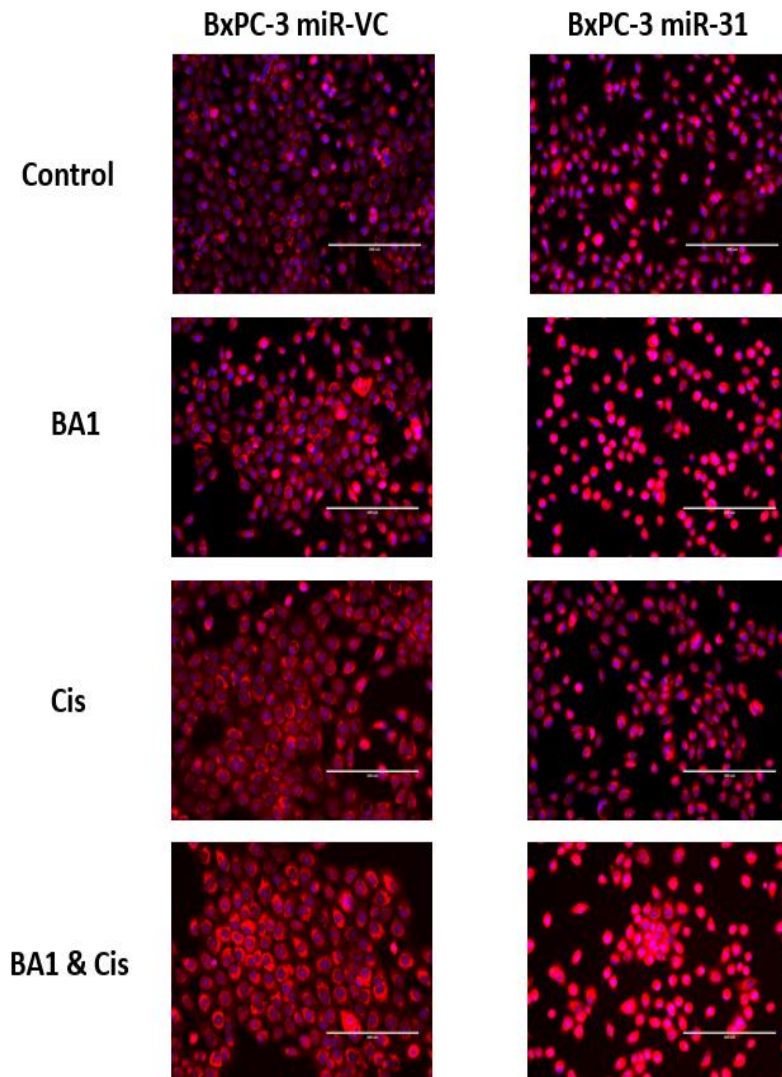


Figure 4.14 Overexpressing miR-31 increases lysosomal pH in BxPC-3 cells. Immunofluorescent images showing lysosomal mass/pH staining (red) in BxPC-3 untreated, bafilomycin A1 (BA1), cisplatin-treated (Cis), and BA1 & Cis treated cells. MiR-31 displayed an increase in lysosomal mass/pH in all treatment conditions. Nuclei (blue) were stained with DAPI. Cells were pre-treated with 10 nM BA1 for 48 hours, followed by IC₅₀ doses of cisplatin the last 24 h.

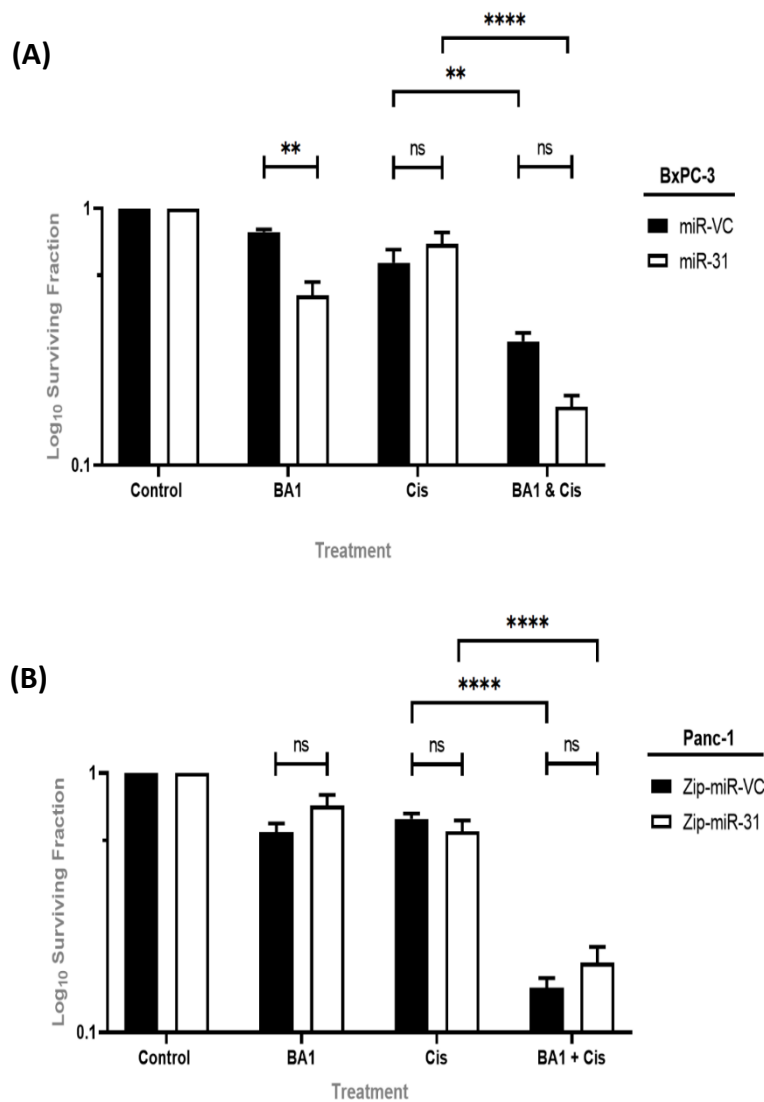


Figure 4.15 Bafilomycin A1 enhances cisplatin sensitivity in PDAC cell lines. **(A)** Overexpressing miR-31 in BxPC-3 cells significantly increases lysosomal mass/pH in untreated cells (* $p = 0.0123$), bafilomycin A1 (BA1) treated cells (**** $p < 0.0001$), cisplatin-treated cells (* $p = 0.0282$), and BA1 with cisplatin-treated cells (***) $p = 0.0006$. **(B)** Suppressing miR-31 in Panc-1 cells displayed no significant differences in lysosomal mass/pH. Cells were pre-treated with 10 nM BA1 for 48 hours, followed by IC_{50} doses of cisplatin for the last 24 h. Data are presented as the mean $\pm$ SEM. A Two-way ANOVA was applied for statistical analysis ($n = 3$).

compared to its Zip-miR-VC equivalent, where the Zip-miR-VC cells displayed more cell death when compared to the cells treated with cisplatin alone (**Figure 4.15**). These results suggest that increasing pH_{Lys} using the V-ATPase inhibitor BA1 does not protect cells against cisplatin in PDAC cell lines but significantly enhances cisplatin sensitivity, particularly in miR-31 expressing cells, which is likely caused by another mechanism that is not associated with lysosomal acidification. Nevertheless, miR-31 could be related to cisplatin resistance by altering lysosomal acidification in BxPC-3 cells.

4.4.7 Manipulating miR-31 alters the lysosomal-bound transporter ABCB9.

An association between miR-31 and the lysosomal-bound transporter ABCB9 was previously established in NSCLC. Here, overexpressing miR-31 in BxPC-3 cells upregulated ABCB9 at the protein level, although no statistically significant reduction was observed upon suppressing miR-31 in Panc-1 cells (**Figure 4.16**). To establish whether an increase in lysosomal quantity may account for the change in ABCB9, the expression of the lysosomal marker LAMP-1 was assessed. No statistically significant differences in LAMP-1 expression were observed, supporting a specific upregulating of the ABCB9 transporter rather than changes in lysosomal numbers (**Figure 4.17**).

4.4.8 Overexpressing miR-31 does not alter the lysosomal packaging of Pt₁₉₅ by increasing ABCB9.

One common route by which cells can traffic cytotoxic drugs, including cisplatin, away from the nucleus is packaging into intracellular vesicles such as lysosomes. Because the lysosomal drug transporter, ABCB9, was significantly greater in miR-31 overexpressed BxPC-3 cells compared to its miR-VC equivalent, both cell types were treated with cisplatin for 24 h, and the lysosomes were isolated to determine if more Pt₁₉₅ were packaged within the lysosomes.

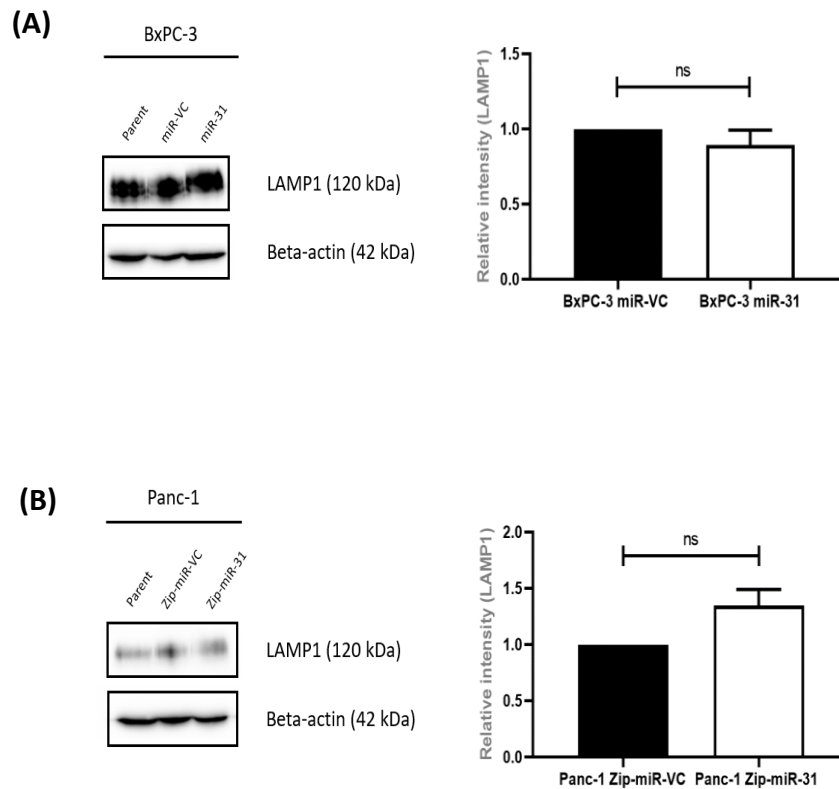


Figure 4.16 Manipulating miR-31 in PDAC cell lines does not significantly alter the expression of LAMP1. (A) Representative western blot illustrating a moderate reduction in lysosomal density as assessed by LAMP1 when miR-31 was overexpressing in BxPC-3 cells. **(B)** Representative western blot illustrating a moderate increase in lysosomal density as assessed by LAMP1 when miR-31 was suppressed in Panc-1 cells. Densitometry analysis was applied, displaying the relative intensity of ABCB9 expression. Data are presented as the mean $\pm$ SEM. BxPC-3 miR-VC/Panc-1 Zip-miR-VC is utilised as the relative control and is set to 1; no error is associated. A one-sampled t.test was applied for statistical analysis ($n = 3$).

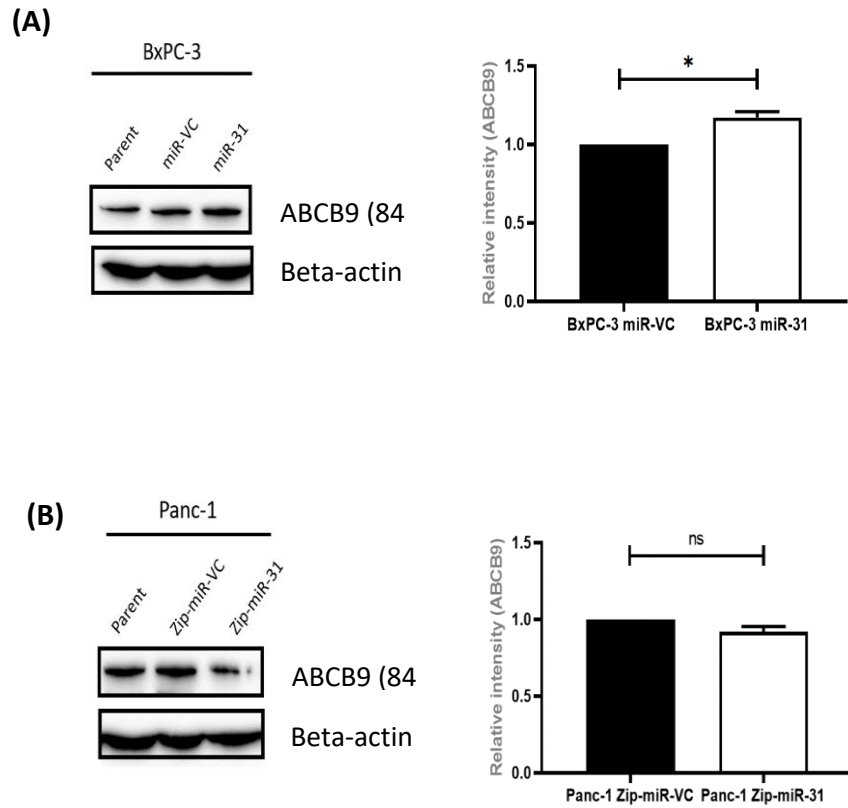


Figure 4.17 Overexpressing mi3-31 significantly increases the lysosomal drug transporter ABCB9. **(A)** Representative western blot ($n = 4$) illustrating a significant increase in ABCB9 expression with miR-31 overexpression in BxPC-3 cells (* $p = 0.0181$). **(B)** Representative western blot ($n = 3$) illustrating a modest reduction of ABCB9 expression with miR-31 suppression in Panc-1 cells. Densitometry analysis was applied, displaying the relative intensity of ABCB9 expression. Data are presented as the mean $\pm$ SEM. BxPC-3 miR-VC/Panc-1 Zip-miR-VC is utilised as the relative control and is set to 1; no error is associated. A one-sampled t.test was applied for statistical analysis.

Surprisingly, ICP-MS analysis displayed a $0.57 \text{ ppb} \pm 0.15 \text{ ppb}$ decrease in Pt_{195} concentration, but no statically significant differences were observed between the lysosomal compartments of the two cell types **(Figure 4.18)**. Indicating that despite miR-31 overexpressed BxPC-3 cells displaying an increase in ABCB9 levels, the lysosomal packaging of Pt_{195} remains unaltered.

4.4.9 Manipulating miR-31 alters the trafficking of cisplatin to the nucleus via ATOX1.

Emerging evidence has revealed that copper-transport proteins play a crucial role in cisplatin activity, including the metal chaperone ATOX1, which binds via its conserved metal-binding motif. Interestingly, ATOX1 has been associated with cisplatin transportation across the cell and the nucleus, potentially contributing to the regulation of cisplatin accumulation. In Silico analysis displayed, ATOX1 is a predictive target of miR-31-3p **(Figure 4.19A)**. To investigate whether miR-31 altered ATOX1 levels, basal ATOX1 was assessed at the protein level in the miR-31 manipulated PDAC models. It was found that overexpressing miR-31 in BxPC-3 cells significantly reduced ATOX1 levels while suppressing miR-31 in Panc-1 cells significantly increased ATOX1 levels **(Figure 4.19B & 4.19C)**.

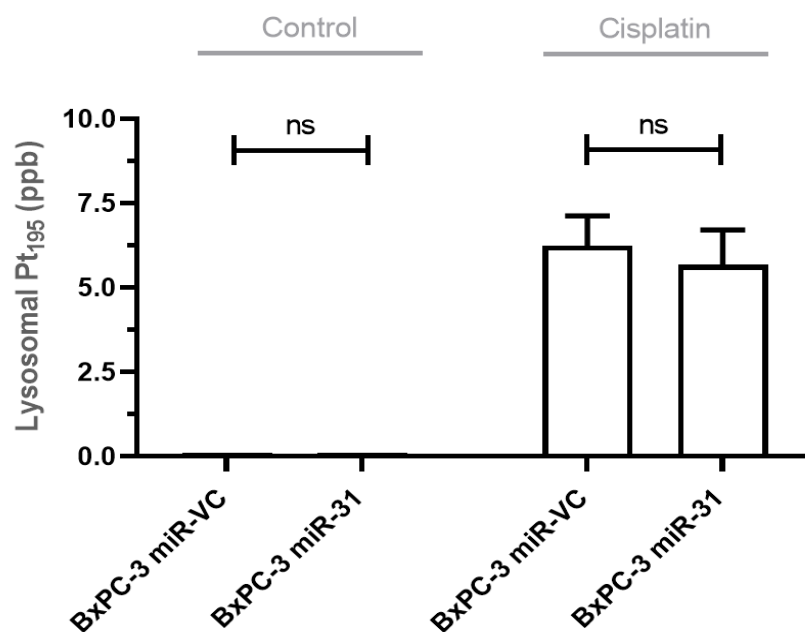


Figure 4.18 Overexpressing miR-31 in BxPC-3 cells does not affect the platinum content of the lysosomal region. ICP-MS of lysosomal isolation following 50 μ M cisplatin treatment for 24 h displayed a trend towards a decrease in platinum (Pt₁₉₅) with miR-31 overexpressed BxPC-3 cells. Data are presented as the mean $\pm$ SEM. A two-tailed paired t.test was applied for statistical analysis ($n = 3$).

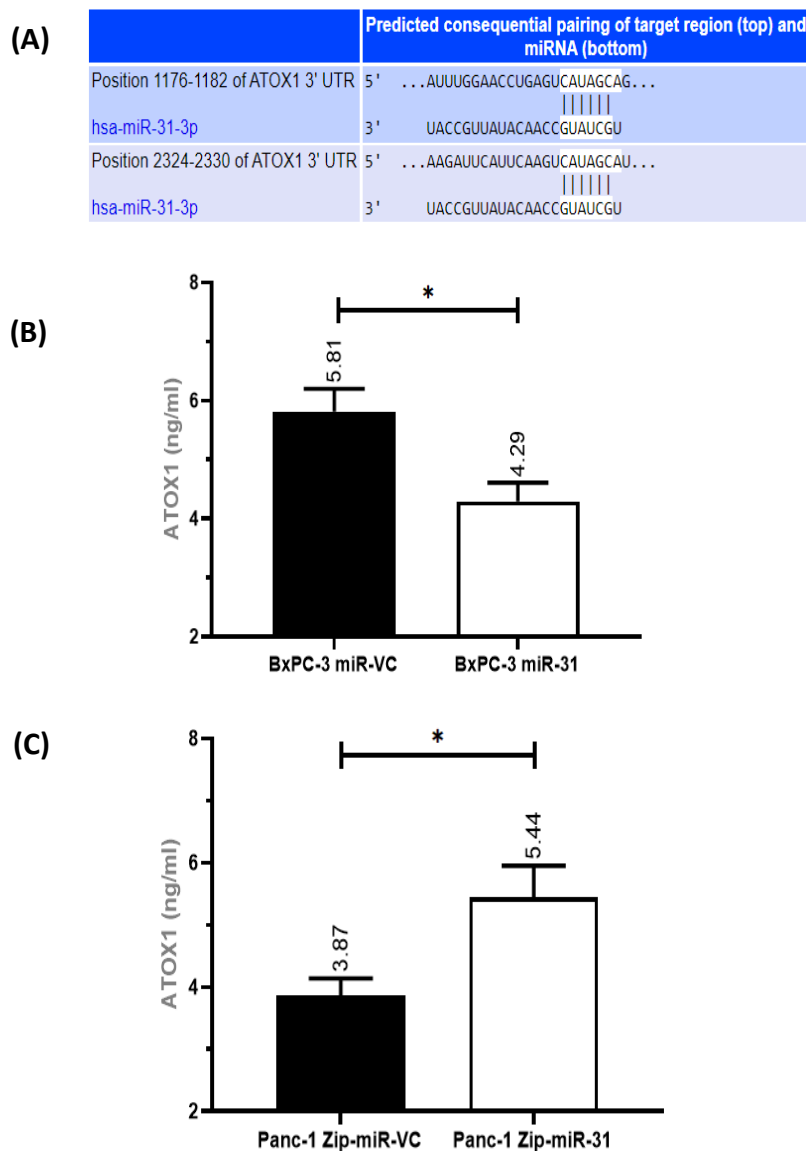


Figure 4.19 Manipulating miR-31 in PDAC cells alters the expression of ATOX1. (A) The communications between ATOX1 transcripts with miR-31-3p recognition sites. (B) Overexpressing miR-31 in BxPC-3 cells significantly reduced ATOX1 levels compared to its vector control equivalent (* $p = 0.0221$). (C) Suppressing miR-31 in Panc-1 cells significantly increased ATOX1 levels compared to its vector control equivalent (* $p = 0.0396$). Data are expressed as the mean $\pm$ SEM and was analysed by a two-tailed paired t.test ($n = 5$).

4.5 Discussion

Cisplatin is one of the most used chemotherapeutic agents in treating patients with various types of cancer, including ovarian, lung, and breast cancer [317]. It is estimated that up to 80% of all cancer patients undergoing chemotherapy will receive cisplatin, representing an essential cancer treatment regimen [317]. Additionally, cisplatin is considered the last option for treating patients with PDAC and is frequently used to treat advanced or metastatic disease, particularly for patients who present with BRAC1/2 or PALB mutations [318]. However, cisplatin resistance remains a significant challenge in cancer, especially PDAC. Therefore, it is vital to understand the molecular mechanisms underlying cellular cisplatin resistance.

In the previous chapter, it was observed that miR-31 expressing cells increased resistance to platinum-based chemotherapeutics, particularly cisplatin. This chapter aimed to explore how miR-31 potentially modulated cisplatin resistance and examined the differences in the trafficking of cisplatin to the nucleus of the cell.

With the potential accumulation of Pt₁₉₅ in miR-31 expressing cells, the cellular flux of chemotherapeutics was investigated. It is well established that copper membrane-bound transporters are essential mediators for the cellular uptake and efflux of platinum-based agents such as cisplatin [308-311]. Reduced influx or increased efflux is associated with decreased intracellular accumulation. In this study, it was found no changes in influx transporter CTR1 or efflux transporters ATP7A and ATP7B, suggesting that miR-31 promotes cisplatin resistance independent of copper transporters. In contrast with the results established here, Feng *et al.* [319] demonstrated that miR-130a reduced CTR1 levels and influenced cisplatin resistance in cervical cancer cells. A recent study evaluated the relationship between CTR1 expression and intratumoral tissue platinum concentrations from NSCLC specimens and found that undetectable CTR1 expression was linked to reduced platinum

concentrations. Despite showing no significant differences, it was shown here that CTR1 appeared to be increased in miR-31 expressing cells, which may explain, at least to some degree, the increase in Pt₁₉₅ concentrations found within the cell. However, it would not explain the role of miR-31 in promoting cisplatin resistance in PDAC cells. Interestingly, Eljack *et al.* [320] revealed that cisplatin could passively diffuse across a lipid bilayer but only in high chloride concentrations in the surrounding medium, suggesting that chloride ions play a role in the cellular accumulation of cisplatin.

Although not explored in this study, CTR2 has also been linked to cisplatin resistance, although CTR2 plays an opposing role compared to CTR1 and functions as an efflux transporter [321]. Higher CTR2 levels have been linked to cisplatin resistance in ovarian cancer cell lines [322]. Additionally, recent studies have indicated that CTR2 can induce CTR1 cleavage, resulting in a reduced influx of cisplatin [323, 324].

The export of cisplatin is achieved by the copper-transporting ATPases ATP7A and ATP7B, which can be found on the plasma membrane and are located on the trans-Golgi network, therefore, regulating the cellular efflux of cisplatin [307, 311]. Wang *et al.* [325] showed that miR-133a reduced ATP7B levels and enhanced cisplatin sensitivity in larynx carcinoma cells. A previous study demonstrated that miR-495 inhibited cisplatin resistance in oesophageal cancer cells by lowering ATP7A expression [326]. Although in this study, it was shown that miR-31 manipulation in PDAC cells does not significantly alter ATP7A and ATP7B.

Drug detoxification is vital in regulating drug resistance and is primarily achieved by antioxidants. Glutathione (GSH) is a common antioxidant found in all human cells and has been strongly associated with cisplatin resistance by sequestration [87, 89]. Because it was shown that miR-31-expressing cells increased cisplatin resistance, a trend toward the increased cellular accumulation of cisplatin was observed. Therefore, we proposed that miR-31 will likely increase drug detoxification and

sequestration by increasing GSH levels. Although, it was found that miR-31 did not alter GSH levels, indicating that antioxidants, particularly GSH, do not play a role in modulating cisplatin resistance in PDAC cells.

The primary target of cisplatin is genomic DNA which results in DNA adducts, consequently stalling replication. Generally, the DNA damage response is activated due to cisplatin-induced DNA damage. Therefore, levels of Pt₁₉₅ were assessed in the cytoplasmic and nuclear compartments. Overexpressing miR-31 in BxPC-3 cells displayed a significant increase of Pt₁₉₅ in the cytoplasmic compartment and a significant reduction of Pt₁₉₅ in the nuclear compartment. Whereas suppressing miR-31 in Panc-1 cells showed the opposite trend. This suggests that the increased resistance to cisplatin following miR-31 overexpression is likely due to the altered trafficking of drugs to the nucleus. This was supported by changes observed in the induction of DNA damage, as measured by gamma-H2A.X.

With the decrease in DNA damage induction upon miR-31 overexpression, it was essential to analyse whether this was a platinum-based therapy-specific response. In clinical practice, 5-FU is an anti-metabolite drug frequently used to treat patients with PDAC. Although not assessed here, it is likely that suppressing miR-31 reduces DNA damage levels post-5-FU treatment because of the resistant phenotype. This would suggest two points. Firstly, that miR-31 is likely to regulate a pathway specific to cisplatin due to differences in DNA damage from the different drugs. Accumulation differences were previously observed in Lanzi *et al.* [327], resulting in decreased DNA platination, contributing to cisplatin resistance in ovarian cancer cells. Secondly, miR-31 will likely increase 5-FU sensitivity by increasing DNA damage repair, although more work is needed to prove this. Interestingly, Lynam-Lennon *et al.* [320] demonstrated that miR-31 overexpression regulated the number of DNA damage repair genes in OAC cell lines and thus may be a potential

route for future investigation when assessing anti-metabolite treatment in PDAC.

Cells can sequester cytotoxic drugs away from the nucleus by packaging them into intracellular vesicles, such as lysosomes which have been reported to be crucial in altering chemo-resistance [313-315]. One previous study showed that restoring miR-194 expression sensitized cells to drug treatment by down-regulating LAMP2 in metastatic renal cell carcinoma [329]. In this study, it was found that the lysosomal burden does not alter cisplatin sensitivity while manipulating miR-31. Moreover, the lysosomal-bound drug transporter ABCB9 has been associated with cisplatin resistance by manipulating miR-31 expression [330]. Therefore, prompting the investigation of whether miR-31 may alter ABCB9 expression, consequently regulating cisplatin transport across the lysosomal membrane. Here it was found that overexpressing miR-31 displayed a significant increase in ABCB9 expression, establishing one possible mechanism for how miR-31 promotes cisplatin resistance in PDAC cells. Theoretically, an increased ABCB9 expression would mean more cisplatin accumulates within the lysosomes. However, ICP-MS analysis revealed that Pt_{195} levels remained unaltered within the lysosomal compartment of miR-31 overexpressed cells, despite increased ABCB9 expression. This implies that ABCB9 upregulation does not contribute to the specific pathway mediated by miR-31 to enhance PDAC chemo-resistance. Furthermore, lysosomal compartments are acidic, achieved by the membrane V-ATPase pumps. A previous study identified a linear relationship between increased lysosomal pH (pH_{Lys}) and cisplatin resistance in head and neck squamous cell carcinoma cell lines [331]. Moreover, Chauhan *et al.* [332] found an increase in pH_{Lys} within a cisplatin-resistant derived human epidermoid carcinoma cell line. Interestingly, it was found that overexpressing miR-31 significantly increased pH_{Lys} . One possible explanation is that miR-31 expressing cells may present fewer V-ATPase pumps on the lysosomal membrane,

increasing the pH_{Lys} . Bafilomycin A1 (BA1) is a frequently used inhibitor of lysosomal function by blocking the V-ATPase pumps [333]. BA1 prevents lysosomal acidification as fewer protons can enter the lysosomal compartment. Treating cisplatin-sensitive cells with BA1 has been previously shown to mimic cisplatin-resistant cells' behaviour [332]. Furthermore, Nilsson *et al.* [331] found that BA1 pre-treatment protects against cell death caused by cisplatin, supporting that the pH_{Lys} are of importance to the cellular sensitivity to cisplatin. Therefore, it is proposed that BA1 treatment would offer protection in PDAC cell lines against cisplatin by increasing pH_{Lys} . In this study, it was found that treating cells with BA1 and cisplatin increased pH_{Lys} in miR-31 expressing cells. However, here it showed increased cisplatin sensitivity rather than its resistance, as expected.

The question remains as to how miR-31 can regulate the trafficking of cisplatin to the nuclear compartment in PDAC cells. Astoundingly, research focussing on nuclear trafficking and the transport of chemotherapy remains poorly understood. Antioxidant 1 (ATOX1) was identified as the first copper chaperone and plays a significant role in copper transportation to the nucleus [334]. Interestingly, copper and cisplatin share the same binding motif found on ATOX1. Our results show that miR-31 expressing cells significantly reduced ATOX1 expression in PDAC cells. Therefore, promoting the investigation if ATOX1 is responsible for the trafficking of cisplatin to the nucleus and regulating resistance to treatment.

This chapter has uncovered a potentially novel mechanism behind miR-31-mediated cisplatin resistance in PDAC, potentially mediated via the modulation of the copper transporter, ATOX1. Therefore, to further investigate the significance of the contribution of ATOX1 to PDAC resistance, in the next chapter, ATOX1 was independently expressed to evaluate its contribution to the miR-31-mediated cisplatin-resistant phenotype.

Chapter Four

Part II

ATOX1 modulates cisplatin sensitivity
in PDAC.

4.6 Introduction

The redox catalyst, copper (Cu), is an essential micronutrient for the human cell, despite displaying lower concentrations than other metals such as sodium and potassium within the human body [335]. Due to the limited amount of Cu that organisms receive in their diet, cells face the challenge of distributing Cu where it is needed to ensure the activity of Cu-dependent proteins for their function. Cu is a cofactor for numerous redox enzymes, including Cu and Zinc dismutase, tyrosinase, and cytochrome c oxidase [335, 336]. It is essential in mitochondrial metabolism, cell proliferation, and antioxidant defense [335, 336]. Regulating Cu within the body is necessary, where low levels have been linked to neurological diseases, including Alzheimer's, and Parkinson's [337]. Higher Cu levels are equally detrimental by degrading macromolecules, including proteins, lipids, and DNA. Nonetheless, accumulated Cu can be detoxified by high amounts of glutathione (GSH) and metallothioneins [338]. It is not surprising that Cu is fundamental for at least three characteristic phenomena involved in cancer: proliferative immortality, angiogenesis, and metastasis [339]. Elevated Cu concentrations have been previously reported in the serum or tumours of patients with many cancer types, such as breast, lung, gastrointestinal, and prostate cancers [339, 340]. Cu concentrations were significantly increased in the serum of patients with pancreatic cancer and potentially present as a marker for pancreatic cancer and possible development [341].

The antioxidant protein 1 (ATOX1) is a small metal-binding protein with a predicted molecular weight of 7 kDa, found in the cytosolic and nuclear regions and has an 85% sequence identity in mammalian species and is highly conserved amongst eukaryotic cells [342]. CopZ, a known orthologue of ATOX1, was identified in bacteria. ATOX1 is considered a vital cytosolic metallochaperone in human cells [343]. ATOX1 was identified as the first copper chaperone; it receives Cu from the CTR1

influx transporters and delivers it to the ATP7A and ATP7B efflux transporters found on the *trans*-Golgi network, thus playing a significant role in Cu transportation and Cu homeostasis. It is well established that ATOX1 is associated with altering oxidative stress via antioxidant function. One recent study revealed overexpressing ATOX1 protected neuronal cells against hydrogen peroxide treatment by reducing cellular ROS levels [344]. It is thought that ATOX1 regulates antioxidant function by supplying Cu cofactors to Cu-dependent enzymes that participate in antioxidant defense [342]. Moreover, recent evidence revealed that ATOX1 is upregulated in breast, colorectal, uterus, and liver tumours, where patients with high ATOX1 levels are at higher risk of metastasis than those with low ATOX1 levels [345].

Platinum (Pt)-based anti-tumour agents, such as cisplatin, have been used to treat various cancer types and, alongside gemcitabine, including PDAC [64]. The cytotoxic target of these drugs is DNA, which induces platinum-DNA adducts, affecting DNA replication and promoting cell death or apoptosis. However, cisplatin resistance remains a significant challenge in anti-cancer therapy [346]. One of the predominant characteristics of cellular resistance to cisplatin is the reduced drug accumulation in the nucleus. It is thought that only 1% of cisplatin reaches the nucleus, where it is needed to carry out its function [347]. Consequently, fewer platinum-DNA adducts are formed, resulting in cancer-cell survival. Therefore, a better understanding of Pt-based drugs by Pt-trafficking proteins may help address challenging issues, such as drug availability to the cancer cell's nuclei and altering chemosensitivity.

Interestingly, substantial evidence indicates that the mechanism of cisplatin transport into the cells and its distribution to different cellular compartments involves copper transporters, at least to some degree. Like Cu, cisplatin can enter or leave the cell via CTR1 and ATP7A/B, respectively [306]. Moreover, it has been revealed that cisplatin can bind to the metal-binding site of ATOX1 [348]. Initially, it was thought that Cu

was unnecessary because cisplatin could still bind to ATOX1 in its absence. However, recent research revealed that the presence of Cu helps attract cisplatin to ATOX1, which potentially explains why cisplatin favours the Cu transport system over other modes of transport [349]. Recently, it has been reported that Cu accumulation was observed within the nuclei in cells with high levels of ATOX1 compared to cells that lack ATOX1, suggesting a role of ATOX1 in transporting Cu to the cell nuclei [350]. But if ATOX1 plays a part in transporting cisplatin to the nucleus remains poorly understood. Furthermore, it has previously been shown that ATOX1 functions as a novel transcription factor. Once activated by Cu, it undergoes nuclear translocation, which alters cell proliferation by targeting the cis-element of the cyclin D1 promoter [334]. Thus, nuclear ATOX1 may be positively correlated with the proliferation rates of cells. However, Kahra *et al.* [351] supported that ATOX1 was translocated to the nucleus but played no role in DNA binding. It appears that ATOX1 mediates transcriptional regulation via unknown proteins.

Therefore, if cisplatin displays a similar mode of transport, it is worth investigating if altering ATOX1 influences transporting cisplatin to the cell's nucleus, thus presenting a therapeutic target for altering chemosensitivity.

This chapter, therefore, aims to clarify the relationship between ATOX1 and resistance to cisplatin within PDAC cell lines; this is to be done independently of miR-31.

4.7 Rationale, Aims, and Objectives

The metallochaperone ATOX1 is downregulated upon miR-31 overexpression and upregulated upon miR-31 suppression in BxPC-3 and Panc-1 cell lines. The downregulation of ATOX1 in association with increased expression of miR-31 suggests that PDAC cells expressing ATOX1 at lower levels than baseline potentially is not as effective in transporting cisplatin to the nucleus. It is hypothesized that ATOX1, as a miR-31 target, is the functional facilitator of the cisplatin-resistant phenotype.

The objectives of this chapter were to 1) determine whether ATOX1, independent of miR-31, contributes to PDAC cisplatin resistance and 2) explore the effect of ATOX1 modulation on DNA damage to identify whether ATOX1 plays a functional role in cisplatin transportation to the nucleus.

4.8 Experimental design

ATOX1 manipulation in PDAC cell lines

ATOX1 was suppressed in the parental BxPC-3 cell line and overexpressed in the Panc-1 cell line via stable plasmid transfection. The approach to independently modulate ATOX1 expression was adopted to view whether ATOX1 contributed to altering cisplatin resistance identified in previous chapters.

The effect of ATOX1 on chemoresistance

The clonogenic assay was utilized to determine the overall effect of chemoresistance with ATOX1 manipulation, with support from analyzing gamma-H2A.X using Western blot. This approach would investigate whether manipulating ATOX1 expression, a drug transporter, altered cisplatin transportation to the nucleus and determine if ATOX1 was the functional facilitator of miR-31 in regulating cisplatin resistance within the PDAC systems studied here.

4.9 Results

4.9.1 Overexpressing ATOX1 enhances cisplatin sensitivity in Panc-1 cells.

With a correlation found between miR-31 and ATOX1 expression possibly modulating chemosensitivity, further experiments were performed to elucidate if ATOX1 alone was the mediator for chemo-sensitizing PDAC cells. This was investigated by overexpressing ATOX1 in Panc-1 parental cells, independent of miR-31 modifications. ATOX1 overexpression was confirmed by western blot (**Figure 4.20A**). Clonogenic analysis revealed that overexpressing ATOX1 significantly reduced the surviving fraction (* $p = 0.0416$) post-cisplatin treatment compared to its vector control equivalent (**Figure 4.20B**). Where overexpressing ATOX1 reduced the surviving fraction by $9.2\% \pm 0.2\%$ in Panc-1 cells.

4.9.2 ATOX1 expression is associated with improved overall survival in PDAC.

PDAC patients with high ATOX1 expression have significantly improved overall survival rates compared to those with low ATOX1 expression (**** $p = 0.00094$, FDR=0.01). Patients with high ATOX1 expression are twice as likely to survive to any given time point compared to low expression (HR=2.08) (**Figure 4.21**). The choice of chemotherapy used was unknown and presents as a limitation.

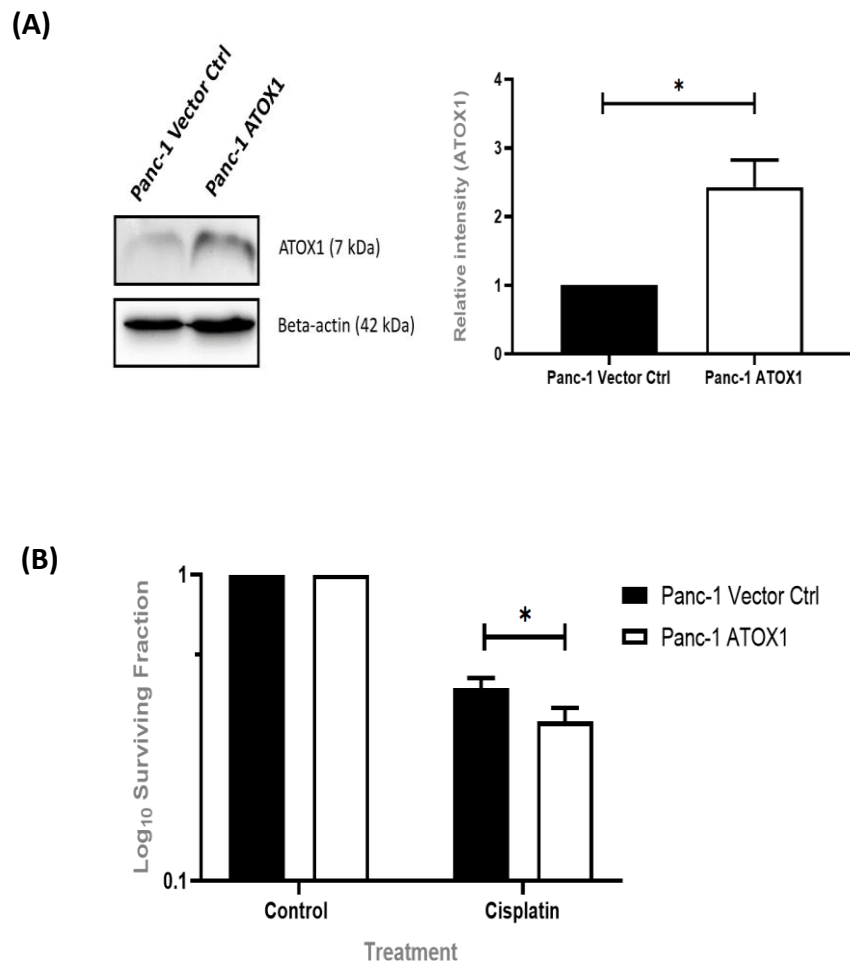


Figure 4.20 Overexpressing ATOX1 in Panc-1 cells enhances cisplatin sensitivity. **(A)** Representative western blot confirming ATOX1 overexpression in Panc-1 cells. Densitometry analysis revealed a significant increase in ATOX1 expression (* $p = 0.0353$) in Panc-1 ATOX1 cells compared to Panc-1 Vector Ctrl cells. Data are expressed as the mean $\pm$ SEM and analysed by a one-sample t.test. **(B)** Cells were treated with 1.38 μ M cisplatin for 24 h. Clonogenic analysis revealed overexpressing ATOX1 in Panc-1 cells significantly reduced surviving fraction (* $p = 0.0416$) compared to its vector control equivalent. Data are expressed as the mean $\pm$ SEM and was analysed by a two-tailed paired t.test ($n = 4$).

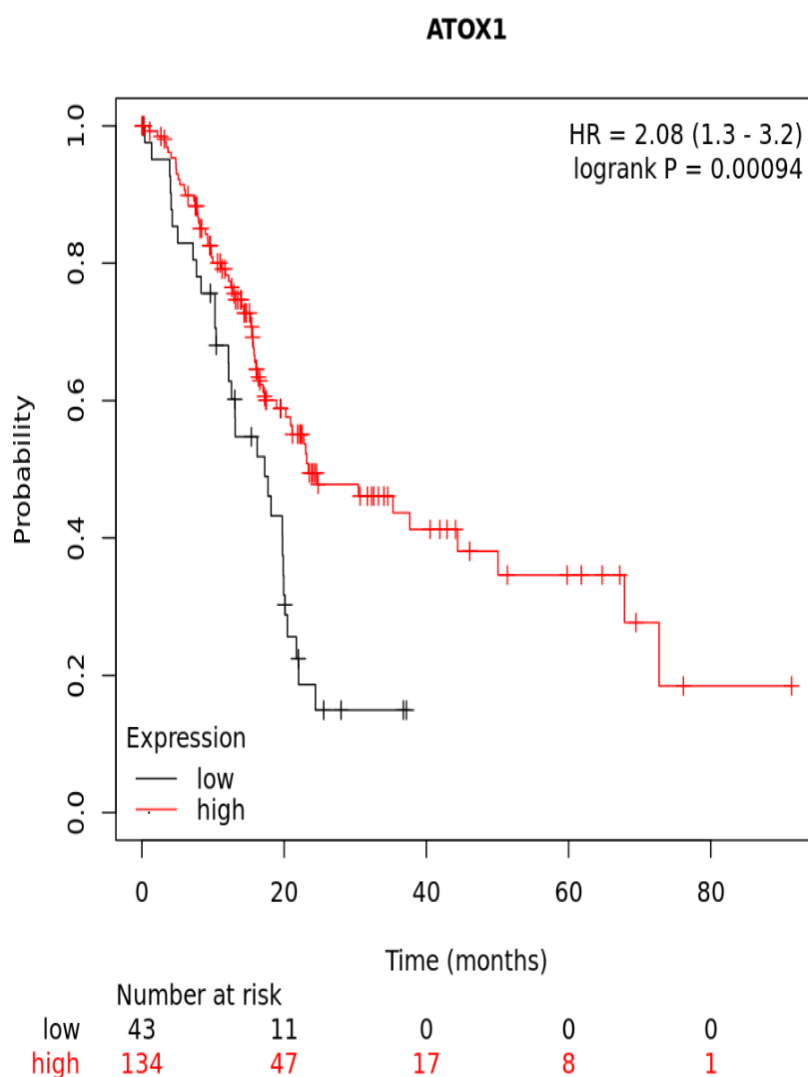


Figure 4.21 The effect of ATOX1 expression on survival in PDAC. The Kaplan-Meier plotter (PAN-cancer) was used to examine the effect of low and high ATOX1 expression on the overall survival of patients with PDAC ($n = 177$). To select the expression cut-off between the groups, all possible cut-off values between the lower and upper quartiles were computed, with the best performing cut-off selected. Cox proportional hazards regression analysis was performed to assess the relationship between ATOX1 expression levels and survival.

4.10 Discussion

Cisplatin treats many human malignancies, including ovarian, lung, and colon cancers [84]. Drug resistance and nephrotoxicity are the significant limitations of this commonly used anti-cancer drug [81, 352]. After being intravenously administrated, a considerable amount of cisplatin will bind with proteins in the blood. It appears that 24 h after infusion, the protein-binding rate for cisplatin is close to 99%, and the protein-platinum complex formed is generally irreversible [353, 354]. *In vitro* studies revealed how cisplatin can bind to serum albumin in blood plasma, haemoglobin, cytochrome c, and CopC [353-357]. Most often, large doses of cisplatin are needed to be administrated in the blood to assure sufficient cell uptake and to make it to the nucleus to target DNA and promote cell death. It is still unclear how much cisplatin reaches the nuclear compartment, but our findings suggest it is in the low micromolar ranges, despite large amounts getting into the cell cytoplasm [347]. It is widely accepted today that cellular copper (Cu) transporting proteins are involved in cisplatin uptake and efflux [82]. In humans, cellular Cu homeostasis is maintained by the Cu chaperone ATOX1, which obtains Cu from CTR1 and then delivers it to metal-binding domains of ATP7A and ATP7B in the secretory pathway [358]. It has been revealed that the metal-binding motif domains of common Cu transporters, such as ATP7B, are similar in structure to ATOX1, which suggests that this metallochaperone will also be able to interact with cisplatin.

Numerous reports revealed that the ATOX1 levels in cells influence their sensitivity to cisplatin. Safaei *et al.* [359] demonstrated that ATOX1-expressing cells (ATOX1 +/+) were slightly more sensitive to the cytotoxic effects of cisplatin than ATOX1 knockout cells (ATOX1 -/-). Additionally, to assess the impact of the loss of ATOX1 on the intracellular distribution of cisplatin, ATOX1 +/+ and ATOX1 -/- cells were exposed to cisplatin for 24 h and then subjected to subcellular fractionation, where the cytosolic and nuclear compartments were isolated, and the levels of Pt was

quantified. Similar levels of Pt were observed in the cytosolic compartments. However, the nuclear fractions of ATOX1 +/+ cells had significantly higher levels of Pt than those of ATOX1 -/- cells [359]. In this study, it was demonstrated that overexpressing PDAC cells with ATOX1 enhances cisplatin sensitivity and is correlated with improved survival; although not assessed here, one possible explanation is the increased cisplatin transport to the nucleus.

Based on the above observations and our findings, targeting copper transporters appear to be an innovative strategy for promoting cellular sensitivity to cisplatin. For instance, ammonium tetra-thiomolybdate (TM) is a copper chelator used to treat Wilson's disease [360]. It has also been shown to inhibit tumour growth due to its ant-angiogenic effect [361]. Mechanistic *in vitro* and *in vivo* investigations indicated that TM modulates copper levels by binding to copper proteins, such as metallothioneins [362]. An X-ray crystal structure displayed that TM binds to ATX1, an analogue of ATOX1 in yeast [363]. Additionally, one previous study showed that TM could enhance cisplatin sensitivity by increasing DNA platination in cancerous cells [364]. It was hypothesised that TM modulates cisplatin resistance by altering CTR1 expression [364, 365]. However, no changes in CTR1 mRNA levels were found, suggesting that other Cu-transporters may play an essential role in modulating cellular sensitivity to cisplatin. Undoubtedly, more research is needed to understand how ATOX1 modulates cisplatin sensitivity and how copper chelators such as TM can be combined with cisplatin to encourage DNA-induced cell death.

Generally, metal binding is believed to significantly lower the reactivity of metalloproteins towards platinum (Pt) compounds, especially when the metal shares similar binding sites as the Pt. Xi *et al.* [366] displayed that the binding of Cu and Pt to ATOX1 is not simply competitive but that Cu binding promotes the interaction of ATOX1 with Pt. Furthermore, the structural investigations on ATOX1 have shown that, upon Cu binding,

both Cys¹² and Cys¹⁵ show decreased dynamics, and their side chains become closer [367]. In addition, the more solvent exposure of Cys¹⁵ thiol in Cu-ATOX1 could make the cysteines more accessible for Pt binding [367]. This suggests that measuring intracellular Cu levels could also be critical to understanding the relationship between Pt-ATOX1 and cisplatin sensitivity.

It has previously been revealed that a crucial transcriptional factor, p53, plays a role in the Cu transport to the nuclei of HCT116 cells, where its accumulation can promote cell death [368]. Beaino *et al.* [350] displayed that p53 may affect the nuclear transport of Cu by increasing ATOX1 levels. Additionally, ATOX1 levels in HCT116 p53 knock-out cells are less than in HCT116 p53 expressing cells [350]. The effect of cisplatin on Cu nuclear localization in HCT116 p53 expressing cells and knockout cells was investigated. Cisplatin treatment increased nuclear localization of Cu in p53-expressing cells, likely due to the increased ATOX1 levels. Moreover, Beaino *et al.* [350] showed that HCT116 p53 +/+ are more sensitive to cisplatin than p53 -/- cells and undergo much more apoptosis and cell death. This study concludes that combining Cu and cisplatin may improve the efficiency of treating resistant tumours with wild-type p53 by increasing ATOX1 expression, therefore presenting an innovative therapeutic target for increasing the effectiveness of platinum agents in cancer, including PDAC.

Ultimately, we have shown that miR-31 modulates cisplatin resistance within *in vitro* studies of PDAC by altering ATOX1 expression (**Figure 4.22**). Additionally, when ATOX1 is overexpressed independently of miR-31, an increase in cisplatin sensitivity is observed, supporting the initial hypothesis. Altogether, this insinuates that ATOX1 expression is essential within the context of PDAC; nevertheless, further investigation is needed to determine if platinum-based agents use ATOX1 as a hitchhiker to the nucleus to promote DNA damage and, consequently, cell death.

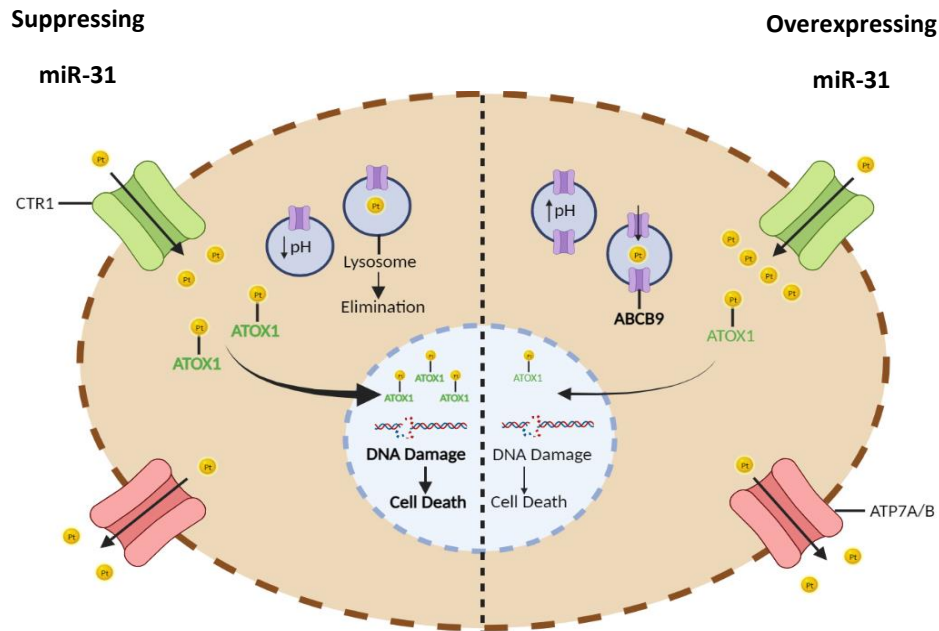


Figure 4.22 Summary of the effect of miR-31 manipulation and ATOX1 overexpression on PDAC cells. The PDAC cell (orange) displayed a chemo-resistant phenotype when miR-31 was overexpressed and a chemo-sensitive phenotype when miR-31 was suppressed. Cells that express higher levels of miR-31 reduced levels of the copper transporter, ATOX1, consequently decreasing the nuclear content of cisplatin (Pt). Overall, it can be noted that the increase in ATOX1 expression is essential within the context of PDAC chemosensitivity.

Chapter Five

MiR-31 regulates oxidative stress and radiosensitivity in PDAC.

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5.1 Introduction

Pancreatic cancer is one of the most lethal forms of human malignancy, having a 5-year survival rate of less than 7% [1, 2]. Pancreatic cancer is expected to become the second most common cause of cancer-related death in the United States by 2030, a trend reflected in Europe [369, 370]. Pancreatic ductal adenocarcinoma (PDAC) accounts for over 90% of all pancreatic cancer cases, with surgery being the only curative treatment. The symptoms associated with PDAC, such as abdominal pain or back pain, are notoriously vague and contribute to these cancers' late diagnosis and subsequent poor survival rates [371]. As a result, only 10–20% of patients are eligible for curative surgery due to late diagnosis [372]. Approximately 35% of patients present with locally advanced PDAC and will receive radiotherapy, an essential component of palliative treatment for patients with metastatic disease [370–372]. Unfortunately, tumour resistance to radiotherapy remains a significant clinical challenge in PDAC treatment and is poorly understood [78, 373]. The features frequently associated with radioresistance include alterations in DNA repair, proliferation, cell-cycle checkpoint control, apoptosis, and altered reactive oxygen species (ROS) biology [374, 375]. As such, elucidating the mechanisms of radioresistance in PDAC is essential for developing new therapeutic approaches to improve treatment efficacy and prolong patient survival. ROS are unstable oxygen-containing substances that display significant oxidative activity [376]. Radiotherapy generates ROS through the radiolysis of water within the cell [377].

The excessive amounts of ROS induced by radiotherapy account for about two-thirds of the DNA damage caused during treatment, resulting in cell damage and death [378, 379]. However, the cell has an antioxidant defense system that protects against oxidative damage caused by elevated ROS levels [380]. Nevertheless, alterations within these antioxidant defense systems have been associated with resistance to radiotherapy by negatively impacting the detoxification of excess ROS

[379-382]. MicroRNAs (miRs) are small (18–22 nucleotides), non-coding RNAs that regulate gene expression at the post-transcriptional level by predominantly targeting the 3' untranslated region (UTR) of target mRNAs, resulting in mRNA degradation and inhibition of protein translation **[163, 164]**. Importantly, due to imperfect complementarity, a single miR molecule has the potential to target multiple mRNAs simultaneously, making them attractive therapeutic targets **[165, 170]**. One of the key genetic events in PDAC development is the inactivation of the p16 tumour suppressor gene **[269]**. The p16 gene is encoded on chromosome 9p21.3, a recognised fragile site in the human genome **[270]**. Interestingly, microRNA-31 (miR-31) is encoded just downstream of p16, and as such, they are frequently co-deleted or co-disrupted together **[382]**. We have previously demonstrated that miR-31 is a useful therapeutic target regulating chemotherapy and radiotherapy sensitivity by altering drug transportation and DNA damage repair genes in other cancer types **[274, 265, 328]**. However, its role in regulating radiosensitivity in PDAC remains to be elucidated. In this study, we examine the role of miR-31 in radioresistance using PDAC cell lines of differing miR-31 statuses. For the first time, our results show that manipulating miR-31 expression in PDAC cells regulates sensitivity to clinically relevant doses of radiation by targeting an antioxidant enzyme, glutathione peroxidase 8 (GPx8), which plays a vital role in ROS detoxification. We demonstrate miR-31 as a suitable therapeutic target in PDAC by regulating sensitivity to radiotherapy via modulation of oxidative stress and DNA damage.

5.2 Rationale, Aims, and Objectives

The antioxidant GPx8 is downregulated upon miR-31 overexpression in BxPC-3 cells. The downregulation of GPx8 in association with increased expression of miR-31 suggests that PDAC cells expressing GPx8 at lower levels than baseline potentially is not as effective as eliminating ROS levels induced by radiotherapy. It is hypothesised that GPx8, as a miR-31 target, is the functional facilitator of the radio-resistant phenotype.

In chapter 3, we investigated the role of miR-31 in modulating sensitivity to radiotherapy and found that miR-31 suppressed the antioxidant GPx8 in BxPC-3 cells. In this chapter, we examine the role of GPx8 in modulating sensitivity to radiotherapy by altering ROS generation and DNA damage induction.

5.3 Experimental design

Silencing GPx8 in PDAC cell lines

GPx8 was silenced in the parental BxPC-3 cell line using si-RNA technology. The approach to independently silence GPx8 expression was adopted to view whether GPx8 contributed to altering radiosensitivity identified in previous chapters.

The effect of GPx8 on radiosensitivity

The clonogenic assay was utilized to determine the overall effect of radioresistance with GPx8 silencing, with support from the analysis of gamma-H2A.X using Western blot. Wholly this approach would facilitate the investigation of whether silencing GPx8, an enzymatic antioxidant, altered ROS elimination and therefore determine if GPx8 was the functional facilitator of miR-31 in regulating radiosensitivity within the PDAC systems studied here.

5.4 Results

5.4.1 Manipulating miR-31 Alters DNA Damage Induction and Repair in PDAC Cell Lines.

Radiation-induced cell death is frequently due to DNA damage, especially to double-strand DNA breaks (DSBs), and alterations in the DNA repair systems have been strongly associated with radioresistance. Having observed the differences in clonogenic survival, we examined the influence of miR-31 on DNA damage induction and repair by investigating the levels of gamma-H2A.X, which occurs at the sites of DSBs. It was found that overexpressing miR-31 in BxPC-3 cells significantly increased the levels of gamma-H2A.X 20 min post-radiation treatment (* $p = 0.0120$), whereas the levels of gamma-H2A.X are reduced at 4 h ($p = 0.932$) and 24 h ($p = 0.939$) post-radiation (**Figure 5.1**). Gamma-H2A.X levels were shown to be decreased in Panc-1 Zip-miR-31 cells; however, no significant differences were observed at 20 min ($p > 0.999$), 4 h ($p = 0.990$), or 24 h post-radiation treatment ($p = 0.664$), despite a trend being observed (**Figure 5.2**). Subsequently, to determine if the levels of DNA damage corresponded to cell death, apoptosis was assessed post-radiation treatment.

5.4.2 Manipulating miR-31 Alters Radiation-Induced Apoptosis in PDAC Cell Lines.

To study a possible cause of cell sensitivity to radiation treatment, we measured caspase 3/7 activity as a marker of apoptosis. Overexpressing miR-31 in BxPC-3 cells displayed no significant changes in caspase 3/7 at 20 min ($p > 0.999$) post-radiation treatment. However, a significant increase in caspase 3/7 activity was observed at 4 h (**** $p < 0.0001$) and 24 h (**** $p < 0.0001$) post-radiation treatment (**Figure 5.3**). Suppressing miR-31 in Panc-1 cells displayed no significant differences at 20 min post-radiation treatment ($p = 0.968$). However, a significant reduction in

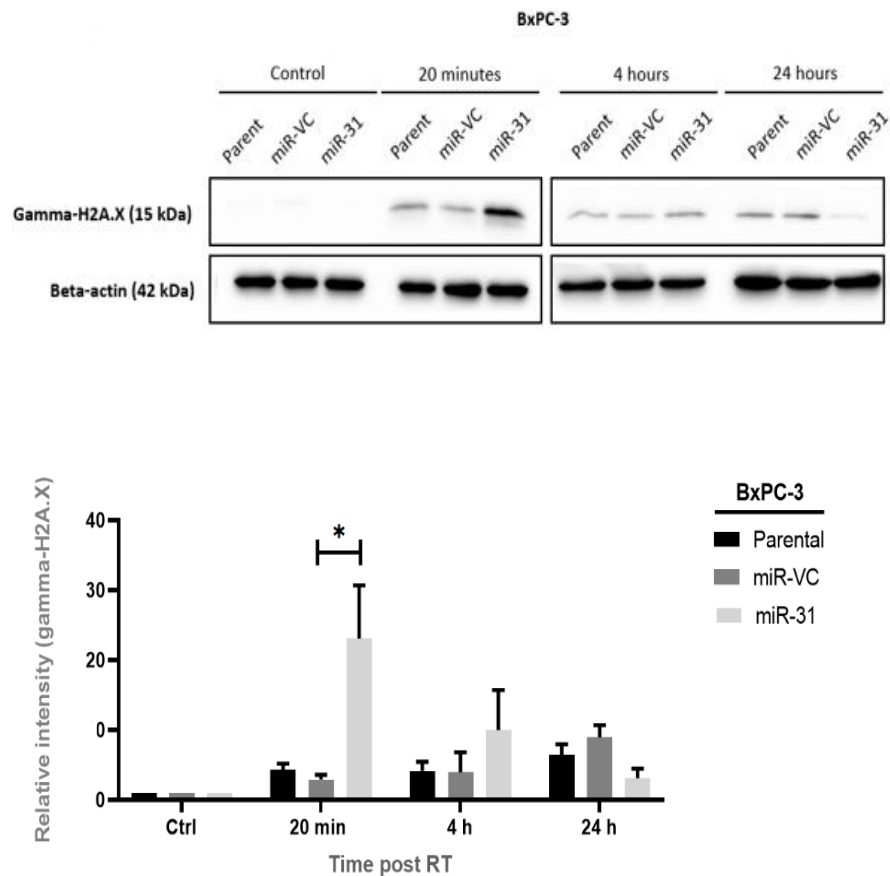


Figure 5.1 The expression of miR-31 positively correlates with DNA damage incurred when treated with radiation in BxPC-3 cells. Representative western blot time course and densitometry analysis for gamma-H2A.X as a marker of DNA damage with radiation treatment (RT) in BxPC-3 cells. A significant increase in gamma-H2A.X levels were observed in the BxPC-3 miR-31 cells 20 min (* $p = 0.0120$) post-RT. Interestingly, levels of gamma-H2A.X were reduced but no significant differences were observed at 4 h ($p = 0.932$) and 24 h ($p = 0.939$) post-RT. Data are represented as the mean $\pm$ SEM. Two-way ANOVA with Tukey's post-hoc test was adopted for statistical analysis ($n = 3$).

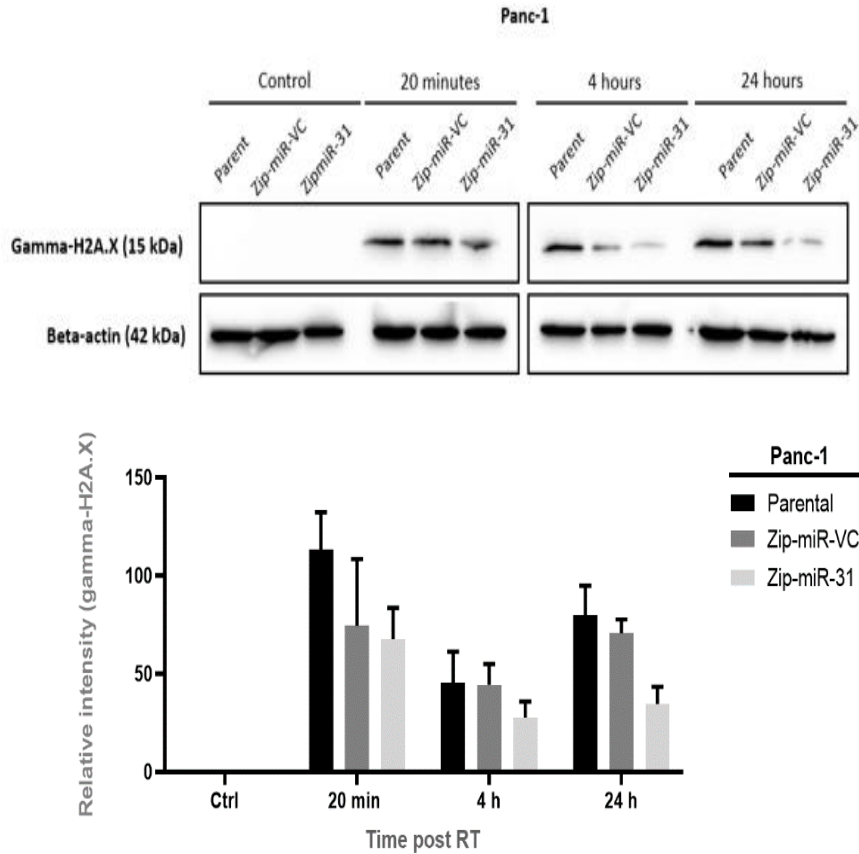


Figure 5.2 The expression of miR-31 positively correlates with DNA damage incurred when treated with radiation in Panc-1 cells. Representative western blot time course and densitometry analysis for gamma-H2A.X as a marker of DNA damage with RT (4 Gy) in Panc-1 cells. It is evident that levels of gamma-H2A.X decreased in Panc-1 Zip-miR-31 cells, however no significant differences were observed 20 min ($p > 0.999$), 4 h ($p = 0.990$), and 24 h post-RT ($p = 0.664$). Data are represented as the mean $\pm$ SEM. Two-way ANOVA with Tukey's post-hoc test was adopted for statistical analysis ($n = 3$).

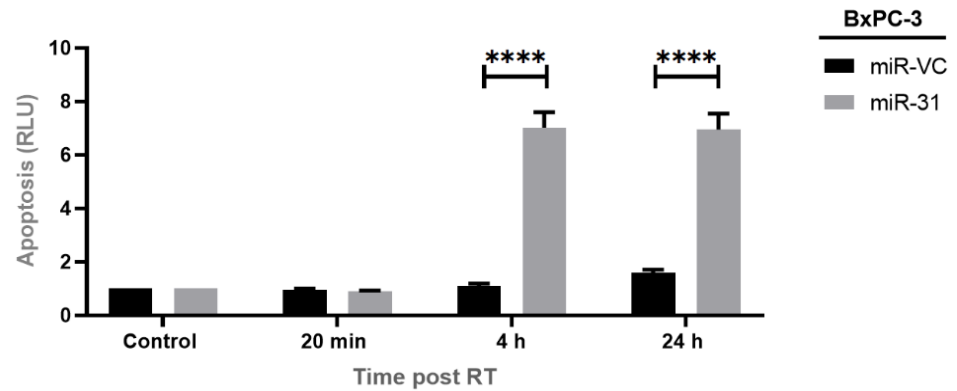


Figure 5.3 Overexpressing miR-31 increases apoptosis levels in BxPC-3 cells. Caspase 3/7 activity was measured as markers of apoptosis at 20 min, 4 h, and 24 h post-radiation treatment (RT). There were no significant differences in apoptosis in 20 min post-RT ($p > 0.999$) between BxPC-3 miR-VC and miR-31 cells. Although overexpressing miR-31 in BxPC-3 cells displayed a significant increase in apoptosis 4 h (**** $p < 0.0001$) and 24 h (**** $p < 0.0001$) post RT. Data are represented as the mean $\pm$ SEM. Two-way ANOVA with Tukey's post-hoc test was adopted for statistical analysis ($n = 3$).

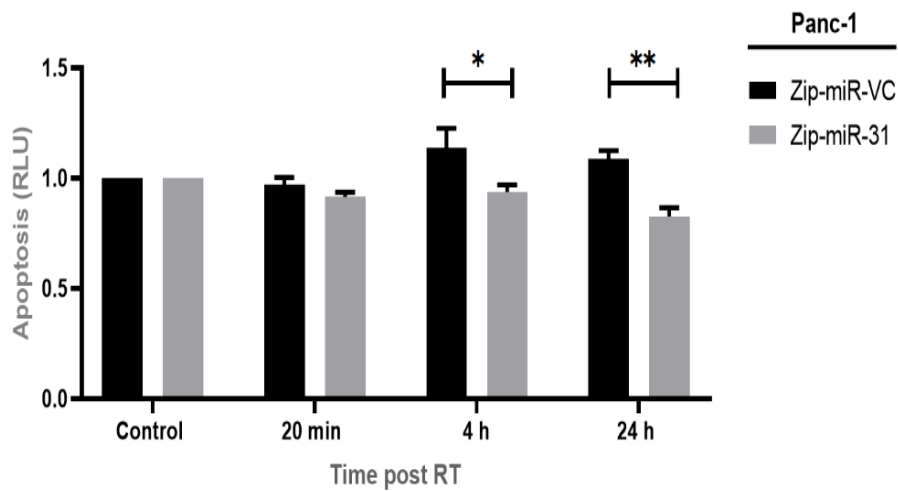


Figure 5.4. Suppressing miR-31 reduces apoptosis levels in Panc-1 cells.

Caspase 3/7 activity was measured as markers of apoptosis at 20 min, 4 h, and 24 h post-radiation treatment (RT). Panc-1 Zip-miR-VC and Zip-miR-31 cells. There are no significant differences in apoptosis 20 min post-RT between Panc-1 Zip-miR-VC and Zip-miR-31 cells ($p = 0.9368$). Although suppressing miR-31 in Panc-1 cells displayed a significant decrease in apoptosis 4 h (* $p = 0.0199$) and 24 h (** $p = 0.00630$) post RT. Data are represented as the mean $\pm$ SEM. Two-way ANOVA with Tukey's post-hoc test adopted for statistical analysis ($n = 3$).

caspace 3/7 activity was observed at 4 h (* $p = 0.0498$) and 24 h (** $p = 0.001$) post-radiation treatment (**Figure 5.4**).

5.4.3 Manipulating miR-31 Alters Reactive Oxygen Species (ROS) Levels in PDAC Cell Lines.

To determine whether the ROS levels contributed to DNA damage and potentially radioresistance within our models, we analysed ROS generation 20 min, 4 h, and 24 h post-radiation treatment and compared this to its untreated cells. It was found that overexpressing miR-31 in BxPC-3 cells resulted in a significant increase in ROS generation when treated with 4 Gy compared to its untreated control at 20 min (**** $p < 0.0001$) and 4 h (* $p = 0.0295$), while no significant increase was observed 24 h post-radiation treatment ($p = 0.0690$). The vector control equivalent displayed a significant increase in ROS generation at 20 min (** $p = 0.00180$) post-radiation treatment only (**Figure 5.5**). Furthermore, suppressing miR31 in Panc-1 cells resulted in a significant increase in ROS generation when treated with 4 Gy compared to the untreated control at 20 min (**** $p < 0.0001$) post-radiation treatment, but no significant increase was displayed at 4 h ($p > 0.999$) post-radiation treatment. Moreover, a significant increase was observed in the vector control equivalent at 20 min (**** $p < 0.0001$), 4 h (** $p = 0.00860$) and 24 h (** $p = 0.00670$) post-radiation treatment (**Figure 5.6**). Overall, these data indicate a role for miR-31-monitored ROS generation post-radiation treatment, subsequently impacting downstream DNA damage. A possible explanation for this is that miR-31 alters the levels of antioxidants, which are essential for scavenging ROS, resulting in their detoxification and elimination.

5.4.4 Manipulating miR-31 Does Not Alter Glutathione (GSH) Levels in PDAC Cell Lines.

The glutathione (GSH) levels were assessed 24 h post-radiation treatment. We observed no significant changes in the GSH between the

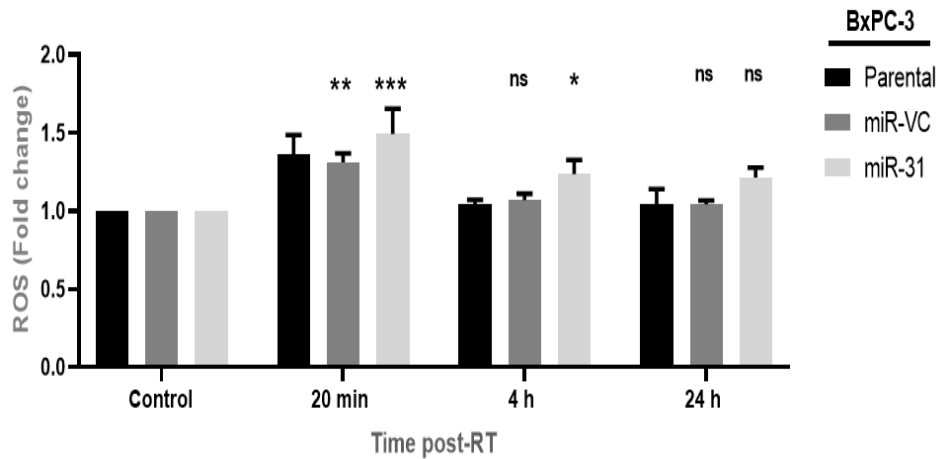


Figure 5.5 Overexpressing miR-31 alters ROS levels in BxPC-3 cells. ROS levels were assessed 20 min, 4 h, and 24 h post-radiation treatment (RT) and compared to its untreated control (0 Gy). Overexpressing miR-31 in BxPC-3 cells displayed a greater significance in ROS levels 20 min (** $p < 0.001$) and 4 h (* $p = 0.0295$) post-RT. Meanwhile, miR-VC cells only displayed a significant increase in ROS levels 20 min (** $p = 0.0018$) post-RT and were restored by displaying no significant differences 4 h and 24 h post-RT. Data are expressed as the mean $\pm$ SEM. Two-way ANOVA with Tukey's post-hoc test was adopted for statistical analysis ($n = 3$).

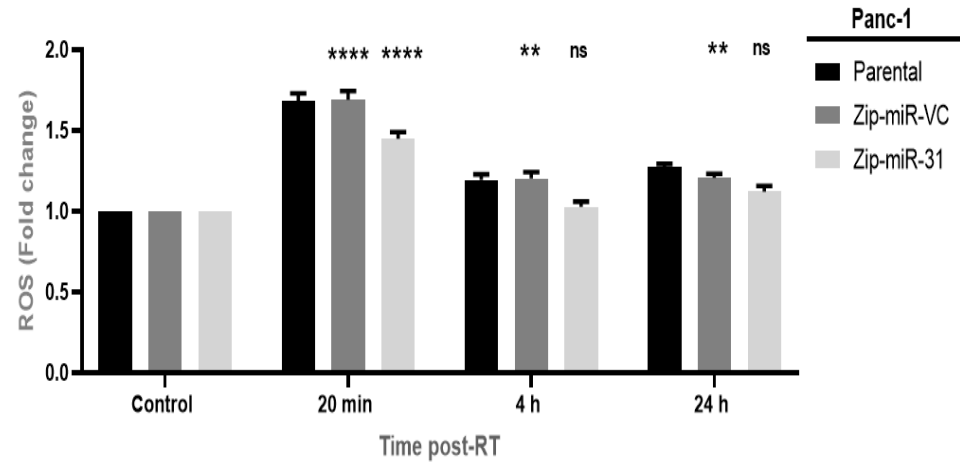


Figure 5.6 Suppressing miR-31 alters ROS levels in Panc-1 cell. ROS levels were assessed 20 min, 4 h, and 24 h post-radiation treatment (RT) and compared to its untreated control (0 Gy). Suppressing miR-31 in Panc-1 cells significantly increased ROS levels 20 min (**** $p < 0.0001$) post-RT but was restored by displaying no significant differences 4 h and 24 h post-RT. Whereas Zip-miR-VC cells displayed a significant increase in ROS levels 20 min (**** $p < 0.0001$), 4 h (** $p = 0.0086$) and 24 h (** $p = 0.0067$) post RT. Data are expressed as the mean $\pm$ SEM. Two-way ANOVA with Tukey's post-hoc test was adopted for statistical analysis ($n = 3$).

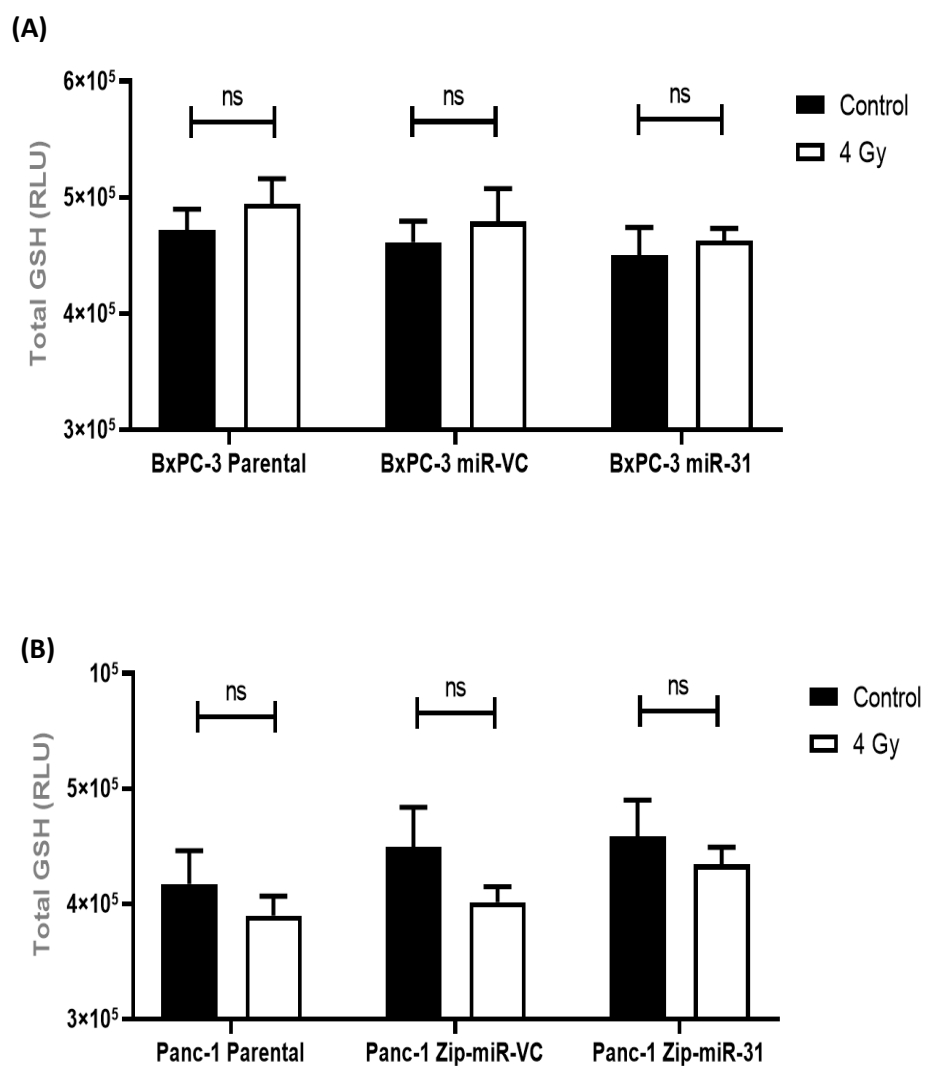


Figure 5.7 Manipulating miR-31 does not alter total glutathione (GSH) levels in PDAC cell lines. Total GSH in (A) BxPC-3 parental, miR-VC, and miR-31 cells, and (B) Panc-1 parent, Zip-miR-VC and Zip-miR-31 cells, post-radiation with 0 Gy and 4 Gy. No significant differences were observed between cells. Data are expressed as the mean $\pm$ SEM and was analysed by a two-tailed paired t.test ($n = 3$).

treated and untreated cells within both the BxPC-3 (**Figure 5.7A**) and Panc-1 (**Figure 5.7B**) models.

5.4.5 Overexpressing miR-31 Alters Glutathione Peroxidase 8 (GPx8) in PDAC Cell Lines.

The potential regulation of sensitivity to radiation treatment by miR-31 is attributed to its ability to alter the expression of its target genes. The miR target prediction algorithm TargetScan (http://www.targetscan.org/vert_72/), miRTargetLink (<https://ccb-web.cs.uni-saarland.de/mirtargetlink/>), and miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>) predicted that the 3'UTR of GPx8 mRNA contained putative miR-31 binding sites (**Figure 5.8A**). To determine whether miR-31 regulates radiosensitivity of PDAC cells by altering GPx8, levels of GPx8 in PDAC models were quantified by western blot. It was shown that overexpressing miR-31 significantly reduced GPx8 expression in BxPC-3 cells (* $p = 0.0279$) (**Figure 5.8B**). Whereas suppressing miR-31 in Panc-1 cells displayed a modest but no significant increase of GPx8 expression ($p = 0.947$) (**Figure 5.8C**).

5.4.6 Silencing GPx8 Enhances Radiosensitivity in BxPC-3 Cells.

With a correlation between the overexpressing miR-31 and GPx8 downregulation, possibly modulating radiosensitivity, further experiments were performed to elucidate if the GPx8 modification alone was sufficient for radio-sensitising PDAC cells. This was investigated by silencing GPx8 in BxPC-3 parental cells, independent of miR-31 modification. GPx8 silencing was confirmed by Western blot (**Figure 9**). The clonogenic analysis revealed that silencing GPx8 significantly reduced the surviving fraction (** $p = 0.00353$) post-radiation treatment compared to its scrambled control (**Figure 10**), indicating an influence of GPx8 on radiosensitivity in PDAC cells.

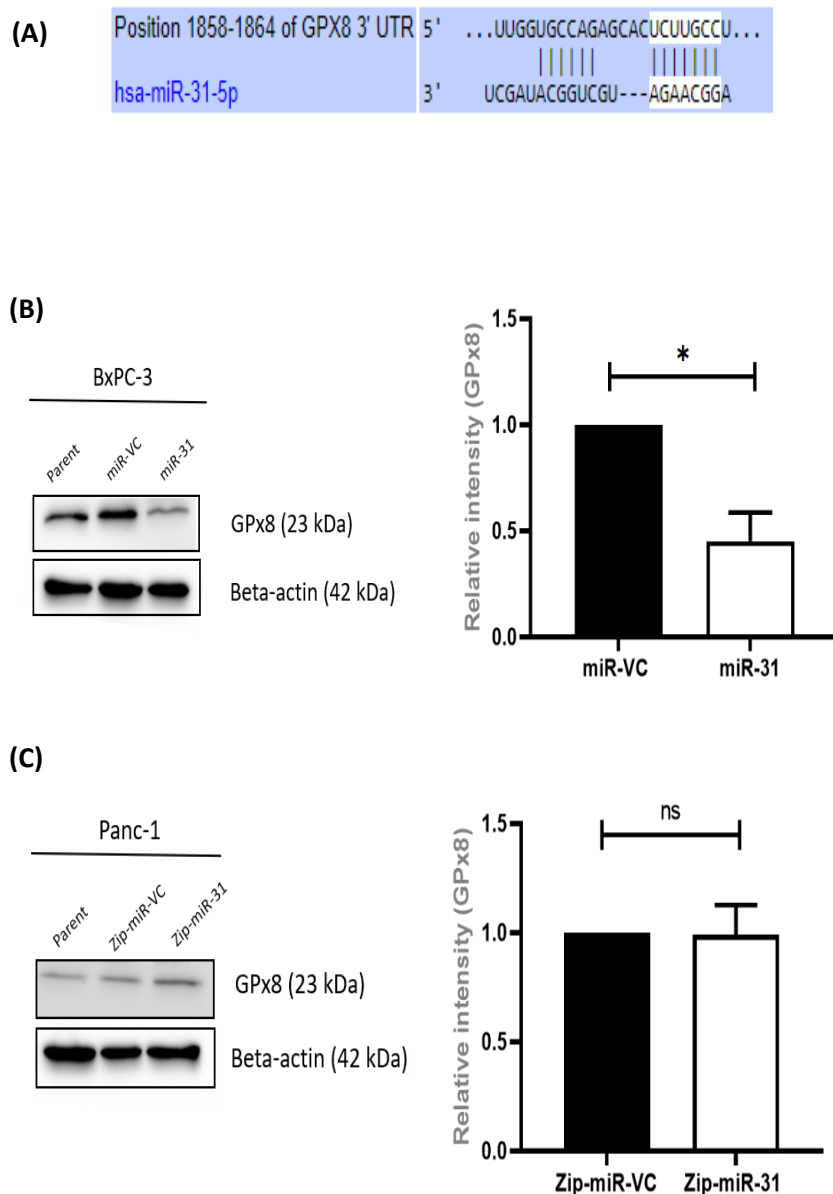


Figure 5.8 Manipulating miR-31 in PDAC cells alters the expression of GPx8.

(A) The communications between *GPx8* transcripts with miR-31-5p recognition sites. **(B)** Representative western blot illustrating GPx8 levels in BxP-C-3 models. Overexpressing miR-31 in BxPC-3 cells significantly reduced GPx8 levels compared to its vector control equivalent (* $p = 0.0279$). **(C)** Representative blot illustrating GPx8 levels in Panc-1 models. Suppressing miR-31 in Panc-1 cells does not significantly alter GPx8 levels compared to its vector control equivalent ($p = 0.947$). Data are expressed as the mean $\pm$ SEM and was analysed by a one-sample t.test ($n = 3$).

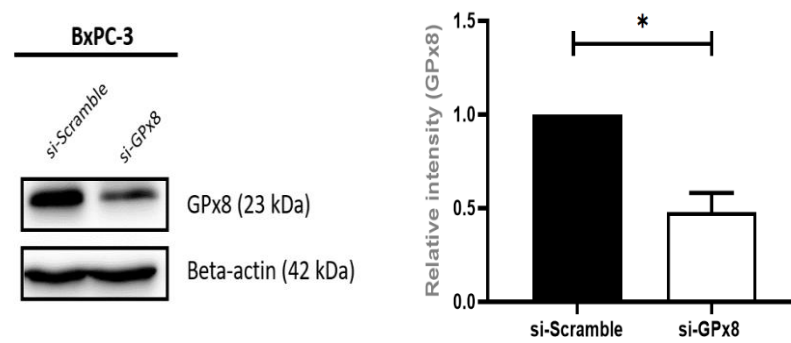


Figure 5.9 Confirmation of GPx8 silencing BxPC-3 cells. Representative western blot confirming GPx8 silencing in BxPC-3 cells. BxPC-3 cells were transiently transfected with either si-Scramble or si-GPx8 for 48 h. Densitometry analysis revealed a significant reduction in GPx8 expression (* $p = 0.0434$) in si-GPx8 cells compared to si-Scramble cells. Data are expressed as the mean $\pm$ SEM and analysed by a one-sample t.test ($n = 4$).

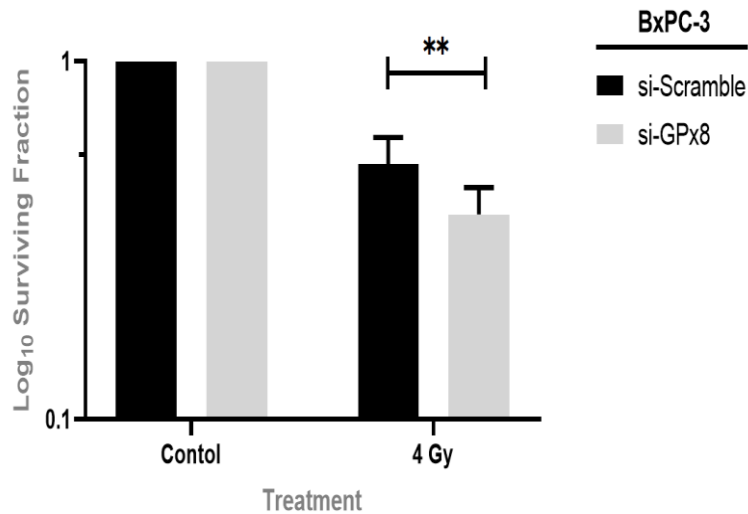


Figure 5.10 Silencing GPx8 in BxPC-3 cells enhances sensitivity to radiation treatment. Clonogenic analysis revealed that silencing GPx8 in BxPC-3 cells significantly reduced surviving fraction (** $p = 0.00353$) when compared to its scramble control. All cells were irradiated with 4 Gy whilst controls were mocked irradiated (0 Gy) 48 h post transfection. Data are expressed as the mean $\pm$ SEM and was analysed by a two-tailed paired t.test ($n = 7$).

5.4.7 Silencing GPx8 Alters Reactive Oxygen Species (ROS) in BxPC-3 Cells.

To determine if GPx8 altered ROS levels and, thus, the radiosensitivity in PDAC cells, we silenced GPx8 in the BxPC-3 parental cells and assessed the ROS levels at 20 min, 4 h, and 24 h post-radiation treatment (**Figure 5.11**). Here it was demonstrated that silencing GPx8 resulted in a significant increase in ROS levels at 20 min (**** $p < 0.0001$) when compared to its untreated control (0 Gy). Similarly, ROS levels were significantly increased in the scrambled control cells at 20 min post-radiation treatment (**** $p < 0.0001$). Interestingly, ROS levels were still significantly increased when silencing GPx8 at 4 h (** $p = 0.0073$) post-radiation treatment, but no significant changes were observed in the scrambled control cells at 4 h ($p = 0.934$) post-radiation treatment. A significant increase in ROS levels was observed at 24 h post-radiation in the si-GPx8 cells (** $p = 0.0004$) and the si-Scramble equivalent (** $p = 0.0002$).

5.4.8 GPx8 Protects BxPC-3 Cells against DNA-Damage Post-Radiation Treatment.

As GPx8 expression was associated with radioresistance, potentially by promoting ROS detoxification compared to the cells with lower GPx8 levels, gamma-H2A.X was assessed at 20 min and 4 h post-radiation treatment to determine if silencing GPx8 affected DNA damage (**Figure 5.12**). A trend indicated that silencing GPx8 in BxPC-3 cells increased the gamma-H2A.X levels compared to its scrambled control 20 min post-radiation treatment. However, no statistical significance was observed. Nevertheless, GPx8 potentially protects cells from radiation treatment by eliminating ROS, which is linked to reduced levels of DNA damage and enhanced cell survival.

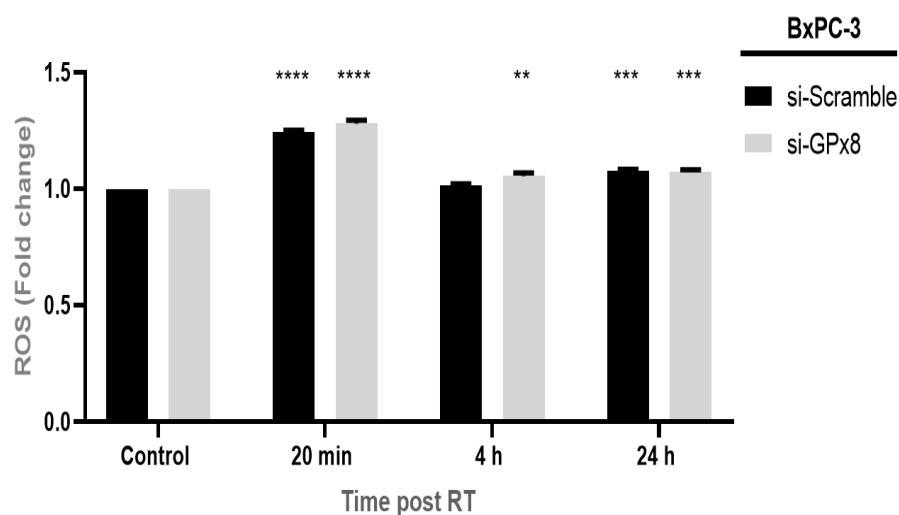


Figure 5.11 Silencing GPx8 in BxPC-3 cells alters ROS levels post-radiation treatment. ROS levels were assessed 20 min, 4 h, and 24 h post-radiation treatment (RT) and compared to its untreated control (0 Gy). Silencing GPx8 in BxPC-3 cells displayed a significant increase in ROS levels 20 min (**** $p < 0.0001$), 4 h (*** $p = 0.0073$), and 24 h (*** $p = 0.0004$) post-RT. The scramble control equivalent displayed a significant increase in ROS levels 20 min (**** $p < 0.0001$) post-RT and displayed no significant increase 4 h ($p = 0.934$); however, a significant increase was observed 24 h (*** $p = 0.0002$) post-RT. Data are expressed as the mean $\pm$ SEM. Two-way ANOVA with Tukey's post-hoc test was adopted for statistical analysis ($n = 3$).

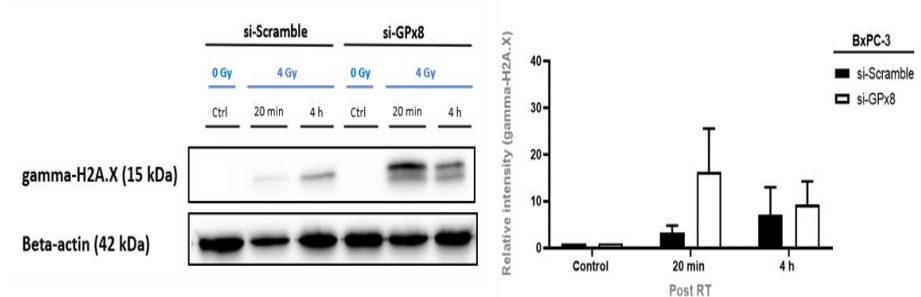


Figure 5.12 Silencing GPx8 in BxPC-3 cells increases gamma-H2A.X levels post-radiation treatment. Representative western blot time course and densitometry analysis for gamma-H2A.X as a marker of DNA damage with radiation treatment (RT) in si-GPx8 and si-Scramble BxPC-3 cells. A trend indicated that gamma-H2A.X levels were increased at 20 min and 4 h post-RT in BxPC-3 cells with GPx8 silenced compared to its scramble control ($n = 3$). However, no statistical significance was found. Two-way ANOVA with Tukey's post-hoc test adopted for statistical analysis, comparing si-GPx8 BxPC-3 cells to si-Scramble BxPC-3 cells at 20 min post-RT ($p = 0.461$) and 4 h post-RT ($p = 0.999$).

5.5 Discussion

Radiotherapy continues to be a central pillar of treatment for all solid tumour types, with over a third of PDAC patients receiving radiotherapy at some point during their disease course [383]. Unfortunately, radioresistance is one of the leading causes of poor prognosis in patients with PDAC, and as such, investigating the mechanisms underlying this radioresistance is crucial for the improvement of treatment strategies and patient survival.

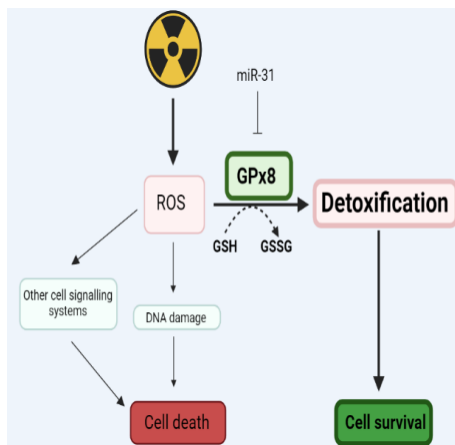
ROS levels play an essential role in cell-cycle progression and proliferation [384]. Additionally, studies have shown how ROS are associated with apoptosis, metabolism, and hypoxic signalling [384, 385]. ROS accumulation can give rise to oxidative stress, resulting in DNA damage and cell death [86]. Moreover, it is well known that ROS-mediated DNA damage is the primary source of cell death caused by radiotherapy [86, 386]. Nevertheless, the cellular antioxidant defence system can help to regulate oxidative stress by reducing excess ROS and promoting DNA repair [387]. However, dysregulation within these defence systems can result in resistance to anti-cancer therapies [388]. Glutathione peroxidases (GPx) are a family of enzymatic antioxidants that play an essential role in ROS detoxification, particularly hydrogen peroxide (H_2O_2), using reduced glutathione (GSH) as its substrate [389]. Additionally, it is well-established that the GPx family protects cells from DNA damage caused by excessive ROS [390]. To date, eight different GPx family members (GPx1-GPx8) have been identified [391], and recent studies have demonstrated that several members of the GPx family play a crucial role in resistance to anti-cancer therapies by altering levels of oxidative stress [392]. GPx8 is a membrane protein located on the endoplasmic reticulum (ER) and is a molecular gatekeeper that plays a vital role in regulating H_2O_2 , where the knockdown of GPx8 in HEK-293 cells encourages ER stress and decreased cellular viability [393]. Zhang *et al.* [394] showed that GPx8 promotes migration and invasion, where high

expression of GPx8 in lung cancer was correlated with a worse clinical outcome and prognosis. Additionally, a recent study demonstrated GPx8 as a critical player in a metabolic-inflammatory pathway that acts as a robust regulator of cancer cell aggressiveness [395]. Despite recent research elucidating the different biological functions of GPx8, its role in regulating radiosensitivity in cancer remains largely unexplored. Emerging evidence has demonstrated miRs as essential regulators of cancer initiation, promotion, progression, and resistance to anti-cancer therapies, including radiotherapy [396]. MiR-31 has been shown to act as either an oncogene or a tumour suppressor gene depending on the cancer type [271] and has been reported to be underexpressed in patients with PDAC [278]. Recent studies have revealed how miR-31 can influence invasion and migration in various cancers [397, 398] and how it plays a vital role in regulating sensitivity to anticancer therapies [274, 275]. However, its role in regulating radiosensitivity in PDAC remains to be investigated. It was shown that modulating miR-31 in PDAC cell lines can regulate radiosensitivity and the levels of DNA damage. Overexpressing miR-31 resulted in a reduction of DNA damage at 24 h post-radiation treatment; this may be explained by the promotion of DNA damage repair in the surviving cells or due to the failure of generating detectable gamma-H2A.X due to a large amount of cell death. Consequently, caspase 3/7 activity was measured as a marker of apoptosis to control for the discrepancy found between radiosensitivity and reduced DNA damage. It was found that overexpressing miR-31 in BxPC3 cells displayed substantial caspase 3/7 activity at 4 h and 24 h post-radiation treatment, indicating that the levels of gamma-H2A.X were difficult to detect and quantify due to the large amounts of cell death occurring at these time points. In comparison, suppressing miR-31 in Panc-1 cells displayed a significant reduction in caspase 3/7 activity 4 h and 24 h post-radiation treatment, indicating that suppressing miR-31 reduces the rates of apoptosis post-radiation treatment. Moreover, this may explain the differences observed within the accumulated cell counts

recorded on day three and day six post-radiation treatment. ROS have been demonstrated as critical regulators of radiosensitivity in cancer and are known to promote DNA damage and cell death. We analysed H_2O_2 generation, a primary type of ROS in PDAC cells. It was demonstrated here that H_2O_2 was elevated 20 min post-radiotherapy but was quickly returned to baseline by 4 h and 24 h post-radiotherapy when suppressing miR-31 in Panc-1 cells. By comparison, the H_2O_2 levels were significantly elevated at 20 min and 4 h post-radiotherapy when overexpressing miR-31 in BxPC-3 cells—indicating that cells with low miR-31 are better equipped at detoxifying ROS post-radiotherapy, thus promoting a radioresistant phenotype. Elevated levels of GSH are known to be associated with radioresistance by detoxifying excessive ROS [399], although it was displayed that levels of GSH remained unaltered across the PDAC cell lines, even post-radiotherapy; suggesting that miR-31 does not affect the GSH levels; therefore, playing no biological role in regulating miR-31-regulated radiosensitivity in PDAC. The mechanisms linking ROS and miR in regulating therapeutic resistance in PDAC are still unclear. However, using specific miRs for targeting antioxidant defence systems has been an area of thriving potential for improving cancer treatments. Pajic *et al.* [400] presented miR-139-5p as a potent modulator of radiotherapy in breast cancer by targeting multiple DNA repair genes and ROS defence pathways. MiR-17-3p has been revealed to target antioxidant enzymes, including GPx2, thus enhancing radiosensitivity in prostate cancer [401]. Furthermore, miR-153 was demonstrated to downregulate GPx1, leading to radioresistance in glioma stem cells [402]. Here, it was revealed that miR-31 alters the expression of the antioxidant enzyme GPx8, where overexpressing miR-31 significantly reduces GPx8 levels, potentially resulting in a loss of its ability to detoxify ROS effectively, thus promoting DNA damage and cell death. However, suppressing miR-31 showed no significant increase in GPx8, despite detoxifying ROS effectively and displaying reduced DNA damage. The potential method by which miR-31 alters GPx8 and

regulates radiosensitivity in PDAC cells is summarised in **Figure 5.13**. Finally, we aimed to determine whether GPx8, independent of miR-31, contributed to PDAC radiosensitivity. It was found that silencing GPx8 in the BxPC-3 parental cells enhanced radiosensitivity. Additionally, GPx8 expression protects cells from radiation treatment by detoxifying ROS more efficiently and is associated with reduced levels of DNA damage. These findings can be used for further research aimed at targeting antioxidants using miRNAs to improve the efficiency of radiotherapy for the treatment of PDAC. This study has assessed miR-31's influence on radiosensitivity in the in vitro PDAC cell models. Analyses of miR-31 expression in pre-treatment patient-derived tumour samples, stratified into good and poor response groups, would considerably add to the impact of this study. The patient-derived samples could be used to evaluate miR-31 and GPx8 expression as predictive biomarkers.

(A)



(B)

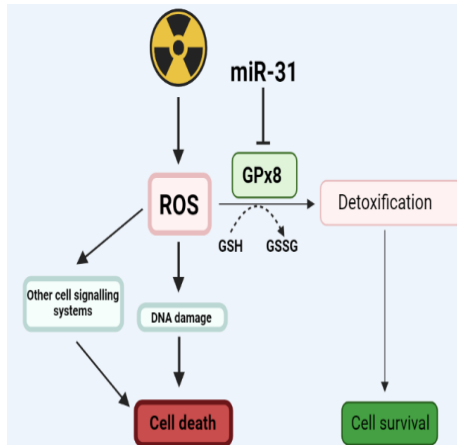


Figure 5.13 An illustration displaying how miR-31 can regulate levels of ROS by targeting GPx8. Radiation treatment can cause an increase in intracellular reactive oxygen species (ROS), which can target specific cell signalling systems and induce DNA damage directly, resulting in cell death. Glutathione peroxidase 8 (GPx8) is an enzymatic antioxidant that helps detoxify excessive ROS by converting reduced glutathione (GSH) into its oxidised form (GSSG), therefore promoting cell survival. **(A)** Low levels of miR-31 in PDAC cells result in an increase in GPx8, supporting ROS detoxification and encouraging cell survival. **(B)** Whereas high levels of miR-31 in PDAC cells can reduce GPx8. Moreover, reducing GPx8 can result in ROS accumulation as ROS is being detoxified less efficiently, so promoting cell death.

Chapter Six

Concluding discussion

6.1 Conclusion discussion

PDAC is a highly malignant tumour with an extremely poor prognosis [1, 369]. Chemotherapy-acquired drug resistance and radioresistance are the main issues facing PDAC patients without surgical opportunities and undergoing postoperative treatments. Therefore, it is of great clinical significance to conduct in-depth research on the molecular mechanism of action, underpin pathways associated with treatment resistance of PDAC, and explore new treatment methods. In this study, we aimed to understand why PDAC cells are resistant to chemotherapy and radiotherapy and to expand a clinically viable route by which sensitivity could be enhanced. A group of noncoding molecules named microRNAs (miRNA/s) are established regulators of many cancer pathways [177]. Studies have shown that miRNAs can induce tumour cell drug resistance through different mechanisms.

An interesting miRNA, miR-31, is encoded on a fragile site and is frequently dysregulated in cancer, including PDAC [271, 279, 382], which led to the potential for miR-31 to be functionally investigated in the context of resistance to chemotherapy and radiotherapy. MiR-31 has been shown to act as either an oncogene or a tumour suppressor gene depending on the cancer type and has been reported to be underexpressed in patients with PDAC [271, 279]. However, miR-31 has also been upregulated in primary PDAC cancers and was recently associated with poor prognosis [271, 279]. The present study has determined that miR-31 expression in PDAC cells promotes resistance to platinum-based therapy, although it enhances sensitivity to anti-metabolite-based therapy and radiotherapy *in vitro*.

Radiotherapy continues to be a central pillar of treatment for all solid tumour types, with over a third of PDAC patients receiving radiotherapy at some point during their disease course [383]. Unfortunately, radioresistance is one of the leading causes of poor prognosis in patients with PDAC. As such, investigating the mechanisms underlying this

radioresistance is crucial for improving treatment strategies and patient survival. GPx is a family of enzymatic antioxidants that play a vital role in ROS detoxification, particularly hydrogen peroxide (H₂O₂) [389]. Additionally, it is well-established that the GPx family protects cells from DNA damage caused by excessive ROS [390]. GPx8 is a membrane protein located on the endoplasmic reticulum (ER) and is considered a molecular gatekeeper that plays a vital role in regulating H₂O₂, where the knockdown of GPx8 in HEK-293 cells encourages ER stress and decreased cellular viability [393]. Zhang *et al.* [394] showed that GPx8 promotes migration and invasion, where high expression of GPx8 in lung cancer was correlated with a worse clinical outcome and prognosis. In this study, it was demonstrated that manipulating miR-31 expression in PDAC cell lines can regulate radiosensitivity and the levels of DNA damage by delaying how fast ROS can be eliminated post-treatment. Furthermore, we reveal that miR-31 alters the expression of the antioxidant enzyme GPx8, where overexpressing miR-31 significantly reduces GPx8 levels, potentially resulting in losing its ability to detoxify ROS effectively, thus promoting DNA damage and cell death. In addition, silencing GPx8 in PDAC cells increased radio sensitivity. These findings can be used for further research to target antioxidants using miRNAs to improve radiotherapy's efficiency in treating PDAC.

Platinum-based drugs are among the most active anticancer agents. They are used as a single agent or in combination with other chemotherapeutic agents and radiotherapy to manage a broad spectrum of human malignancies, including PDAC [64, 84]. Although most patients initially respond well to platinum-based chemotherapy, many develop drug resistance and relapse and succumb to their disease. Platinum resistance is considered multi-factorial and includes both mechanisms that limit the formation of platinum–DNA adducts and mechanisms which prevent cell death following drug treatment. It is well established that reduced cellular accumulation of platinum, either by impaired uptake or increased

efflux, is often found in cells selected for drug resistance, both *in vivo* and *in vitro*, and is generally considered one of the most consistent characteristics of platinum-resistant cells [304].

In this study, a higher amount of platinum in miR-31 expressing cells and a reduction in the concentration of platinum in the nuclear fraction were observed. Yet, the question remained as to how cells could survive an increased intracellular concentration of cisplatin. The movement of platinum-based agents within the intracellular environment is generally characterised by the copper transporters CTR1, ATP7A, and ATP7B, which are known to play significant trafficking roles [311, 322]. Although no significant changes are observed here for the influx and efflux transporters CTR1, ATP7A, and ATP7B, there may still be contributions to the overall phenotypic resistance observed with miR-31 overexpression.

Substantial evidence supports that miRNAs can mediate cellular sequestration by altering lysosomal activity [313-315]. Here, a modest change in lysosomal accumulation was observed; this promoted the investigation of possible drug transporters bound to lysosomes and encouraged the study of whether miR-31 expressing cells had a higher aggregate burden of lysosomes. Pennati *et al.* [403] showed that miR-205 replacement in prostate cancer cells caused an enhancement of cisplatin cytotoxic activity *in vitro* and *in vivo* because of down-regulated lysosome function and protein trafficking, leading to alterations in the autophagic flux of cells. Drayton *et al.* [404] demonstrated that overexpressing miR-27a in cisplatin-resistant bladder cancer cells reduced the expression of the cysteine and glutamate exchanger SLC7A11. This supports the findings that miRNA can regulate cellular transporters, signifying cellular chemoresistance regulation.

Additionally, the lysosomally bound drug transporter ABCB9 has been identified as a modulator of resistance, with the dysregulation of the protein enhancing or reducing response to therapeutics [311, 330]. In this study, ABCB9 appears to be increased with the miR-31 overexpressing

more cisplatin-resistant phenotype, although no differences in cisplatin accumulation were found in ABCB9 high-expressing cells compared to ABCB9 low-expressing cells; indicating that ABCB9 modulating drug resistance is cancer-specific or that the current technology is limiting and is too challenging to determine the lysosomal content of cisplatin in PDAC cells accurately.

Moreover, lysosomes are acidic organelles, and a strict pH is required to function correctly. A previous study demonstrated that a low pH within lysosomes is linked to cisplatin sensitivity. Here It was found that miR-31-expressing cells have a higher pH within lysosomes and are associated with a cisplatin-resistant phenotype, raising the question of whether preventing lysosomal acidification protects cells against cisplatin treatment. However, it was found that pre-treating cells with BA1, a V-ATPase inhibitor responsible for pumping protons into the lysosomal compartment, thus reducing the pH, enhanced cisplatin sensitivity. This finding suggests that BA1 may alter other cisplatin-resistant pathways, such as autophagy [405]. Nevertheless, miR-31-expressing cells appeared to be more sensitive to BA1 treatment and may present an area for future research.

Emerging evidence has revealed that multiple members of the nucleocytoplasmic transport system are deregulated in cancers and malignant tissues [406] and may present an attractive research area for understanding the mechanisms behind drug resistance, particularly platinum-based drugs. For example, Wang *et al.* [407] showed that more cisplatin accumulated within the nuclei of parental cisplatin-sensitive ovarian cancer cell lines compared to cisplatin-resistance cell lines, highlighting the importance of transportation of drugs from the cytoplasmic to the nuclear compartment and its involvement with resistance to therapy. Additionally, our findings show that miR-31 enhances sensitivity to radiation treatment, 5-FU, and gemcitabine,

whereas miR-31 promotes resistance to cisplatin. This suggests that miR-31 is affecting a pathway specific to platinum-based agents.

It has been reported that less than 1% of cisplatin reaches the nucleus of cancer cells to have an impact, suggesting that drug trafficking and sequestration play a vital role in promoting cisplatin resistance [347]. ATOX1 is a copper chaperone and has been reported to shuttle copper to the nucleus [348]. Interestingly, it has been revealed that cisplatin can bind to the copper-binding site of ATOX1. Initially, it was thought copper was unnecessary because cisplatin could still bind to ATOX1 in its absence. However, recent research revealed that the presence of copper helps form the cisplatin-ATOX1 complex, which may explain why cisplatin favours the copper transport system over other modes of transport. In this study, it was found that miR-31 reduces levels of ATOX1, and overexpressing ATOX1 in PDAC cells reduced the survival fraction post cisplatin treatment.

We have shown that miR-31 is an innovative and suitable therapeutic target for overcoming resistance to treatment in PDAC. Although miR-31 presents itself as a double-edged sword, it can enhance sensitivity to radiation treatment, 5-FU, and gemcitabine, while increasing platinum-based agents' resistance, particularly cisplatin. Nonetheless, a potentially novel mechanism behind enhanced resistance, which may potentiate a modified treatment strategy in the future, has been uncovered. Many PDAC patients are inherently resistant to treatment, and most have a poor prognosis; this has driven the field to find an alternative therapy or indeed enhance the ability of the readily available therapeutics to combat this disease [373]. Prospectively, the consequence of further investigating this mechanism within an *in vivo* system may lead to the ability to screen patients for miR-31 status. Patients who express high levels of miR-31 could potentially be stratified to have an antagomir administered to suppress miR-31 expression, which could mean the efficiency of platinum-based chemotherapy cytotoxicity would be

enhanced. But in the context of radiation and anti-metabolite treatment, patients who express low levels of miR-31 could be administered mimics to overexpress miR-31, which could increase cell death.

6.2 Future work

Firstly, with the observed increase in resistance to platinum-based agents and increased sensitivity to radiotherapy and anti-metabolite agents with miR-31 overexpression in PDAC *in vitro*, it would be beneficial to develop a 3- dimensional based system to mimic that of the 2-dimensional model utilised within this study, with the potential to move forward to an *in vivo* model that could explicate cisplatin and radiation sensitivity at a more clinically relevant level. Additionally, this allows *in vivo* studies to better visualise potential interactions, improving their safety, toxicity, and efficacy predictions.

Secondly, analyses of miR-31 expression in pre-treatment patient-derived tumour samples stratified into good and poor response groups would considerably add to the impact of this study. The patient-derived samples could be used to evaluate miR-31 and GPx8/ATOX1 expression as predictive biomarkers of response to cancer therapy.

Furthermore, a screen for miR-31-regulated genes and proteins by transcriptome and proteome analysis, respectively, may be ideal. This investigation would broadly identify miR-31 targets, which could be further analysed by pathway analysis to identify potential new avenues to chemoresistance and radioresistance.

Because miR-31 has the potential to target multiple pathways to modulate sensitivity to chemotherapy and radiotherapy, other pathways which can regulate resistance to treatment are still unknown; pathways that could be potentially more effective than the mechanisms investigated in this study. Exploring the lysosomal and cytosolic pH in more depth is an attractive area of research to understand the pharmacodynamics of drugs used to treat PDAC and to investigate the

acid dissociation constant (pKa) of these drugs, which can alter their function due to changes in pH.

Moreover, further research is needed to understand the mechanisms behind 5-FU and gemcitabine resistance, which frequently treat PDAC tumours. Recent evidence suggests that deficiency in DNA repair proteins confers susceptibility to DNA damage, making cancer cells vulnerable to various cancer chemotherapies. 5-FU is an anticancer nucleoside analogue that inhibits thymidylate synthase and causes DNA damage via the misincorporation of FdUTP and dUTP into DNA under the conditions of dTTP depletion. Interestingly, miRNAs, particularly miR-31, can inhibit thymidylate synthase and dUTPase function, thus increasing cell death by promoting uracil misincorporation.

References

- [1]. Rawla, P., Sunkara, T., & Gaduputi, V. (2019). **Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors.** *World journal of oncology*, 10(1), 10–27. <https://doi.org/10.14740/wjon1166>
- [2]. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). **Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries.** *CA: a cancer journal for clinicians*, 71(3), 209–249. <https://doi.org/10.3322/caac.21660>
- [3]. Rahib, L., Smith, B. D., Aizenberg, R., Rosenzweig, A. B., Fleshman, J. M., & Matrisian, L. M. (2014). **Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States.** *Cancer research*, 74(11), 2913–2921. <https://doi.org/10.1158/0008-5472.CAN-14-0155>
- [4]. McGuigan, A., Kelly, P., Turkington, R. C., Jones, C., Coleman, H. G., & McCain, R. S. (2018). **Pancreatic cancer: A review of clinical diagnosis, epidemiology, treatment, and outcomes.** *World journal of gastroenterology*, 24(43), 4846–4861. <https://doi.org/10.3748/wjg.v24.i43.4846>
- [5]. Oberstein, P. E., & Olive, K. P. (2013). **Pancreatic cancer: why is it so hard to treat?** *Therapeutic advances in gastroenterology*, 6(4), 321–337. <https://doi.org/10.1177/1756283X13478680>
- [6]. Li D. (2012). **Diabetes and pancreatic cancer.** *Molecular carcinogenesis*, 51(1), 64–74. <https://doi.org/10.1002/mc.20771>
- [7]. Berrington de González, A., Spencer, E. A., Bueno-de-Mesquita, H. B., Roddam, A., Stolzenberg-Solomon, R., Halkjaer, J., Tjønneland, A., Overvad, K., Clavel-Chapelon, F., Boutron-Ruault, M. C., Boeing, H., Pischon, T., Linseisen, J., Rohrmann, S., Trichopoulou, A., Benetou, V., Papadimitriou, A., Pala, V., Palli, D., Panico, S., Riboli, E. (2006). **Anthropometry, physical activity, and the risk of**

pancreatic cancer in the European prospective investigation into cancer and nutrition. *Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 15(5), 879–885. <https://doi.org/10.1158/1055-9965.EPI-05-0800>

- [8]. Lin, R. T., Chen, P. L., Yang, C. Y., Yeh, C. C., Lin, C. C., Huang, W. H., Chung, A. K., & Lin, J. T. (2022). **Risk factors related to age at diagnosis of pancreatic cancer: a retrospective cohort pilot study.** *BMC gastroenterology*, 22(1), 243. <https://doi.org/10.1186/s12876-022-02325-7>
- [9]. Yu, J., Blackford, A. L., Dal Molin, M., Wolfgang, C. L., & Goggins, M. (2015). **Time to progression of pancreatic ductal adenocarcinoma from low-to-high tumour stages.** *Gut*, 64(11), 1783–1789. <https://doi.org/10.1136/gutjnl-2014-308653>
- [10]. Midha, S., Chawla, S., & Garg, P. K. (2016). **Modifiable and non-modifiable risk factors for pancreatic cancer: A review.** *Cancer letters*, 381(1), 269–277. <https://doi.org/10.1016/j.canlet.2016.07.022>
- [11]. Wahi, M. M., Shah, N., Schrock, C. E., Rosemurgy, A. S., & Goldin, S. B. (2009). **Reproductive factors and risk of pancreatic cancer in women: a review of the literature.** *Annals of epidemiology*, 19(2), 103–111. <https://doi.org/10.1016/j.annepidem.2008.11.003>
- [12]. Benzel, J., & Fendrich, V. (2018). **Familial Pancreatic Cancer.** *Oncology research and treatment*, 41(10), 611–618. <https://doi.org/10.1159/000493473>
- [13]. Amundadottir, L. T., Thorvaldsson, S., Gudbjartsson, D. F., Sulem, P., Kristjansson, K., Arnason, S., Gulcher, J. R., Bjornsson, J., Kong, A., Thorsteinsdottir, U., & Stefansson, K. (2004). **Cancer as a complex phenotype: pattern of cancer distribution within and**

- beyond the nuclear family. *PLoS medicine*, 1(3), e65.
<https://doi.org/10.1371/journal.pmed.0010065>
- [14]. Porter, N., Laheru, D., Lau, B., He, J., Zheng, L., Narang, A., Roberts, N. J., Canto, M. I., Lennon, A. M., Goggins, M. G., Hruban, R. H., & Klein, A. P. (2022). **Risk of Pancreatic Cancer in the Long-Term Prospective Follow-Up of Familial Pancreatic Cancer Kindreds.** *Journal of the National Cancer Institute*, djac167. Advance online publication. <https://doi.org/10.1093/jnci/djac167>
- [15]. Permuth-Wey, J., & Egan, K. M. (2009). **Family history is a significant risk factor for pancreatic cancer: results from a systematic review and meta-analysis.** *Familial cancer*, 8(2), 109–117. <https://doi.org/10.1007/s10689-008-9214-8>
- [16]. Rosen, M. N., Goodwin, R. A., & Vickers, M. M. (2021). **BRCA mutated pancreatic cancer: A change is coming.** *World journal of gastroenterology*, 27(17), 1943–1958. <https://doi.org/10.3748/wjg.v27.i17.1943>
- [17]. Hofstatter, E. W., Domchek, S. M., Miron, A., Garber, J., Wang, M., Compton, K., Boghossian, L., Miron, P. L., Nathanson, K. L., & Tung, N. (2011). **PALB2 mutations in familial breast and pancreatic cancer.** *Familial cancer*, 10(2), 225–231. <https://doi.org/10.1007/s10689-011-9426-1>
- [18]. Bright R. (1833). **Cases and Observations connected with Disease of the Pancreas and Duodenum.** *Medico-chirurgical transactions*, 18(Pt 1), 1–56. <https://doi.org/10.1177/09595287330180p102>
- [19]. Roy, A., Sahoo, J., Kamalanathan, S., Naik, D., Mohan, P., & Kalayarasan, R. (2021). **Diabetes and pancreatic cancer: Exploring**

the two-way traffic. *World journal of gastroenterology*, 27(30), 4939–4962. <https://doi.org/10.3748/wjg.v27.i30.4939>

- [20]. Andersen, D. K., Korc, M., Petersen, G. M., Eibl, G., Li, D., Rickels, M. R., Chari, S. T., & Abbruzzese, J. L. (2017). Diabetes, Pancreatogenic Diabetes, and Pancreatic Cancer. *Diabetes*, 66(5), 1103–1110. <https://doi.org/10.2337/db16-1477>
- [21]. Wang, Y. T., Gou, Y. W., Jin, W. W., Xiao, M., & Fang, H. Y. (2016). **Association between alcohol intake and the risk of pancreatic cancer: a dose-response meta-analysis of cohort studies.** *BMC cancer*, 16, 212. <https://doi.org/10.1186/s12885-016-2241-1>
- [22]. Genkinger, J. M., Spiegelman, D., Anderson, K. E., Bergkvist, L., Bernstein, L., van den Brandt, P. A., English, D. R., Freudenheim, J. L., Fuchs, C. S., Giles, G. G., Giovannucci, E., Hankinson, S. E., Horn-Ross, P. L., Leitzmann, M., Männistö, S., Marshall, J. R., McCullough, M. L., Miller, A. B., Reding, D. J., Robien, K., Smith-Warner, S. A. (2009). **Alcohol intake and pancreatic cancer risk: a pooled analysis of fourteen cohort studies.** *Cancer epidemiology, biomarkers & prevention: a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, 18(3), 765–776. <https://doi.org/10.1158/1055-9965.EPI-08-0880>
- [23]. Bosetti, C., Lucenteforte, E., Silverman, D. T., Petersen, G., Bracci, P. M., Ji, B. T., Negri, E., Li, D., Risch, H. A., Olson, S. H., Gallinger, S., Miller, A. B., Bueno-de-Mesquita, H. B., Talamini, R., Polesel, J., Ghadirian, P., Baghurst, P. A., Zatonski, W., Fontham, E., Bamlet, W. R., ... La Vecchia, C. (2012). **Cigarette smoking and pancreatic cancer: an analysis from the International Pancreatic Cancer Case-Control Consortium (Panc4).** *Annals of oncology: official journal of the European Society for Medical Oncology*, 23(7), 1880–1888. <https://doi.org/10.1093/annonc/mdr541>

- [24]. Machicado, J. D., & Yadav, D. (2017). **Epidemiology of Recurrent Acute and Chronic Pancreatitis: Similarities and Differences.** *Digestive diseases and sciences*, 62(7), 1683–1691.
<https://doi.org/10.1007/s10620-017-4510-5>
- [25]. Raimondi, S., Lowenfels, A. B., Morselli-Labate, A. M., Maisonneuve, P., & Pezzilli, R. (2010). **Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection.** *Best practice & research. Clinical gastroenterology*, 24(3), 349–358.
<https://doi.org/10.1016/j.bpg.2010.02.007>
- [26]. Esposito, I., Konukiewitz, B., Schlitter, A. M., & Klöppel, G. (2014). **Pathology of pancreatic ductal adenocarcinoma: facts, challenges and future developments.** *World journal of gastroenterology*, 20(38), 13833–13841.
<https://doi.org/10.3748/wjg.v20.i38.13833>
- [27]. Tanase, C. P., Neagu, A. I., Necula, L. G., Mambet, C., Enciu, A. M., Calenic, B., Cruceru, M. L., & Albulescu, R. (2014). **Cancer stem cells: involvement in pancreatic cancer pathogenesis and perspectives on cancer therapeutics.** *World journal of gastroenterology*, 20(31), 10790–10801.
<https://doi.org/10.3748/wjg.v20.i31.10790>
- [28]. Hruban, R. H., Adsay, N. V., Albores-Saavedra, J., Compton, C., Garrett, E. S., Goodman, S. N., Kern, S. E., Klimstra, D. S., Klöppel, G., Longnecker, D. S., Lüttges, J., & Offerhaus, G. J. (2001). **Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions.** *The American journal of surgical pathology*, 25(5), 579–586.
<https://doi.org/10.1097/00000478-200105000-00003>

- [29]. Basturk, O., Hong, S. M., Wood, L. D., Adsay, N. V., Albores-Saavedra, J., Biankin, A. V., Brosens, L. A., Fukushima, N., Goggins, M., Hruban, R. H., Kato, Y., Klimstra, D. S., Klöppel, G., Krasinskas, A., Longnecker, D. S., Matthaei, H., Offerhaus, G. J., Shimizu, M., Takaori, K., Terris, B., ... Baltimore Consensus Meeting (2015). **A Revised Classification System and Recommendations From the Baltimore Consensus Meeting for Neoplastic Precursor Lesions in the Pancreas.** *The American journal of surgical pathology*, 39(12), 1730–1741.
<https://doi.org/10.1097/PAS.0000000000000533>
- [30]. Matsuda, Y., Furukawa, T., Yachida, S., Nishimura, M., Seki, A., Nonaka, K., Aida, J., Takubo, K., Ishiwata, T., Kimura, W., Arai, T., & Mino-Kenudson, M. (2017). **The Prevalence and Clinicopathological Characteristics of High-Grade Pancreatic Intraepithelial Neoplasia: Autopsy Study Evaluating the Entire Pancreatic Parenchyma.** *Pancreas*, 46(5), 658–664.
<https://doi.org/10.1097/MPA.0000000000000786>
- [31]. Andea, A., Sarkar, F., & Adsay, V. N. (2003). **Clinicopathological correlates of pancreatic intraepithelial neoplasia: a comparative analysis of 82 cases with and 152 cases without pancreatic ductal adenocarcinoma.** *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc*, 16(10), 996–1006.
<https://doi.org/10.1097/01.MP.0000087422.24733.62>
- [32]. Peters, M., Eckel, A., Mueller, P. P., Tramontano, A. C., Weaver, D. T., Lietz, A., Hur, C., Kong, C. Y., & Pandharipande, P. V. (2018). **Progression to pancreatic ductal adenocarcinoma from pancreatic intraepithelial neoplasia: Results of a simulation model.** *Pancreatology: official journal of the International*

- Association of Pancreatology (IAP) [et al.]*, 18(8), 928–934.
<https://doi.org/10.1016/j.pan.2018.07.009>
- [33]. Machado, N. O., Al Qadhi, H., & Al Wahibi, K. (2015). **Intraductal Papillary Mucinous Neoplasm of Pancreas**. *North American journal of medical sciences*, 7(5), 160–175.
<https://doi.org/10.4103/1947-2714.157477>
- [34]. Hwang, D. W., Jang, J. Y., Lee, S. E., Lim, C. S., Lee, K. U., & Kim, S. W. (2012). **Clinicopathologic analysis of surgically proven intraductal papillary mucinous neoplasms of the pancreas in SNUH: a 15-year experience at a single academic institution**. *Langenbeck's archives of surgery*, 397(1), 93–102.
<https://doi.org/10.1007/s00423-010-0674-6>
- [35]. Crippa, S., Fernández-Del Castillo, C., Salvia, R., Finkelstein, D., Bassi, C., Domínguez, I., Muzikansky, A., Thayer, S. P., Falconi, M., Mino-Kenudson, M., Capelli, P., Lauwers, G. Y., Partelli, S., Pederzoli, P., & Warshaw, A. L. (2010). **Mucin-producing neoplasms of the pancreas: an analysis of distinguishing clinical and epidemiologic characteristics**. *Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association*, 8(2), 213–219.
<https://doi.org/10.1016/j.cgh.2009.10.001>
- [36]. Schnelldorfer, T., Sarr, M. G., Nagorney, D. M., Zhang, L., Smyrk, T. C., Qin, R., Chari, S. T., & Farnell, M. B. (2008). **Experience with 208 resections for intraductal papillary mucinous neoplasm of the pancreas**. *Archives of surgery (Chicago, Ill.: 1960)*, 143(7), 639–646. <https://doi.org/10.1001/archsurg.143.7.639>
- [37]. Reddy, R. P., Smyrk, T. C., Zapiach, M., Levy, M. J., Pearson, R. K., Clain, J. E., Farnell, M. B., Sarr, M. G., & Chari, S. T. (2004). **Pancreatic mucinous cystic neoplasm defined by ovarian stroma:**

demographics, clinical features, and prevalence of cancer. *Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association*, 2(11), 1026–1031. [https://doi.org/10.1016/s1542-3565\(04\)00450-1](https://doi.org/10.1016/s1542-3565(04)00450-1)

- [38]. Hruban, R. H., Takaori, K., Klimstra, D. S., Adsay, N. V., Albores-Saavedra, J., Biankin, A. V., Biankin, S. A., Compton, C., Fukushima, N., Furukawa, T., Goggins, M., Kato, Y., Klöppel, G., Longnecker, D. S., Lüttges, J., Maitra, A., Offerhaus, G. J., Shimizu, M., & Yonezawa, S. (2004). **An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms.** *The American journal of surgical pathology*, 28(8), 977–987. <https://doi.org/10.1097/01.pas.0000126675.59108.80>
- [39]. Hassid, B. G., Lucas, A. L., Salomao, M., Weng, C., Liu, F., Khanna, L. G., Kumar, S., Hwang, C., Chabot, J. A., & Frucht, H. (2014). **Absence of pancreatic intraepithelial neoplasia predicts poor survival after resection of pancreatic cancer.** *Pancreas*, 43(7), 1073–1077. <https://doi.org/10.1097/MPA.0000000000000161>
- [40]. Furukawa, T., Klöppel, G., Volkan Adsay, N., Albores-Saavedra, J., Fukushima, N., Horii, A., Hruban, R. H., Kato, Y., Klimstra, D. S., Longnecker, D. S., Lüttges, J., Offerhaus, G. J., Shimizu, M., Sunamura, M., Suriawinata, A., Takaori, K., & Yonezawa, S. (2005). **Classification of types of intraductal papillary-mucinous neoplasm of the pancreas: a consensus study.** *Virchows Archiv: an international journal of pathology*, 447(5), 794–799. <https://doi.org/10.1007/s00428-005-0039-7>
- [41]. Yamao, K., Yanagisawa, A., Takahashi, K., Kimura, W., Doi, R., Fukushima, N., Ohike, N., Shimizu, M., Hatori, T., Nobukawa, B., Hifumi, M., Kobayashi, Y., Tobita, K., Tanno, S., Sugiyama, M.,

- Miyasaka, Y., Nakagohri, T., Yamaguchi, T., Hanada, K., Abe, H., ... Tanaka, M. (2011). **Clinicopathological features and prognosis of mucinous cystic neoplasm with ovarian-type stroma: a multi-institutional study of the Japan pancreas society.** *Pancreas*, 40(1), 67–71.
<https://doi.org/10.1097/MPA.0b013e3181f749d3>
- [42]. Wood, L. D., & Brosens, L. A. (2016). **The Changing Landscape of Pancreatic Pathology.** *Surgical pathology clinics*, 9(4), xiii.
<https://doi.org/10.1016/j.path.2016.10.001>
- [43]. Elbanna, K. Y., Jang, H. J., & Kim, T. K. (2020). **Imaging diagnosis and staging of pancreatic ductal adenocarcinoma: a comprehensive review.** *Insights into imaging*, 11(1), 58.
<https://doi.org/10.1186/s13244-020-00861-y>
- [44]. Roalsø, M., Aunan, J. R., & Søreide, K. (2020). **Refined TNM-staging for pancreatic adenocarcinoma - Real progress or much ado about nothing?** *European journal of surgical oncology: the journal of the European Society of Surgical Oncology and the British Association of Surgical Oncology*, 46(8), 1554–1557.
<https://doi.org/10.1016/j.ejso.2020.02.014>
- [45]. Henry, N. L., & Hayes, D. F. (2012). **Cancer biomarkers.** *Molecular oncology*, 6(2), 140–146.
<https://doi.org/10.1016/j.molonc.2012.01.010>
- [46]. Sturm, N., Ettrich, T. J., & Perkhofer, L. (2022). **The Impact of Biomarkers in Pancreatic Ductal Adenocarcinoma on Diagnosis, Surveillance and Therapy.** *Cancers*, 14(1), 217.
<https://doi.org/10.3390/cancers14010217>
- [47]. Poruk, K. E., Gay, D. Z., Brown, K., Mulvihill, J. D., Boucher, K. M., Scaife, C. L., Firpo, M. A., & Mulvihill, S. J. (2013). **The clinical utility**

of CA 19-9 in pancreatic adenocarcinoma: diagnostic and prognostic updates. *Current molecular medicine*, 13(3), 340–351.
<https://doi.org/10.2174/1566524011313030003>

- [48]. Ballehaninna, U. K., & Chamberlain, R. S. (2011). **Serum CA 19-9 as a Biomarker for Pancreatic Cancer-A Comprehensive Review.** *Indian journal of surgical oncology*, 2(2), 88–100.
<https://doi.org/10.1007/s13193-011-0042-1>
- [49]. Bünger, S., Laubert, T., Roblick, U. J., & Habermann, J. K. (2011). **Serum biomarkers for improved diagnostic of pancreatic cancer: a current overview.** *Journal of cancer research and clinical oncology*, 137(3), 375–389. <https://doi.org/10.1007/s00432-010-0965-x>
- [50]. Zhao, J. Z., & Wu, B. H. (1997). **Clinical significance of CA19-9 in diagnosis of digestive tract tumors.** *World journal of gastroenterology*, 3(4), 253–254.
<https://doi.org/10.3748/wjg.v3.i4.253>
- [51]. Gao, Y., Wang, J., Zhou, Y., Sheng, S., Qian, S. Y., & Huo, X. (2018). **Evaluation of Serum CEA, CA19-9, CA72-4, CA125 and Ferritin as Diagnostic Markers and Factors of Clinical Parameters for Colorectal Cancer.** *Scientific reports*, 8(1), 2732.
<https://doi.org/10.1038/s41598-018-21048-y>
- [52]. Waters, A. M., & Der, C. J. (2018). KRAS: The Critical Driver and Therapeutic Target for Pancreatic Cancer. *Cold Spring Harbor perspectives in medicine*, 8(9), a031435.
<https://doi.org/10.1101/cshperspect.a031435>
- [53]. Yanagisawa, A., Ohtake, K., Ohashi, K., Hori, M., Kitagawa, T., Sugano, H., & Kato, Y. (1993). **Frequent c-Ki-ras oncogene**

activation in mucous cell hyperplasias of pancreas suffering from chronic inflammation. *Cancer research*, 53(5), 953–956.

- [54]. Logsdon, C. D., & Ji, B. (2009). **Ras activity in acinar cells links chronic pancreatitis and pancreatic cancer.** *Clinical gastroenterology and hepatology: the official clinical practice journal of the American Gastroenterological Association*, 7(11 Suppl), S40–S43. <https://doi.org/10.1016/j.cgh.2009.07.040>
- [55]. Löhr, M., Klöppel, G., Maisonneuve, P., Lowenfels, A. B., & Lüttges, J. (2005). **Frequency of K-ras mutations in pancreatic intraductal neoplasias associated with pancreatic ductal adenocarcinoma and chronic pancreatitis: a meta-analysis.** *Neoplasia (New York, N.Y.)*, 7(1), 17–23. <https://doi.org/10.1593/neo.04445>
- [56]. Mulcahy, H. E., Lyautey, J., Lederrey, C., qi Chen, X., Anker, P., Alstead, E. M., Ballinger, A., Farthing, M. J., & Stroun, M. (1998). **A prospective study of K-ras mutations in the plasma of pancreatic cancer patients.** *Clinical cancer research: an official journal of the American Association for Cancer Research*, 4(2), 271–275.
- [57]. Buanes T. A. (2017). **Role of surgery in pancreatic cancer.** *World journal of gastroenterology*, 23(21), 3765–3770. <https://doi.org/10.3748/wjg.v23.i21.3765>
- [58]. Changazi, S. H., Ahmed, Q., Bhatti, S., Siddique, S., Abdul Raffay, E., Farooka, M. W., & Ayyaz, M. (2020). **Whipple Procedure: A Five-Year Clinical Experience in Tertiary Care Center.** *Cureus*, 12(11), e11466. <https://doi.org/10.7759/cureus.11466>
- [59]. Burris, H. A., 3rd, Moore, M. J., Andersen, J., Green, M. R., Rothenberg, M. L., Modiano, M. R., Cripps, M. C., Portenoy, R. K., Storniolo, A. M., Tarassoff, P., Nelson, R., Dorr, F. A., Stephens, C.

- D., & Von Hoff, D. D. (1997). **Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial.** *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*, 15(6), 2403–2413.
<https://doi.org/10.1200/JCO.1997.15.6.2403>
- [60]. Conroy, T., Desseigne, F., Ychou, M., Bouché, O., Guimbaud, R., Bécouarn, Y., Adenis, A., Raoul, J. L., Gourgou-Bourgade, S., de la Fouchardière, C., Bennouna, J., Bachet, J. B., Khemissa-Akouz, F., Péré-Vergé, D., Delbaldo, C., Assenat, E., Chauffert, B., Michel, P., Montoto-Grillot, C., Ducreux, M. (2011). **FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer.** *The New England journal of medicine*, 364(19), 1817–1825.
<https://doi.org/10.1056/NEJMoa1011923>
- [61]. Conroy, T., Hammel, P., Hebbar, M., Ben Abdelghani, M., Wei, A. C., Raoul, J. L., Choné, L., Francois, E., Artru, P., Biagi, J. J., Lecomte, T., Assenat, E., Faroux, R., Ychou, M., Volet, J., Sauvanet, A., Breysacher, G., Di Fiore, F., Cripps, C., Kavan, P. Canadian Cancer Trials Group and the Unicancer-GI-PRODIGE Group (2018). **FOLFIRINOX or Gemcitabine as Adjuvant Therapy for Pancreatic Cancer.** *The New England journal of medicine*, 379(25), 2395–2406. <https://doi.org/10.1056/NEJMoa1809775>
- [62]. Wang, Y., Golesworthy, B., Cuggia, A., Domecq, C., Chaudhury, P., Barkun, J., Metrakos, P., Asselah, J., Bouganim, N., Gao, Z. H., Chong, G., Foulkes, W. D., & Zogopoulos, G. (2022). **Oncology clinic-based germline genetic testing for exocrine pancreatic cancer enables timely return of results and unveils low uptake of cascade testing.** *Journal of medical genetics*, 59(8), 793–800.
<https://doi.org/10.1136/jmedgenet-2021-108054>

- [63]. Prakash, R., Zhang, Y., Feng, W., & Jasin, M. (2015). **Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins.** *Cold Spring Harbor perspectives in biology*, 7(4), a016600. <https://doi.org/10.1101/cshperspect.a016600>
- [64]. Fazio N. (2020). **Cisplatin Plus Gemcitabine as Standard of Care for Germline BRCA/PALB2-Mutated Pancreatic Adenocarcinoma: Are We Moving Too Fast?** *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*, 38(21), 2466–2467. <https://doi.org/10.1200/JCO.20.00419>
- [65]. Nehlsen, A. D., & Goodman, K. A. (2021). **Controversies in radiotherapy for pancreas cancer.** *Journal of surgical oncology*, 123(6), 1460–1466. <https://doi.org/10.1002/jso.26313>
- [66]. Oba, A., Ho, F., Bao, Q. R., Al-Musawi, M. H., Schulick, R. D., & Del Chiaro, M. (2020). **Neoadjuvant Treatment in Pancreatic Cancer.** *Frontiers in oncology*, 10, 245. <https://doi.org/10.3389/fonc.2020.00245>
- [67]. Hirai, T., Matsumoto, H., Yamashita, K., Urakami, A., Iki, K., Yamamura, M., & Tsunoda, T. (2005). **Surgical oncotaxis--excessive surgical stress and postoperative complications contribute to enhancing tumor metastasis, resulting in a poor prognosis for cancer patients.** *Annals of thoracic and cardiovascular surgery: official journal of the Association of Thoracic and Cardiovascular Surgeons of Asia*, 11(1), 4–6.
- [68]. Gillen, S., Schuster, T., Meyer Zum Büschenfelde, C., Friess, H., & Kleeff, J. (2010). **Preoperative/neoadjuvant therapy in pancreatic cancer: a systematic review and meta-analysis of**

response and resection percentages. *PLoS medicine*, 7(4), e1000267. <https://doi.org/10.1371/journal.pmed.1000267>

- [69]. Kalser, M. H., & Ellenberg, S. S. (1985). **Pancreatic cancer. Adjuvant combined radiation and chemotherapy following curative resection.** *Archives of surgery*, 120(8), 899–903. <https://doi.org/10.1001/archsurg.1985.01390320023003>
- [70]. Klinkenbijl, J. H., Jeekel, J., Sahmoud, T., van Pel, R., Couvreur, M. L., Veenhof, C. H., Arnaud, J. P., Gonzalez, D. G., de Wit, L. T., Hennisman, A., & Wils, J. (1999). **Adjuvant radiotherapy and 5-fluorouracil after curative resection of cancer of the pancreas and periampullary region: phase III trial of the EORTC gastrointestinal tract cancer cooperative group.** *Annals of surgery*, 230(6), 776–784. <https://doi.org/10.1097/00000658-199912000-00006>
- [71]. Rose, M., Burgess, J. T., O'Byrne, K., Richard, D. J., & Bolderson, E. (2020). **PARP Inhibitors: Clinical Relevance, Mechanisms of Action and Tumor Resistance.** *Frontiers in cell and developmental biology*, 8, 564601. <https://doi.org/10.3389/fcell.2020.564601>
- [72]. Kindler, H. L., Hammel, P., Reni, M., Van Cutsem, E., Macarulla, T., Hall, M. J., Park, J. O., Hochhauser, D., Arnold, D., Oh, D. Y., Reinacher-Schick, A., Tortora, G., Algül, H., O'Reilly, E. M., Bordia, S., McGuinness, D., Cui, K., Locker, G. Y., & Golan, T. (2022). **Overall Survival Results From the POLO Trial: A Phase III Study of Active Maintenance Olaparib Versus Placebo for Germline BRCA-Mutated Metastatic Pancreatic Cancer.** *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*, 40(34), 3929–3939. <https://doi.org/10.1200/JCO.21.01604>

- [73]. Simanshu, D. K., Nissley, D. V., & McCormick, F. (2017). **RAS Proteins and Their Regulators in Human Disease**. *Cell*, 170(1), 17–33. <https://doi.org/10.1016/j.cell.2017.06.009>
- [74]. Shen, H., Lundy, J., Strickland, A. H., Harris, M., Swan, M., Desmond, C., Jenkins, B. J., & Croagh, D. (2022). **KRAS G12D Mutation Subtype in Pancreatic Ductal Adenocarcinoma: Does It Influence Prognosis or Stage of Disease at Presentation?** *Cells*, 11(19), 3175. <https://doi.org/10.3390/cells11193175>
- [75]. Wang, X., Allen, S., Blake, J. F., Bowcut, V., Briere, D. M., Calinisan, A., Dahlke, J. R., Fell, J. B., Fischer, J. P., Gunn, R. J., Hallin, J., Laguer, J., Lawson, J. D., Medwid, J., Newhouse, B., Nguyen, P., O'Leary, J. M., Olson, P., Pajk, S., Rahbaek, L., ... Marx, M. A. (2022). **Identification of MRTX1133, a Noncovalent, Potent, and Selective KRAS^{G12D} Inhibitor**. *Journal of medicinal chemistry*, 65(4), 3123–3133. <https://doi.org/10.1021/acs.jmedchem.1c01688>
- [76]. Adamska, A., Domenichini, A., & Falasca, M. (2017). **Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies**. *International journal of molecular sciences*, 18(7), 1338. <https://doi.org/10.3390/ijms18071338>
- [77]. Lin, X., Kong, D., & Chen, Z. S. (2022). **Editorial: Chemo-Radiation-Resistance in Cancer Therapy**. *Frontiers in pharmacology*, 13, 904063. <https://doi.org/10.3389/fphar.2022.904063>
- [78]. Quiñonero, F., Mesas, C., Doello, K., Cabeza, L., Perazzoli, G., Jimenez-Luna, C., Rama, A. R., Melguizo, C., & Prados, J. (2019). **The challenge of drug resistance in pancreatic ductal adenocarcinoma: a current overview**. *Cancer biology &*

medicine, 16(4), 688–699. <https://doi.org/10.20892/j.issn.2095-3941.2019.0252>

- [79]. Mezencev, R., Matyunina, L. V., Wagner, G. T., & McDonald, J. F. (2016). **Acquired resistance of pancreatic cancer cells to cisplatin is multifactorial with cell context-dependent involvement of resistance genes.** *Cancer gene therapy*, 23(12), 446–453. <https://doi.org/10.1038/cgt.2016.71>
- [80]. Schirmacher V. (2019). **From chemotherapy to biological therapy: A review of novel concepts to reduce the side effects of systemic cancer treatment (Review).** *International journal of oncology*, 54(2), 407–419. <https://doi.org/10.3892/ijo.2018.4661>
- [81]. Bukowski, K., Kciuk, M., & Kontek, R. (2020). **Mechanisms of Multidrug Resistance in Cancer Chemotherapy.** *International journal of molecular sciences*, 21(9), 3233. <https://doi.org/10.3390/ijms21093233>
- [82]. Kilari, D., Guancial, E., & Kim, E. S. (2016). **Role of copper transporters in platinum resistance.** *World journal of clinical oncology*, 7(1), 106–113. <https://doi.org/10.5306/wjco.v7.i1.106>
- [83]. Choi C. H. (2005). **ABC transporters as multidrug resistance mechanisms and the development of chemosensitizers for their reversal.** *Cancer cell international*, 5, 30. <https://doi.org/10.1186/1475-2867-5-30>
- [84]. Dasari, S., & Tchounwou, P. B. (2014). **Cisplatin in cancer therapy: molecular mechanisms of action.** *European journal of pharmacology*, 740, 364–378. <https://doi.org/10.1016/j.ejphar.2014.07.025>
- [85]. Shirmanova, M. V., Druzhkova, I. N., Lukina, M. M., Dudenkova, V. V., Ignatova, N. I., Snopova, L. B., Shcheslavskiy, V. I., Belousov, V.

- V., & Zagaynova, E. V. (2017). **Chemotherapy with cisplatin: insights into intracellular pH and metabolic landscape of cancer cells in vitro and in vivo.** *Scientific reports*, 7(1), 8911. <https://doi.org/10.1038/s41598-017-09426-4>
- [86]. Srinivas, U. S., Tan, B., Vellayappan, B. A., & Jeyasekharan, A. D. (2019). **ROS and the DNA damage response in cancer.** *Redox biology*, 25, 101084. <https://doi.org/10.1016/j.redox.2018.101084>
- [87]. Traverso, N., Ricciarelli, R., Nitti, M., Marengo, B., Furfaro, A. L., Pronzato, M. A., Marinari, U. M., & Domenicotti, C. (2013). **Role of glutathione in cancer progression and chemoresistance.** *Oxidative medicine and cellular longevity*, 2013, 972913. <https://doi.org/10.1155/2013/972913>
- [88]. Shimura, T., Nakashiro, C., Fujiwara, K., Shiga, R., Sasatani, M., Kamiya, K., & Ushiyama, A. (2022). **Radiation affects glutathione redox reaction by reduced glutathione peroxidase activity in human fibroblasts.** *Journal of radiation research*, 63(2), 183–191. <https://doi.org/10.1093/jrr/rrab122>
- [89]. Zheng, Z. G., Xu, H., Suo, S. S., Xu, X. L., Ni, M. W., Gu, L. H., Chen, W., Wang, L. Y., Zhao, Y., Tian, B., & Hua, Y. J. (2016). **The Essential Role of H19 Contributing to Cisplatin Resistance by Regulating Glutathione Metabolism in High-Grade Serous Ovarian Cancer.** *Scientific reports*, 6, 26093. <https://doi.org/10.1038/srep26093>
- [90]. Hosoya, N., & Miyagawa, K. (2014). **Targeting DNA damage response in cancer therapy.** *Cancer science*, 105(4), 370–388. <https://doi.org/10.1111/cas.12366>

- [91]. Jackson, S. P., & Bartek, J. (2009). **The DNA-damage response in human biology and disease.** *Nature*, 461(7267), 1071–1078.
<https://doi.org/10.1038/nature08467>
- [92]. Rocha, C., Silva, M. M., Quinet, A., Cabral-Neto, J. B., & Menck, C. (2018). **DNA repair pathways and cisplatin resistance: an intimate relationship.** *Clinics*, e478s.
<https://doi.org/10.6061/clinics/2018/e478s>
- [93]. Mishina, Y., Duguid, E. M., & He, C. (2006). **Direct reversal of DNA alkylation damage.** *Chemical reviews*, 106(2), 215–232.
<https://doi.org/10.1021/cr0404702>
- [94]. Chan, K. K., Zhang, Q. M., & Dianov, G. L. (2006). **Base excision repair fidelity in normal and cancer cells.** *Mutagenesis*, 21(3), 173–178. <https://doi.org/10.1093/mutage/gel020>
- [95]. Krasikova, Y., Rechkunova, N., & Lavrik, O. (2021). **Nucleotide Excision Repair: From Molecular Defects to Neurological Abnormalities.** *International journal of molecular sciences*, 22(12), 6220. <https://doi.org/10.3390/ijms22126220>
- [96]. Fishel R. (2015). **Mismatch repair.** *The Journal of biological chemistry*, 290(44), 26395–26403.
<https://doi.org/10.1074/jbc.R115.660142>
- [97]. Aebi, S., Kurdi-Haidar, B., Gordon, R., Cenni, B., Zheng, H., Fink, D., Christen, R. D., Boland, C. R., Koi, M., Fishel, R., & Howell, S. B. (1996). **Loss of DNA mismatch repair in acquired resistance to cisplatin.** *Cancer research*, 56(13), 3087–3090.
- [98]. Jacob, S., Aguado, M., Fallik, D., & Praz, F. (2001). **The role of the DNA mismatch repair system in the cytotoxicity of the topoisomerase inhibitors camptothecin and etoposide to human colorectal cancer cells.** *Cancer research*, 61(17), 6555–6562.

- [99]. Chernikova, S. B., Game, J. C., & Brown, J. M. (2012). **Inhibiting homologous recombination for cancer therapy**. *Cancer biology & therapy*, 13(2), 61–68. <https://doi.org/10.4161/cbt.13.2.18872>
- [100]. Li, Y. H., Wang, X., Pan, Y., Lee, D. H., Chowdhury, D., & Kimmelman, A. C. (2012). **Inhibition of non-homologous end joining repair impairs pancreatic cancer growth and enhances radiation response**. *PloS one*, 7(6), e39588. <https://doi.org/10.1371/journal.pone.0039588>
- [101]. Zhao, X., Wei, C., Li, J., Xing, P., Li, J., Zheng, S., & Chen, X. (2017). **Cell cycle-dependent control of homologous recombination**. *Acta biochimica et biophysica Sinica*, 49(8), 655–668. <https://doi.org/10.1093/abbs/gmx055>
- [102]. Li, X., & Heyer, W. D. (2008). **Homologous recombination in DNA repair and DNA damage tolerance**. *Cell research*, 18(1), 99–113. <https://doi.org/10.1038/cr.2008.1>
- [103]. Mao, Z., Bozzella, M., Seluanov, A., & Gorbunova, V. (2008). **DNA repair by nonhomologous end joining and homologous recombination during cell cycle in human cells**. *Cell cycle (Georgetown, Tex.)*, 7(18), 2902–2906. <https://doi.org/10.4161/cc.7.18.6679>
- [104]. Hunt, T., Nasmyth, K., & Novák, B. (2011). **The cell cycle**. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 366(1584), 3494–3497. <https://doi.org/10.1098/rstb.2011.0274>
- [105]. Alimbetov, D., Askarova, S., Umbayev, B., Davis, T., & Kipling, D. (2018). **Pharmacological Targeting of Cell Cycle, Apoptotic and Cell Adhesion Signaling Pathways Implicated in Chemoresistance**

of Cancer Cells. *International journal of molecular sciences*, 19(6), 1690. <https://doi.org/10.3390/ijms19061690>

- [106]. Pawlik, T. M., & Keyomarsi, K. (2004). **Role of cell cycle in mediating sensitivity to radiotherapy.** *International journal of radiation oncology, biology, physics*, 59(4), 928–942. <https://doi.org/10.1016/j.ijrobp.2004.03.005>
- [107]. Zhang, N., Yin, Y., Xu, S. J., & Chen, W. S. (2008). **5-Fluorouracil: mechanisms of resistance and reversal strategies.** *Molecules (Basel, Switzerland)*, 13(8), 1551–1569. <https://doi.org/10.3390/molecules13081551>
- [108]. Un F. (2007). **G1 arrest induction represents a critical determinant for cisplatin cytotoxicity in G1 checkpoint-retaining human cancers.** *Anti-cancer drugs*, 18(4), 411–417. <https://doi.org/10.1097/CAD.0b013e32801429ed>
- [109]. Hanahan, D., & Weinberg, R. A. (2000). **The hallmarks of cancer.** *Cell*, 100(1), 57–70. [https://doi.org/10.1016/s0092-8674\(00\)81683-9](https://doi.org/10.1016/s0092-8674(00)81683-9)
- [110]. Hanahan, D., & Weinberg, R. A. (2011). **Hallmarks of cancer: the next generation.** *Cell*, 144(5), 646–674. <https://doi.org/10.1016/j.cell.2011.02.013>
- [111]. Hanahan D. (2022). **Hallmarks of Cancer: New Dimensions.** *Cancer discovery*, 12(1), 31–46. <https://doi.org/10.1158/2159-8290.CD-21-1059>
- [112]. Feitelson, M. A., Arzumanyan, A., Kulathinal, R. J., Blain, S. W., Holcombe, R. F., Mahajna, J., Marino, M., Martinez-Chantar, M. L., Nawroth, R., Sanchez-Garcia, I., Sharma, D., Saxena, N. K., Singh, N., Vlachostergios, P. J., Guo, S., Honoki, K., Fujii, H., Georgakilas, A. G., Bilsland, A., Amedei, A., ... Nowsheen, S. (2015). **Sustained**

- proliferation in cancer: Mechanisms and novel therapeutic targets.** *Seminars in cancer biology*, 35 Suppl(Suppl), S25–S54.
<https://doi.org/10.1016/j.semcan.2015.02.006>
- [113]. Sporn, M. B., & Roberts, A. B. (1985). **Autocrine growth factors and cancer.** *Nature*, 313(6005), 745–747.
<https://doi.org/10.1038/313745a0>
- [114]. Braicu, C., Buse, M., Busuioc, C., Drula, R., Gulei, D., Raduly, L., Rusu, A., Irimie, A., Atanasov, A. G., Slaby, O., Ionescu, C., & Berindan-Neagoe, I. (2019). **A Comprehensive Review on MAPK: A Promising Therapeutic Target in Cancer.** *Cancers*, 11(10), 1618.
<https://doi.org/10.3390/cancers11101618>
- [115]. Levy, D. S., Kahana, J. A., & Kumar, R. (2009). **AKT inhibitor, GSK690693, induces growth inhibition and apoptosis in acute lymphoblastic leukemia cell lines.** *Blood*, 113(8), 1723–1729.
<https://doi.org/10.1182/blood-2008-02-137737>
- [116]. Wu, R., Hu, T. C., Rehemtulla, A., Fearon, E. R., & Cho, K. R. (2011). **Preclinical testing of PI3K/AKT/mTOR signaling inhibitors in a mouse model of ovarian endometrioid adenocarcinoma.** *Clinical cancer research: an official journal of the American Association for Cancer Research*, 17(23), 7359–7372.
<https://doi.org/10.1158/1078-0432.CCR-11-1388>
- [117]. Vélez-Cruz, R., & Johnson, D. G. (2017). **The Retinoblastoma (RB) Tumor Suppressor: Pushing Back against Genome Instability on Multiple Fronts.** *International journal of molecular sciences*, 18(8), 1776. <https://doi.org/10.3390/ijms18081776>
- [118]. Chen J. (2016). **The Cell-Cycle Arrest and Apoptotic Functions of p53 in Tumor Initiation and Progression.** *Cold Spring Harbor*

perspectives in medicine, 6(3), a026104.
<https://doi.org/10.1101/cshperspect.a026104>

- [119]. Engel, B. E., Cress, W. D., & Santiago-Cardona, P. G. (2015). **THE RETINOBLASTOMA PROTEIN: A MASTER TUMOR SUPPRESSOR ACTS AS A LINK BETWEEN CELL CYCLE AND CELL ADHESION.** *Cell health and cytoskeleton*, 7, 1–10.
<https://doi.org/10.2147/CHC.S28079>
- [120]. Maddalena, M., Mallel, G., Nataraj, N. B., Shreberk-Shaked, M., Hassin, O., Mukherjee, S., Arandkar, S., Rotkopf, R., Kapsack, A., Lambiase, G., Pellegrino, B., Ben-Isaac, E., Golani, O., Addadi, Y., Hajaj, E., Eilam, R., Straussman, R., Yarden, Y., Lotem, M., & Oren, M. (2021). **TP53 missense mutations in PDAC are associated with enhanced fibrosis and an immunosuppressive microenvironment.** *Proceedings of the National Academy of Sciences of the United States of America*, 118(23), e2025631118.
<https://doi.org/10.1073/pnas.2025631118>
- [121]. Thomas, N. A., Abraham, R. G., Dedi, B., & Krucher, N. A. (2019). **Targeting retinoblastoma protein phosphorylation in combination with EGFR inhibition in pancreatic cancer cells.** *International journal of oncology*, 54(2), 527–536.
<https://doi.org/10.3892/ijo.2018.4658>
- [122]. Morton, J. P., Timpson, P., Karim, S. A., Ridgway, R. A., Athineos, D., Doyle, B., Jamieson, N. B., Oien, K. A., Lowy, A. M., Brunton, V. G., Frame, M. C., Evans, T. R., & Sansom, O. J. (2010). **Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer.** *Proceedings of the National Academy of Sciences of the United States of America*, 107(1), 246–251.
<https://doi.org/10.1073/pnas.0908428107>

- [123]. Grochola, L. F., Taubert, H., Greither, T., Bhanot, U., Udelnow, A., & Würfl, P. (2011). **Elevated transcript levels from the MDM2 P1 promoter and low p53 transcript levels are associated with poor prognosis in human pancreatic ductal adenocarcinoma.** *Pancreas*, 40(2), 265–270.
<https://doi.org/10.1097/mpa.0b013e3181f95104>
- [124]. DeBerardinis, R. J., & Chandel, N. S. (2016). **Fundamentals of cancer metabolism.** *Science advances*, 2(5), e1600200.
<https://doi.org/10.1126/sciadv.1600200>
- [125]. Fernandez-de-Cossio-Diaz, J., & Vazquez, A. (2017). **Limits of aerobic metabolism in cancer cells.** *Scientific reports*, 7(1), 13488.
<https://doi.org/10.1038/s41598-017-14071-y>
- [126]. Liberti, M. V., & Locasale, J. W. (2016). **The Warburg Effect: How Does it Benefit Cancer Cells?** *Trends in biochemical sciences*, 41(3), 211–218.
<https://doi.org/10.1016/j.tibs.2015.12.001>
- [127]. Achalandabaso Boira, M., Di Martino, M., Gordillo, C., Adrados, M., & Martín-Pérez, E. (2020). **GLUT-1 as a predictor of worse prognosis in pancreatic adenocarcinoma: immunohistochemistry study showing the correlation between expression and survival.** *BMC cancer*, 20(1), 909.
<https://doi.org/10.1186/s12885-020-07409-9>
- [128]. Yan, L., Tu, B., Yao, J., Gong, J., Carugo, A., Bristow, C. A., Wang, Q., Zhu, C., Dai, B., Kang, Y., Han, L., Feng, N., Jin, Y., Fleming, J., Heffernan, T. P., Yao, W., & Ying, H. (2021). **Targeting Glucose Metabolism Sensitizes Pancreatic Cancer to MEK Inhibition.** *Cancer research*, 81(15), 4054–4065.
<https://doi.org/10.1158/0008-5472.CAN-20-3792>

- [129]. Elmore S. (2007). **Apoptosis: a review of programmed cell death.** *Toxicologic pathology*, 35(4), 495–516.
<https://doi.org/10.1080/01926230701320337>
- [130]. Hardwick, J. M., & Soane, L. (2013). **Multiple functions of BCL-2 family proteins.** *Cold Spring Harbor perspectives in biology*, 5(2), a008722. <https://doi.org/10.1101/cshperspect.a008722>
- [131]. Faust, J. B., & Meeker, T. C. (1992). **Amplification and expression of the bcl-1 gene in human solid tumor cell lines.** *Cancer research*, 52(9), 2460–2463.
- [132]. Song, S., Wang, B., Gu, S., Li, X., & Sun, S. (2017). **Expression of Beclin 1 and Bcl-2 in pancreatic neoplasms and its effect on pancreatic ductal adenocarcinoma prognosis.** *Oncology letters*, 14(6), 7849–7861. <https://doi.org/10.3892/ol.2017.7218>
- [133]. Masood, A., Azmi, A. S., & Mohammad, R. M. (2011). **Small molecule inhibitors of bcl-2 family proteins for pancreatic cancer therapy.** *Cancers*, 3(2), 1527–1549.
<https://doi.org/10.3390/cancers3021527>
- [134]. Shurin M. R. (2012). **Cancer as an immune-mediated disease.** *ImmunoTargets and therapy*, 1, 1–6.
<https://doi.org/10.2147/ITT.S29834>
- [135]. Travis, M. A., & Sheppard, D. (2014). **TGF- β activation and function in immunity.** *Annual review of immunology*, 32, 51–82.
<https://doi.org/10.1146/annurev-immunol-032713-120257>
- [136]. Shen, W., Tao, G. Q., Zhang, Y., Cai, B., Sun, J., & Tian, Z. Q. (2017). **TGF- β in pancreatic cancer initiation and progression: two sides of the same coin.** *Cell & bioscience*, 7, 39.
<https://doi.org/10.1186/s13578-017-0168-0>

- [137]. Hussain, S. M., Kansal, R. G., Alvarez, M. A., Hollingsworth, T. J., Elahi, A., Miranda-Carboni, G., Hendrick, L. E., Pingili, A. K., Albritton, L. M., Dickson, P. V., Deneve, J. L., Yakoub, D., Hayes, D. N., Kurosu, M., Shibata, D., Makowski, L., & Glazer, E. S. (2021). **Role of TGF- β in pancreatic ductal adenocarcinoma progression and PD-L1 expression.** *Cellular oncology (Dordrecht)*, 44(3), 673–687. <https://doi.org/10.1007/s13402-021-00594-0>
- [138]. Li, K. Y., Yuan, J. L., Trafton, D., Wang, J. X., Niu, N., Yuan, C. H., Liu, X. B., & Zheng, L. (2020). **Pancreatic ductal adenocarcinoma immune microenvironment and immunotherapy prospects.** *Chronic diseases and translational medicine*, 6(1), 6–17. <https://doi.org/10.1016/j.cdtm.2020.01.002>
- [139]. Yao, Y., & Dai, W. (2014). **Genomic Instability and Cancer.** *Journal of carcinogenesis & mutagenesis*, 5, 1000165. <https://doi.org/10.4172/2157-2518.1000165>
- [140]. Armstrong, S. A., Schultz, C. W., Azimi-Sadjadi, A., Brody, J. R., & Pishvaian, M. J. (2019). **ATM Dysfunction in Pancreatic Adenocarcinoma and Associated Therapeutic Implications.** *Molecular cancer therapeutics*, 18(11), 1899–1908. <https://doi.org/10.1158/1535-7163.MCT-19-0208>
- [141]. Yoshida, K., & Miki, Y. (2004). **Role of BRCA1 and BRCA2 as regulators of DNA repair, transcription, and cell cycle in response to DNA damage.** *Cancer science*, 95(11), 866–871. <https://doi.org/10.1111/j.1349-7006.2004.tb02195.x>
- [142]. Bonilla, B., Hengel, S. R., Grundy, M. K., & Bernstein, K. A. (2020). **RAD51 Gene Family Structure and Function.** *Annual review of genetics*, 54, 25–46. <https://doi.org/10.1146/annurev-genet-021920-092410>

- [143]. Principe D. R. (2022). **Precision Medicine for *BRCA/PALB2*-Mutated Pancreatic Cancer and Emerging Strategies to Improve Therapeutic Responses to PARP Inhibition.** *Cancers*, 14(4), 897.
<https://doi.org/10.3390/cancers14040897>
- [144]. Symington L. S. (2005). **Focus on recombinational DNA repair.** *EMBO reports*, 6(6), 512–517.
<https://doi.org/10.1038/sj.embor.7400438>
- [145]. Sahin, I. H., Lowery, M. A., Stadler, Z. K., Salo-Mullen, E., Iacobuzio-Donahue, C. A., Kelsen, D. P., & O'Reilly, E. M. (2016). **Genomic instability in pancreatic adenocarcinoma: a new step towards precision medicine and novel therapeutic approaches.** *Expert review of gastroenterology & hepatology*, 10(8), 893–905.
<https://doi.org/10.1586/17474124.2016.1153424>
- [146]. Ko, H. L., & Ren, E. C. (2012). **Functional Aspects of PARP1 in DNA Repair and Transcription.** *Biomolecules*, 2(4), 524–548.
<https://doi.org/10.3390/biom2040524>
- [147]. Demin, A. A., Hirota, K., Tsuda, M., Adamowicz, M., Hailstone, R., Brazina, J., Gittens, W., Kalasova, I., Shao, Z., Zha, S., Sasanuma, H., Hanzlikova, H., Takeda, S., & Caldecott, K. W. (2021). **XRCC1 prevents toxic PARP1 trapping during DNA base excision repair.** *Molecular cell*, 81(14), 3018–3030.e5.
<https://doi.org/10.1016/j.molcel.2021.05.009>
- [148]. Audebert, M., Salles, B., & Calsou, P. (2008). **Effect of double-strand break DNA sequence on the PARP-1 NHEJ pathway.** *Biochemical and biophysical research communications*, 369(3), 982–988.
<https://doi.org/10.1016/j.bbrc.2007.11.132>

- [149]. Michels, J., Vitale, I., Senovilla, L., Enot, D. P., Garcia, P., Lissa, D., Olausson, K. A., Brenner, C., Soria, J. C., Castedo, M., & Kroemer, G. (2013). **Synergistic interaction between cisplatin and PARP inhibitors in non-small cell lung cancer.** *Cell cycle (Georgetown, Tex.)*, 12(6), 877–883. <https://doi.org/10.4161/cc.24034>
- [150]. Chi, J., Chung, S. Y., Parakrama, R., Fayyaz, F., Jose, J., & Saif, M. W. (2021). **The role of PARP inhibitors in BRCA mutated pancreatic cancer.** *Therapeutic advances in gastroenterology*, 14, 17562848211014818. <https://doi.org/10.1177/17562848211014818>
- [151]. Teleanu, R. I., Chircov, C., Grumezescu, A. M., & Teleanu, D. M. (2019). **Tumor Angiogenesis and Anti-Angiogenic Strategies for Cancer Treatment.** *Journal of clinical medicine*, 9(1), 84. <https://doi.org/10.3390/jcm9010084>
- [152]. Zhang, X., Kazerounian, S., Duquette, M., Perruzzi, C., Nagy, J. A., Dvorak, H. F., Parangi, S., & Lawler, J. (2009). **Thrombospondin-1 modulates vascular endothelial growth factor activity at the receptor level.** *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, 23(10), 3368–3376. <https://doi.org/10.1096/fj.09-131649>
- [153]. Yoo, P. S., Mulkeen, A. L., & Cha, C. H. (2006). **Post-transcriptional regulation of vascular endothelial growth factor: implications for tumor angiogenesis.** *World journal of gastroenterology*, 12(31), 4937–4942. <https://doi.org/10.3748/wjg.v12.i31.4937>
- [154]. Whatcott, C. J., Diep, C. H., Jiang, P., Watanabe, A., LoBello, J., Sima, C., Hostetter, G., Shepard, H. M., Von Hoff, D. D., & Han, H. (2015). **Desmoplasia in Primary Tumors and Metastatic Lesions of Pancreatic Cancer.** *Clinical cancer research: an official journal*

of the American Association for Cancer Research, 21(15), 3561–3568. <https://doi.org/10.1158/1078-0432.CCR-14-1051>

- [155]. Schober, M., Jesenofsky, R., Faissner, R., Weidenauer, C., Hagmann, W., Michl, P., Heuchel, R. L., Haas, S. L., & Löhr, J. M. (2014). **Desmoplasia and chemoresistance in pancreatic cancer.** *Cancers*, 6(4), 2137–2154. <https://doi.org/10.3390/cancers6042137>
- [156]. Escudier, B., Michaelson, M. D., Motzer, R. J., Hutson, T. E., Clark, J. I., Lim, H. Y., Porfiri, E., Zalewski, P., Kannourakis, G., Staehler, M., Tarazi, J., Rosbrook, B., Cisar, L., Hariharan, S., Kim, S., & Rini, B. I. (2014). **Axitinib versus sorafenib in advanced renal cell carcinoma: subanalyses by prior therapy from a randomised phase III trial.** *British journal of cancer*, 110(12), 2821–2828. <https://doi.org/10.1038/bjc.2014.244>
- [157]. Ribatti, D., Tamma, R., & Annese, T. (2020). **Epithelial-Mesenchymal Transition in Cancer: A Historical Overview.** *Translational oncology*, 13(6), 100773. <https://doi.org/10.1016/j.tranon.2020.100773>
- [158]. Sommariva, M., & Gagliano, N. (2020). **E-Cadherin in Pancreatic Ductal Adenocarcinoma: A Multifaceted Actor during EMT.** *Cells*, 9(4), 1040. <https://doi.org/10.3390/cells9041040>
- [159]. Liu, M., Hancock, S. E., Sultani, G., Wilkins, B. P., Ding, E., Osborne, B., Quek, L. E., & Turner, N. (2019). **Snail-Overexpression Induces Epithelial-mesenchymal Transition and Metabolic Reprogramming in Human Pancreatic Ductal Adenocarcinoma and Non-tumorigenic Ductal Cells.** *Journal of clinical medicine*, 8(6), 822. <https://doi.org/10.3390/jcm8060822>

- [160]. Aydemir Çoban, E., Tecimel, D., Kaşıkci, E., Bayrak, Ö. F., & Şahin, F. (2020). **E-cadherin might be a stage-dependent modulator in aggressiveness in pancreatic cancer cells.** *Turkish journal of biology*, 44(5), 230–237. <https://doi.org/10.3906/biy-1912-60>
- [161]. O'Brien, J., Hayder, H., Zayed, Y., & Peng, C. (2018). **Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation.** *Frontiers in endocrinology*, 9, 402. <https://doi.org/10.3389/fendo.2018.00402>
- [162]. Sun, W., Julie Li, Y. S., Huang, H. D., Shyy, J. Y., & Chien, S. (2010). **microRNA: a master regulator of cellular processes for bioengineering systems.** *Annual review of biomedical engineering*, 12, 1–27. <https://doi.org/10.1146/annurev-bioeng-070909-105314>
- [163]. Ardekani, A. M., & Naeini, M. M. (2010). **The Role of MicroRNAs in Human Diseases.** *Avicenna journal of medical biotechnology*, 2(4), 161–179.
- [164]. Bhaskaran, M., & Mohan, M. (2014). **MicroRNAs: history, biogenesis, and their evolving role in animal development and disease.** *Veterinary pathology*, 51(4), 759–774. <https://doi.org/10.1177/0300985813502820>
- [165]. Finnegan, E. F., & Pasquinelli, A. E. (2013). **MicroRNA biogenesis: regulating the regulators.** *Critical reviews in biochemistry and molecular biology*, 48(1), 51–68. <https://doi.org/10.3109/10409238.2012.738643>
- [166]. Krol, J., Loedige, I., & Filipowicz, W. (2010). **The widespread regulation of microRNA biogenesis, function and decay.** *Nature reviews. Genetics*, 11(9), 597–610. <https://doi.org/10.1038/nrg2843>

- [167]. Ha, M., & Kim, V. N. (2014). **Regulation of microRNA biogenesis.** *Nature reviews. Molecular cell biology*, 15(8), 509–524. <https://doi.org/10.1038/nrm3838>
- [168]. Gregory, R. I., Chendrimada, T. P., Cooch, N., & Shiekhattar, R. (2005). **Human RISC couples microRNA biogenesis and posttranscriptional gene silencing.** *Cell*, 123(4), 631–640. <https://doi.org/10.1016/j.cell.2005.10.022>
- [169]. Winter, J., & Diederichs, S. (2011). **Argonaute proteins regulate microRNA stability: Increased microRNA abundance by Argonaute proteins is due to microRNA stabilization.** *RNA biology*, 8(6), 1149–1157. <https://doi.org/10.4161/rna.8.6.17665>
- [170]. Kehl, T., Backes, C., Kern, F., Fehlmann, T., Ludwig, N., Meese, E., Lenhof, H. P., & Keller, A. (2017). **About miRNAs, miRNA seeds, target genes and target pathways.** *Oncotarget*, 8(63), 107167–107175. <https://doi.org/10.18632/oncotarget.22363>
- [171]. Grimson, A., Farh, K. K., Johnston, W. K., Garrett-Engle, P., Lim, L. P., & Bartel, D. P. (2007). **MicroRNA targeting specificity in mammals: determinants beyond seed pairing.** *Molecular cell*, 27(1), 91–105. <https://doi.org/10.1016/j.molcel.2007.06.017>
- [172]. Lai, E. C., Wiel, C., & Rubin, G. M. (2004). **Complementary miRNA pairs suggest a regulatory role for miRNA:miRNA duplexes.** *RNA (New York, N.Y.)*, 10(2), 171–175. <https://doi.org/10.1261/rna.5191904>
- [173]. Wilczynska, A., & Bushell, M. (2015). **The complexity of miRNA-mediated repression.** *Cell death and differentiation*, 22(1), 22–33. <https://doi.org/10.1038/cdd.2014.112>
- [174]. Granados-López, A. J., Ruiz-Carrillo, J. L., Servín-González, L. S., Martínez-Rodríguez, J. L., Reyes-Estrada, C. A., Gutiérrez-

- Hernández, R., & López, J. A. (2017). **Use of Mature miRNA Strand Selection in miRNAs Families in Cervical Cancer Development.** *International journal of molecular sciences*, 18(2), 407. <https://doi.org/10.3390/ijms18020407>
- [175]. Du, Z. Q., Yang, C. X., Rothschild, M. F., & Ross, J. W. (2013). **Novel microRNA families expanded in the human genome.** *BMC genomics*, 14, 98. <https://doi.org/10.1186/1471-2164-14-98>
- [176]. Gao, Y., Schug, J., McKenna, L. B., Le Lay, J., Kaestner, K. H., & Greenbaum, L. E. (2011). **Tissue-specific regulation of mouse microRNA genes in endoderm-derived tissues.** *Nucleic acids research*, 39(2), 454–463. <https://doi.org/10.1093/nar/gkq782>
- [177]. Peng, Y., & Croce, C. M. (2016). **The role of MicroRNAs in human cancer.** *Signal transduction and targeted therapy*, 1, 15004. <https://doi.org/10.1038/sigtrans.2015.4>
- [178]. Calin, G. A., Dumitru, C. D., Shimizu, M., Bichi, R., Zupo, S., Noch, E., Aldler, H., Rattan, S., Keating, M., Rai, K., Rassenti, L., Kipps, T., Negrini, M., Bullrich, F., & Croce, C. M. (2002). **Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia.** *Proceedings of the National Academy of Sciences of the United States of America*, 99(24), 15524–15529. <https://doi.org/10.1073/pnas.242606799>
- [179]. Calin, G. A., Sevignani, C., Dumitru, C. D., Hyslop, T., Noch, E., Yendamuri, S., Shimizu, M., Rattan, S., Bullrich, F., Negrini, M., & Croce, C. M. (2004). **Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers.** *Proceedings of the National Academy of Sciences of the United States of America*, 101(9), 2999–3004. <https://doi.org/10.1073/pnas.0307323101>

- [180]. Rupaimoole, R., Calin, G. A., Lopez-Berestein, G., & Sood, A. K. (2016). **miRNA Deregulation in Cancer Cells and the Tumor Microenvironment.** *Cancer discovery*, 6(3), 235–246.
<https://doi.org/10.1158/2159-8290.CD-15-0893>
- [181]. Zuberi, M., Mir, R., Das, J., Ahmad, I., Javid, J., Yadav, P., Masroor, M., Ahmad, S., Ray, P. C., & Saxena, A. (2015). **Expression of serum miR-200a, miR-200b, and miR-200c as candidate biomarkers in epithelial ovarian cancer and their association with clinicopathological features.** *Clinical & translational oncology: official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico*, 17(10), 779–787.
<https://doi.org/10.1007/s12094-015-1303-1>
- [182]. Wilczynski, M., Kielbik, M., Senderowska, D., Krawczyk, T., Szymanska, B., Klink, M., Bieńkiewicz, J., Romanowicz, H., Frühauf, F., & Malinowski, A. (2020). **MiRNA-103/107 in Primary High-Grade Serous Ovarian Cancer and Its Clinical Significance.** *Cancers*, 12(9), 2680.
<https://doi.org/10.3390/cancers12092680>
- [183]. Kajdasz, A., Majer, W., Kluzek, K., Sobkowiak, J., Milecki, T., Derebecka, N., Kwias, Z., Bluysen, H., & Wesoly, J. (2021). **Identification of RCC Subtype-Specific microRNAs-Meta-Analysis of High-Throughput RCC Tumor microRNA Expression Data.** *Cancers*, 13(3), 548.
<https://doi.org/10.3390/cancers13030548>
- [184]. Svoronos, A. A., Engelman, D. M., & Slack, F. J. (2016). **OncomiR or Tumor Suppressor? The Duplicity of MicroRNAs in Cancer.** *Cancer research*, 76(13), 3666–3670.
<https://doi.org/10.1158/0008-5472.CAN-16-0359>

- [185]. Cho W. C. (2007). **OncomiRs: the discovery and progress of microRNAs in cancers.** *Molecular cancer*, 6, 60. <https://doi.org/10.1186/1476-4598-6-60>
- [186]. Stenvang, J., Petri, A., Lindow, M., Obad, S., & Kauppinen, S. (2012). **Inhibition of microRNA function by anti-miR oligonucleotides.** *Silence*, 3(1), 1. <https://doi.org/10.1186/1758-907X-3-1>
- [187]. Baumann, V., & Winkler, J. (2014). **miRNA-based therapies: strategies and delivery platforms for oligonucleotide and non-oligonucleotide agents.** *Future medicinal chemistry*, 6(17), 1967–1984. <https://doi.org/10.4155/fmc.14.116>
- [188]. Simonson, B., & Das, S. (2015). **MicroRNA Therapeutics: the Next Magic Bullet?** *Mini reviews in medicinal chemistry*, 15(6), 467–474. <https://doi.org/10.2174/1389557515666150324123208>
- [189]. Szczepanek, J., Skorupa, M., & Tretyn, A. (2022). **MicroRNA as a Potential Therapeutic Molecule in Cancer.** *Cells*, 11(6), 1008. <https://doi.org/10.3390/cells11061008>
- [190]. Zhang, Y., Wang, Z., & Gemeinhart, R. A. (2013). **Progress in microRNA delivery.** *Journal of controlled release: official journal of the Controlled Release Society*, 172(3), 962–974. <https://doi.org/10.1016/j.jconrel.2013.09.015>
- [191]. Geisler, A., & Fechner, H. (2016). **MicroRNA-regulated viral vectors for gene therapy.** *World journal of experimental medicine*, 6(2), 37–54. <https://doi.org/10.5493/wjem.v6.i2.37>
- [192]. Sharma, A., Tandon, M., Bangari, D. S., & Mittal, S. K. (2009). **Adenoviral vector-based strategies for cancer therapy.** *Current drug therapy*, 4(2), 117–138. <https://doi.org/10.2174/157488509788185123>

- [193]. Wold, W. S., & Toth, K. (2013). Adenovirus vectors for gene therapy, vaccination and cancer gene therapy. *Current gene therapy*, 13(6), 421–433. <https://doi.org/10.2174/1566523213666131125095046>
- [194]. Daya, S., & Berns, K. I. (2008). **Gene therapy using adeno-associated virus vectors**. *Clinical microbiology reviews*, 21(4), 583–593. <https://doi.org/10.1128/CMR.00008-08>
- [195]. Santiago-Ortiz, J. L., & Schaffer, D. V. (2016). **Adeno-associated virus (AAV) vectors in cancer gene therapy**. *Journal of controlled release: official journal of the Controlled Release Society*, 240, 287–301. <https://doi.org/10.1016/j.jconrel.2016.01.001>
- [196]. Anson D. S. (2004). **The use of retroviral vectors for gene therapy- what are the risks? A review of retroviral pathogenesis and its relevance to retroviral vector-mediated gene delivery**. *Genetic vaccines and therapy*, 2(1), 9. <https://doi.org/10.1186/1479-0556-2-9>
- [197]. Dufait, I., Liechtenstein, T., Lanna, A., Bricogne, C., Laranga, R., Padella, A., Breckpot, K., & Escors, D. (2012). **Retroviral and lentiviral vectors for the induction of immunological tolerance**. *Scientifica*, 2012, 694137. <https://doi.org/10.6064/2012/694137>
- [198]. Escors, D., & Breckpot, K. (2010). **Lentiviral vectors in gene therapy: their current status and future potential**. *Archivum immunologiae et therapiae experimentalis*, 58(2), 107–119. <https://doi.org/10.1007/s00005-010-0063-4>
- [199]. Jiang, Y., Huo, S., Hardie, J., Liang, X. J., & Rotello, V. M. (2016). **Progress and perspective of inorganic nanoparticle-based siRNA**

- delivery systems.** *Expert opinion on drug delivery*, 13(4), 547–559.
<https://doi.org/10.1517/17425247.2016.1134486>
- [200]. Zare, M., Pemmada, R., Madhavan, M., Shailaja, A., Ramakrishna, S., Kandiyil, S. P., Donahue, J. M., & Thomas, V. (2022). **Encapsulation of miRNA and siRNA into Nanomaterials for Cancer Therapeutics.** *Pharmaceutics*, 14(8), 1620.
<https://doi.org/10.3390/pharmaceutics14081620>
- [201]. Lee, S., Paoletti, C., Campisi, M., Osaki, T., Adriani, G., Kamm, R. D., Mattu, C., & Chiono, V. (2019). **MicroRNA delivery through nanoparticles.** *Journal of controlled release: official journal of the Controlled Release Society*, 313, 80–95.
<https://doi.org/10.1016/j.jconrel.2019.10.007>
- [202]. Moodley, T., & Singh, M. (2021). **Current Stimuli-Responsive Mesoporous Silica Nanoparticles for Cancer Therapy.** *Pharmaceutics*, 13(1), 71.
<https://doi.org/10.3390/pharmaceutics13010071>
- [203]. Shafiee, A., Iravani, S., & Varma, R. S. (2022). **Graphene and graphene oxide with anticancer applications: Challenges and future perspectives.** *MedComm*, 3(1), e118.
<https://doi.org/10.1002/mco2.118>
- [204]. Grilli, F., Hajimohammadi Gohari, P., & Zou, S. (2022). **Characteristics of Graphene Oxide for Gene Transfection and Controlled Release in Breast Cancer Cells.** *International journal of molecular sciences*, 23(12), 6802.
<https://doi.org/10.3390/ijms23126802>
- [205]. Dadfar, S. M., Roemhild, K., Drude, N. I., von Stillfried, S., Knüchel, R., Kiessling, F., & Lammers, T. (2019). **Iron oxide nanoparticles: Diagnostic, therapeutic and theranostic applications.** *Advanced*

drug delivery reviews, 138, 302–325.
<https://doi.org/10.1016/j.addr.2019.01.005>

- [206]. Sukumar, U. K., Bose, R., Malhotra, M., Babikir, H. A., Afjei, R., Robinson, E., Zeng, Y., Chang, E., Habte, F., Sinclair, R., Gambhir, S. S., Massoud, T. F., & Paulmurugan, R. (2019). **Intranasal delivery of targeted polyfunctional gold-iron oxide nanoparticles loaded with therapeutic microRNAs for combined theranostic multimodality imaging and presensitization of glioblastoma to temozolomide.** *Biomaterials*, 218, 119342.
<https://doi.org/10.1016/j.biomaterials.2019.119342>
- [207]. Xia, W., Tao, Z., Zhu, B., Zhang, W., Liu, C., Chen, S., & Song, M. (2021). **Targeted Delivery of Drugs and Genes Using Polymer Nanocarriers for Cancer Therapy.** *International journal of molecular sciences*, 22(17), 9118.
<https://doi.org/10.3390/ijms22179118>
- [208]. Dasgupta, I., & Chatterjee, A. (2021). **Recent Advances in miRNA Delivery Systems.** *Methods and protocols*, 4(1), 10.
<https://doi.org/10.3390/mps4010010>
- [209]. Deng, R., Yue, Y., Jin, F., Chen, Y., Kung, H. F., Lin, M. C., & Wu, C. (2009). **Revisit the complexation of PEI and DNA - how to make low cytotoxic and highly efficient PEI gene transfection non-viral vectors with a controllable chain length and structure?** *Journal of controlled release : official journal of the Controlled Release Society*, 140(1), 40–46.
<https://doi.org/10.1016/j.jconrel.2009.07.009>
- [210]. Croy, S. R., & Kwon, G. S. (2006). **Polymeric micelles for drug delivery.** *Current pharmaceutical design*, 12(36), 4669–4684.
<https://doi.org/10.2174/138161206779026245>

- [211]. Ghosh, B., & Biswas, S. (2021). **Polymeric micelles in cancer therapy: State of the art.** *Journal of controlled release: official journal of the Controlled Release Society*, 332, 127–147. <https://doi.org/10.1016/j.jconrel.2021.02.016>
- [212]. Cheung, R. C., Ng, T. B., Wong, J. H., & Chan, W. Y. (2015). **Chitosan: An Update on Potential Biomedical and Pharmaceutical Applications.** *Marine drugs*, 13(8), 5156–5186. <https://doi.org/10.3390/md13085156>
- [213]. Denizli, M., Aslan, B., Mangala, L. S., Jiang, D., Rodriguez-Aguayo, C., Lopez-Berestein, G., & Sood, A. K. (2017). **Chitosan Nanoparticles for miRNA Delivery.** *Methods in molecular biology (Clifton, N.J.)*, 1632, 219–230. https://doi.org/10.1007/978-1-4939-7138-1_14
- [214]. Zaro J. L. (2015). **Lipid-based drug carriers for prodrugs to enhance drug delivery.** *The AAPS journal*, 17(1), 83–92. <https://doi.org/10.1208/s12248-014-9670-z>
- [215]. Sercombe, L., Veerati, T., Moheimani, F., Wu, S. Y., Sood, A. K., & Hua, S. (2015). **Advances and Challenges of Liposome Assisted Drug Delivery.** *Frontiers in pharmacology*, 6, 286. <https://doi.org/10.3389/fphar.2015.00286>
- [216]. Mishra, V., Bansal, K. K., Verma, A., Yadav, N., Thakur, S., Sudhakar, K., & Rosenholm, J. M. (2018). **Solid Lipid Nanoparticles: Emerging Colloidal Nano Drug Delivery Systems.** *Pharmaceutics*, 10(4), 191. <https://doi.org/10.3390/pharmaceutics10040191>
- [217]. Duan, Y., Dhar, A., Patel, C., Khimani, M., Neogi, S., Sharma, P., Siva Kumar, N., & Vekariya, R. L. (2020). **A brief review on solid lipid nanoparticles: part and parcel of contemporary drug**

delivery systems. *RSC advances*, 10(45), 26777–26791.
<https://doi.org/10.1039/d0ra03491f>

- [218]. Elmowafy, M., & Al-Sanea, M. M. (2021). **Nanostructured lipid carriers (NLCs) as drug delivery platform: Advances in formulation and delivery strategies.** *Saudi pharmaceutical journal: SPJ: the official publication of the Saudi Pharmaceutical Society*, 29(9), 999–1012.
<https://doi.org/10.1016/j.jsps.2021.07.015>
- [219]. Haider, M., Abdin, S. M., Kamal, L., & Orive, G. (2020). **Nanostructured Lipid Carriers for Delivery of Chemotherapeutics: A Review.** *Pharmaceutics*, 12(3), 288.
<https://doi.org/10.3390/pharmaceutics12030288>
- [220]. Zhang, Y., Liu, Y., Liu, H., & Tang, W. H. (2019). **Exosomes: biogenesis, biologic function and clinical potential.** *Cell & bioscience*, 9, 19. <https://doi.org/10.1186/s13578-019-0282-2>
- [221]. Kumar, D. N., Chaudhuri, A., Aqil, F., Dehari, D., Munagala, R., Singh, S., Gupta, R. C., & Agrawal, A. K. (2022). **Exosomes as Emerging Drug Delivery and Diagnostic Modality for Breast Cancer: Recent Advances in Isolation and Application.** *Cancers*, 14(6), 1435.
<https://doi.org/10.3390/cancers14061435>
- [222]. Hu, G., Drescher, K. M., & Chen, X. M. (2012). **Exosomal miRNAs: Biological Properties and Therapeutic Potential.** *Frontiers in genetics*, 3, 56. <https://doi.org/10.3389/fgene.2012.00056>
- [223]. Zhao, D., Tao, W., Li, S., Chen, Y., Sun, Y., He, Z., Sun, B., & Sun, J. (2021). **Apoptotic body-mediated intercellular delivery for enhanced drug penetration and whole tumor**

- destruction.** *Science advances*, 7(16), eabg0880.
<https://doi.org/10.1126/sciadv.abg0880>
- [224]. Mussbacher, M., Pirabe, A., Brunnthaler, L., Schrottmaier, W. C., & Assinger, A. (2021). **Horizontal MicroRNA Transfer by Platelets - Evidence and Implications.** *Frontiers in physiology*, 12, 678362.
<https://doi.org/10.3389/fphys.2021.678362>
- [225]. Vigata, M., Meinert, C., Hutmacher, D. W., & Bock, N. (2020). **Hydrogels as Drug Delivery Systems: A Review of Current Characterization and Evaluation Techniques.** *Pharmaceutics*, 12(12), 1188.
<https://doi.org/10.3390/pharmaceutics12121188>
- [226]. Hernandez, M. J., Gaetani, R., Pieters, V. M., Ng, N. W., Chang, A. E., Martin, T. R., van Ingen, E., Mol, E. A., Sluijter, J., & Christman, K. L. (2018). **Decellularized Extracellular Matrix Hydrogels as a Delivery Platform for MicroRNA and Extracellular Vesicle Therapeutics.** *Advanced therapeutics*, 1(3), 1800032.
<https://doi.org/10.1002/adtp.201800032>
- [227]. Weidhaas, J. B., Babar, I., Nallur, S. M., Trang, P., Roush, S., Boehm, M., Gillespie, E., & Slack, F. J. (2007). **MicroRNAs as potential agents to alter resistance to cytotoxic anticancer therapy.** *Cancer research*, 67(23), 11111–11116. <https://doi.org/10.1158/0008-5472.CAN-07-2858>
- [228]. Dhayat, S. A., Mardin, W. A., Seggewiß, J., Ströse, A. J., Matuszcak, C., Hummel, R., Senninger, N., Mees, S. T., & Haier, J. (2015). **MicroRNA Profiling Implies New Markers of Gemcitabine Chemoresistance in Mutant p53 Pancreatic Ductal Adenocarcinoma.** *PloS one*, 10(11), e0143755.
<https://doi.org/10.1371/journal.pone.0143755>

- [229]. Wei, X., Wang, W., Wang, L., Zhang, Y., Zhang, X., Chen, M., Wang, F., Yu, J., Ma, Y., & Sun, G. (2016). **MicroRNA-21 induces 5-fluorouracil resistance in human pancreatic cancer cells by regulating PTEN and PDCD4.** *Cancer medicine*, 5(4), 693–702. <https://doi.org/10.1002/cam4.626>
- [230]. Dong, J., Zhao, Y. P., Zhou, L., Zhang, T. P., & Chen, G. (2011). **Bcl-2 upregulation induced by miR-21 via a direct interaction is associated with apoptosis and chemoresistance in MIA PaCa-2 pancreatic cancer cells.** *Archives of medical research*, 42(1), 8–14. <https://doi.org/10.1016/j.arcmed.2011.01.006>
- [231]. Xiong, J., Wang, D., Wei, A., Ke, N., Wang, Y., Tang, J., He, S., Hu, W., & Liu, X. (2017). **MicroRNA-410-3p attenuates gemcitabine resistance in pancreatic ductal adenocarcinoma by inhibiting HMGB1-mediated autophagy.** *Oncotarget*, 8(64), 107500–107512. <https://doi.org/10.18632/oncotarget.22494>
- [232]. Zhao, Y., Zhao, L., Ischenko, I., Bao, Q., Schwarz, B., Nieß, H., Wang, Y., Renner, A., Mysliwietz, J., Jauch, K. W., Nelson, P. J., Ellwart, J. W., Bruns, C. J., & Camaj, P. (2015). Antisense inhibition of microRNA-21 and microRNA-221 in tumor-initiating stem-like cells modulates tumorigenesis, metastasis, and chemotherapy resistance in pancreatic cancer. *Targeted oncology*, 10(4), 535–548. <https://doi.org/10.1007/s11523-015-0360-2>
- [233]. Nagano, H., Tomimaru, Y., Eguchi, H., Hama, N., Wada, H., Kawamoto, K., Kobayashi, S., Mori, M., & Doki, Y. (2013). MicroRNA-29a induces resistance to gemcitabine through the Wnt/ β -catenin signaling pathway in pancreatic cancer cells. *International journal of oncology*, 43(4), 1066–1072. <https://doi.org/10.3892/ijo.2013.2037>

- [234]. Yu, G., Jia, B., Cheng, Y., Zhou, L., Qian, B., Liu, Z., & Wang, Y. (2017). **MicroRNA-429 sensitizes pancreatic cancer cells to gemcitabine through regulation of PDCD4.** *American journal of translational research*, 9(11), 5048–5055.
- [235]. Li, Z., Li, X., Yu, C., Wang, M., Peng, F., Xiao, J., Tian, R., Jiang, J., & Sun, C. (2014). **MicroRNA-100 regulates pancreatic cancer cells growth and sensitivity to chemotherapy through targeting FGFR3.** *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine*, 35(12), 11751–11759. <https://doi.org/10.1007/s13277-014-2271-8>
- [236]. Schreiber, R., Mezencev, R., Matyunina, L. V., & McDonald, J. F. (2016). **Evidence for the role of microRNA 374b in acquired cisplatin resistance in pancreatic cancer cells.** *Cancer gene therapy*, 23(8), 241–245. <https://doi.org/10.1038/cgt.2016.23>
- [237]. Zhang, J., Gao, S., Zhang, Y., Yi, H., Xu, M., Xu, J., Liu, H., Ding, Z., He, H., Wang, H., Hao, Z., Sun, L., Liu, Y., & Wei, F. (2020). **MiR-216a-5p inhibits tumorigenesis in Pancreatic Cancer by targeting TPT1/mTORC1 and is mediated by LINC01133.** *International journal of biological sciences*, 16(14), 2612–2627. <https://doi.org/10.7150/ijbs.46822>
- [238]. Wang, P., Zhang, J., Zhang, L., Zhu, Z., Fan, J., Chen, L., Zhuang, L., Luo, J., Chen, H., Liu, L., Chen, Z., & Meng, Z. (2013). **MicroRNA 23b regulates autophagy associated with radioresistance of pancreatic cancer cells.** *Gastroenterology*, 145(5), 1133–1143.e12. <https://doi.org/10.1053/j.gastro.2013.07.048>
- [239]. Fu, X., & Calin, G. A. (2018). **miR-122 and hepatocellular carcinoma: from molecular biology to therapeutics.** *EBioMedicine*, 37, 17–18. <https://doi.org/10.1016/j.ebiom.2018.10.032>

- [240]. Hong, D. S., Kang, Y. K., Borad, M., Sachdev, J., Ejadi, S., Lim, H. Y., Brenner, A. J., Park, K., Lee, J. L., Kim, T. Y., Shin, S., Becerra, C. R., Falchook, G., Stoudemire, J., Martin, D., Kelnar, K., Peltier, H., Bonato, V., Bader, A. G., Smith, S., Beg, M. S. (2020). **Phase 1 study of MRX34, a liposomal miR-34a mimic, in patients with advanced solid tumours.** *British journal of cancer*, 122(11), 1630–1637. <https://doi.org/10.1038/s41416-020-0802-1>
- [241]. Hennessy, E. J., & Moore, K. J. (2013). **Using microRNA as an alternative treatment for hyperlipidemia and cardiovascular disease: cardio-miRs in the pipeline.** *Journal of cardiovascular pharmacology*, 62(3), 247–254. <https://doi.org/10.1097/FJC.0b013e31829d48bf>
- [242]. Li, M. M., Addepalli, B., Tu, M. J., Chen, Q. X., Wang, W. P., Limbach, P. A., LaSalle, J. M., Zeng, S., Huang, M., & Yu, A. M. (2015). **Chimeric MicroRNA-1291 Biosynthesized Efficiently in Escherichia coli Is Effective to Reduce Target Gene Expression in Human Carcinoma Cells and Improve Chemosensitivity.** *Drug metabolism and disposition: the biological fate of chemicals*, 43(7), 1129–1136. <https://doi.org/10.1124/dmd.115.064493>
- [243]. Amponsah, P. S., Fan, P., Bauer, N., Zhao, Z., Gladkich, J., Fellenberg, J., & Herr, I. (2017). **microRNA-210 overexpression inhibits tumor growth and potentially reverses gemcitabine resistance in pancreatic cancer.** *Cancer letters*, 388, 107–117. <https://doi.org/10.1016/j.canlet.2016.11.035>
- [244]. Lai, I. L., Chou, C. C., Lai, P. T., Fang, C. S., Shirley, L. A., Yan, R., Mo, X., Bloomston, M., Kulp, S. K., Bekaii-Saab, T., & Chen, C. S. (2014). **Targeting the Warburg effect with a novel glucose transporter**

- inhibitor to overcome gemcitabine resistance in pancreatic cancer cells.** *Carcinogenesis*, 35(10), 2203–2213. <https://doi.org/10.1093/carcin/bgu124> (Retraction published *Carcinogenesis*. 2019 Apr 29;40(2):e16)
- [245]. Hu, H., He, Y., Wang, Y., Chen, W., Hu, B., & Gu, Y. (2017). **micorRNA-101 silences DNA-PKcs and sensitizes pancreatic cancer cells to gemcitabine.** *Biochemical and biophysical research communications*, 483(1), 725–731. <https://doi.org/10.1016/j.bbrc.2016.12.074>
- [246]. Fan, P., Liu, L., Yin, Y., Zhao, Z., Zhang, Y., Amponsah, P. S., Xiao, X., Bauer, N., Abukiwan, A., Nwaeburu, C. C., Gladkich, J., Gao, C., Schemmer, P., Gross, W., & Herr, I. (2016). **MicroRNA-101-3p reverses gemcitabine resistance by inhibition of ribonucleotide reductase M1 in pancreatic cancer.** *Cancer letters*, 373(1), 130–137. <https://doi.org/10.1016/j.canlet.2016.01.038>
- [247]. Lu, H., Lu, S., Yang, D., Zhang, L., Ye, J., Li, M., & Hu, W. (2019). **MiR-20a-5p regulates gemcitabine chemosensitivity by targeting RRM2 in pancreatic cancer cells and serves as a predictor for gemcitabine-based chemotherapy.** *Bioscience reports*, 39(5), BSR20181374. <https://doi.org/10.1042/BSR20181374>
- [248]. Ma, J., Fang, B., Zeng, F., Ma, C., Pang, H., Cheng, L., Shi, Y., Wang, H., Yin, B., Xia, J., & Wang, Z. (2015). **Down-regulation of miR-223 reverses epithelial-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells.** *Oncotarget*, 6(3), 1740–1749. <https://doi.org/10.18632/oncotarget.2714>
- [249]. Li, Y., VandenBoom, T. G., 2nd, Kong, D., Wang, Z., Ali, S., Philip, P. A., & Sarkar, F. H. (2009). **Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic**

cancer cells. *Cancer research*, 69(16), 6704–6712.
<https://doi.org/10.1158/0008-5472.CAN-09-1298>

- [250]. Wang, J., Zheng, Y., Bai, B., Song, Y., Zheng, K., Xiao, J., Liang, Y., Bao, L., Zhou, Q., Ji, L., & Feng, X. (2020). **MicroRNA-125a-3p participates in odontoblastic differentiation of dental pulp stem cells by targeting Fyn.** *Cytotechnology*, 72(1), 69–79.
<https://doi.org/10.1007/s10616-019-00358-7>
- [251]. Yang, R. M., Zhan, M., Xu, S. W., Long, M. M., Yang, L. H., Chen, W., Huang, S., Liu, Q., Zhou, J., Zhu, J., & Wang, J. (2017). **miR-3656 expression enhances the chemosensitivity of pancreatic cancer to gemcitabine through modulation of the RHO/EMT axis.** *Cell death & disease*, 8(10), e3129.
<https://doi.org/10.1038/cddis.2017.530>
- [252]. Liu, F., Liu, B., Qian, J., Wu, G., Li, J., & Ma, Z. (2017). **miR-153 enhances the therapeutic effect of gemcitabine by targeting Snail in pancreatic cancer.** *Acta biochimica et biophysica Sinica*, 49(6), 520–529. <https://doi.org/10.1093/abbs/gmx039>
- [253]. Fesler, A., & Ju, J. (2019). **Development of microRNA-based therapy for pancreatic cancer.** *Journal of pancreatology*, 2(4), 147–151. <https://doi.org/10.1097/jp9.0000000000000029>
- [254]. Park, J. K., Lee, E. J., Esau, C., & Schmittgen, T. D. (2009). **Antisense inhibition of microRNA-21 or -221 arrests cell cycle, induces apoptosis, and sensitizes the effects of gemcitabine in pancreatic adenocarcinoma.** *Pancreas*, 38(7), e190–e199.
<https://doi.org/10.1097/MPA.0b013e3181ba82e1>
- [255]. Gu, J., Wang, D., Zhang, J., Zhu, Y., Li, Y., Chen, H., Shi, M., Wang, X., Shen, B., Deng, X., Zhan, Q., Wei, G., & Peng, C. (2016). **GFRα2 prompts cell growth and chemoresistance through down-**

- regulating tumor suppressor gene PTEN via Mir-17-5p in pancreatic cancer. *Cancer letters*, 380(2), 434–441. <https://doi.org/10.1016/j.canlet.2016.06.016>
- [256]. Song, W. F., Wang, L., Huang, W. Y., Cai, X., Cui, J. J., & Wang, L. W. (2013). **MiR-21 upregulation induced by promoter zone histone acetylation is associated with chemoresistance to gemcitabine and enhanced malignancy of pancreatic cancer cells.** *Asian Pacific journal of cancer prevention: APJCP*, 14(12), 7529–7536. <https://doi.org/10.7314/apjcp.2013.14.12.7529>
- [257]. Shen, H., Zhu, F., Liu, J., Xu, T., Pei, D., Wang, R., Qian, Y., Li, Q., Wang, L., Shi, Z., Zheng, J., Chen, Q., Jiang, B., & Shu, Y. (2014). **Alteration in Mir-21/PTEN expression modulates gefitinib resistance in non-small cell lung cancer.** *PloS one*, 9(7), e103305. <https://doi.org/10.1371/journal.pone.0103305>
- [258]. Wei, F., Liu, Y., Guo, Y., Xiang, A., Wang, G., Xue, X., & Lu, Z. (2013). **miR-99b-targeted mTOR induction contributes to irradiation resistance in pancreatic cancer.** *Molecular cancer*, 12, 81. <https://doi.org/10.1186/1476-4598-12-81>
- [259]. Fang, C., Dai, C. Y., Mei, Z., Jiang, M. J., Gu, D. N., Huang, Q., & Tian, L. (2018). **microRNA-193a stimulates pancreatic cancer cell repopulation and metastasis through modulating TGF- β 2/TGF- β RIII signalings.** *Journal of experimental & clinical cancer research: CR*, 37(1), 25. <https://doi.org/10.1186/s13046-018-0697-3>
- [260]. Huang, X., Taeb, S., Jahangiri, S., Korpela, E., Cadonic, I., Yu, N., Krylov, S. N., Fokas, E., Boutros, P. C., & Liu, S. K. (2015). **miR-620 promotes tumor radioresistance by targeting 15-hydroxyprostaglandin dehydrogenase**

(HPGD). *Oncotarget*, 6(26), 22439–22451.
<https://doi.org/10.18632/oncotarget.4210>

- [261]. Takiuchi, D., Eguchi, H., Nagano, H., Iwagami, Y., Tomimaru, Y., Wada, H., Kawamoto, K., Kobayashi, S., Marubashi, S., Tanemura, M., Mori, M., & Doki, Y. (2013). **Involvement of microRNA-181b in the gemcitabine resistance of pancreatic cancer cells.** *Pancreatology: official journal of the International Association of Pancreatology (IAP)*, 13(5), 517–523.
<https://doi.org/10.1016/j.pan.2013.06.007>
- [262]. Mikamori, M., Yamada, D., Eguchi, H., Hasegawa, S., Kishimoto, T., Tomimaru, Y., Asaoka, T., Noda, T., Wada, H., Kawamoto, K., Gotoh, K., Takeda, Y., Tanemura, M., Mori, M., & Doki, Y. (2017). **MicroRNA-155 Controls Exosome Synthesis and Promotes Gemcitabine Resistance in Pancreatic Ductal Adenocarcinoma.** *Scientific reports*, 7, 42339.
<https://doi.org/10.1038/srep42339>
- [263]. Yang, D., Hu, Z., Xu, J., Tang, Y., Wang, Y., Cai, Q., & Zhu, Z. (2019). **MiR-760 enhances sensitivity of pancreatic cancer cells to gemcitabine through modulating Integrin $\beta 1$.** *Bioscience reports*, 39(11), BSR20192358.
<https://doi.org/10.1042/BSR20192358>
- [264]. Sun, D., Wang, X., Sui, G., Chen, S., Yu, M., & Zhang, P. (2018). **Downregulation of miR-374b-5p promotes chemotherapeutic resistance in pancreatic cancer by upregulating multiple anti-apoptotic proteins.** *International journal of oncology*, 52(5), 1491–1503. <https://doi.org/10.3892/ijo.2018.4315>
- [265]. Livak, K. J., & Schmittgen, T. D. (2001). **Analysis of relative gene expression data using real-time quantitative PCR and the 2(-**

- Delta Delta C(T)) Method.** *Methods (San Diego, Calif.)*, 25(4), 402–408. <https://doi.org/10.1006/meth.2001.1262>
- [266]. Beger, H. G., Rau, B., Gansauge, F., Leder, G., Schwarz, M., & Poch, B. (2008). **Pancreatic cancer--low survival rates.** *Deutsches Arzteblatt international*, 105(14), 255–262. <https://doi.org/10.3238/arztebl.2008.0255>
- [267]. Calin, G. A., & Croce, C. M. (2006). **MicroRNA-cancer connection: the beginning of a new tale.** *Cancer research*, 66(15), 7390–7394. <https://doi.org/10.1158/0008-5472.CAN-06-0800>
- [268]. Laurila, E. M., & Kallioniemi, A. (2013). **The diverse role of miR-31 in regulating cancer associated phenotypes.** *Genes, chromosomes & cancer*, 52(12), 1103–1113. <https://doi.org/10.1002/gcc.22107>
- [269]. Iwatate, Y., Hoshino, I., Ishige, F., Itami, M., Chiba, S., Arimitsu, H., Yanagibashi, H., Nagase, H., Yokota, H., & Takayama, W. (2020). **Prognostic significance of p16 protein in pancreatic ductal adenocarcinoma.** *Molecular and clinical oncology*, 13(1), 83–91. <https://doi.org/10.3892/mco.2020.2047>
- [270]. Sherborne, A. L., Hosking, F. J., Prasad, R. B., Kumar, R., Koehler, R., Vijayakrishnan, J., Papaemmanuil, E., Bartram, C. R., Stanulla, M., Schrappe, M., Gast, A., Dobbins, S. E., Ma, Y., Sheridan, E., Taylor, M., Kinsey, S. E., Lightfoot, T., Roman, E., Irving, J. A., Allan, J. M., Houlston, R. S. (2010). **Variation in CDKN2A at 9p21.3 influences childhood acute lymphoblastic leukemia risk.** *Nature genetics*, 42(6), 492–494. <https://doi.org/10.1038/ng.585>
- [271]. Yu, T., Ma, P., Wu, D., Shu, Y., & Gao, W. (2018). **Functions and mechanisms of microRNA-31 in human cancers.** *Biomedicine &*

pharmacotherapy = Biomedecine & pharmacotherapie, 108, 1162–1169. <https://doi.org/10.1016/j.biopha.2018.09.132>

- [272]. Asangani, I. A., Harms, P. W., Dodson, L., Pandhi, M., Kunju, L. P., Maher, C. A., Fullen, D. R., Johnson, T. M., Giordano, T. J., Palanisamy, N., & Chinnaiyan, A. M. (2012). **Genetic and epigenetic loss of microRNA-31 leads to feed-forward expression of EZH2 in melanoma.** *Oncotarget*, 3(9), 1011–1025. <https://doi.org/10.18632/oncotarget.622>
- [273]. Kent, O. A., Mendell, J. T., & Rottapel, R. (2016). **Transcriptional Regulation of miR-31 by Oncogenic KRAS Mediates Metastatic Phenotypes by Repressing RASA1.** *Molecular cancer research: MCR*, 14(3), 267–277. <https://doi.org/10.1158/1541-7786.MCR-15-0456>
- [274]. Moody, H. L., Lind, M. J., & Maher, S. G. (2017). **MicroRNA-31 Regulates Chemosensitivity in Malignant Pleural Mesothelioma.** *Molecular therapy. Nucleic acids*, 8, 317–329. <https://doi.org/10.1016/j.omtn.2017.07.001>
- [275]. Lynam-Lennon, N., Reynolds, J. V., Marignol, L., Sheils, O. M., Pidgeon, G. P., & Maher, S. G. (2012). **MicroRNA-31 modulates tumour sensitivity to radiation in oesophageal adenocarcinoma.** *Journal of molecular medicine (Berlin, Germany)*, 90(12), 1449–1458. <https://doi.org/10.1007/s00109-012-0924-x>
- [276]. Wang, L. L., Li, H. X., Yang, Y. Y., Su, Y. L., Lian, J. S., Li, T., Xu, J., Wang, X. N., Jin, N., & Liu, X. F. (2018). **MiR-31 is a potential biomarker for diagnosis of head and neck squamous cell carcinoma.** *International journal of clinical and experimental pathology*, 11(9), 4339–4345.

- [277]. Prinz, C., Fehring, L., & Frese, R. (2022). **MicroRNAs as Indicators of Malignancy in Pancreatic Ductal Adenocarcinoma (PDAC) and Cystic Pancreatic Lesions.** *Cells*, 11(15), 2374. <https://doi.org/10.3390/cells11152374>
- [278]. Szafranska, A. E., Davison, T. S., John, J., Cannon, T., Sipos, B., Maghnouj, A., Labourier, E., & Hahn, S. A. (2007). **MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma.** *Oncogene*, 26(30), 4442–4452. <https://doi.org/10.1038/sj.onc.1210228>
- [279]. Papaconstantinou, I. G., Manta, A., Gazouli, M., Lyberopoulou, A., Lykoudis, P. M., Polymeneas, G., & Voros, D. (2013). **Expression of microRNAs in patients with pancreatic cancer and its prognostic significance.** *Pancreas*, 42(1), 67–71. <https://doi.org/10.1097/MPA.0b013e3182592ba7>
- [280]. Amrutkar, M., Vethe, N. T., Verbeke, C. S., Aasrum, M., Finstadsveen, A. V., Sántha, P., & Gladhaug, I. P. (2020). **Differential Gemcitabine Sensitivity in Primary Human Pancreatic Cancer Cells and Paired Stellate Cells Is Driven by Heterogenous Drug Uptake and Processing.** *Cancers*, 12(12), 3628. <https://doi.org/10.3390/cancers12123628>
- [281]. Ciccolini, J., Serdjebi, C., Peters, G. J., & Giovannetti, E. (2016). **Pharmacokinetics and pharmacogenetics of Gemcitabine as a mainstay in adult and pediatric oncology: an EORTC-PAMM perspective.** *Cancer chemotherapy and pharmacology*, 78(1), 1–12. <https://doi.org/10.1007/s00280-016-3003-0>
- [282]. Zhang, N., Yin, Y., Xu, S. J., & Chen, W. S. (2008). **5-Fluorouracil: mechanisms of resistance and reversal strategies.** *Molecules*

(Basel, Switzerland), 13(8), 1551–1569.
<https://doi.org/10.3390/molecules13081551>

- [283]. Kamran, M. Z., Ranjan, A., Kaur, N., Sur, S., & Tandon, V. (2016). **Radioprotective Agents: Strategies and Translational Advances.** *Medicinal research reviews*, 36(3), 461–493.
<https://doi.org/10.1002/med.21386>
- [284]. Alizadeh, E., Sanz, A. G., García, G., & Sanche, L. (2013). **Radiation Damage to DNA: The Indirect Effect of Low Energy Electrons.** *The journal of physical chemistry letters*, 4(5), 820–825.
<https://doi.org/10.1021/jz4000998>
- [285]. Buckley, A. M., Bibby, B. A., Dunne, M. R., Kennedy, S. A., Davern, M. B., Kennedy, B. N., Maher, S. G., & O'Sullivan, J. (2019). **Characterisation of an Isogenic Model of Cisplatin Resistance in Oesophageal Adenocarcinoma Cells.** *Pharmaceuticals (Basel, Switzerland)*, 12(1), 33. <https://doi.org/10.3390/ph12010033>
- [286]. Hummel, R., Sie, C., Watson, D. I., Wang, T., Ansar, A., Michael, M. Z., Van der Hoek, M., Haier, J., & Hussey, D. J. (2014). **MicroRNA signatures in chemotherapy resistant esophageal cancer cell lines.** *World journal of gastroenterology*, 20(40), 14904–14912.
<https://doi.org/10.3748/wjg.v20.i40.14904>
- [287]. Lokich, J., & Anderson, N. (1998). **Carboplatin versus cisplatin in solid tumors: an analysis of the literature.** *Annals of oncology: official journal of the European Society for Medical Oncology*, 9(1), 13–21. <https://doi.org/10.1023/a:1008215213739>
- [288]. Stewart D. J. (2007). **Mechanisms of resistance to cisplatin and carboplatin.** *Critical reviews in oncology/hematology*, 63(1), 12–31. <https://doi.org/10.1016/j.critrevonc.2007.02.001>

- [289]. Bruno, P. M., Liu, Y., Park, G. Y., Murai, J., Koch, C. E., Eisen, T. J., Pritchard, J. R., Pommier, Y., Lippard, S. J., & Hemann, M. T. (2017). **A subset of platinum-containing chemotherapeutic agents kills cells by inducing ribosome biogenesis stress.** *Nature medicine*, 23(4), 461–471. <https://doi.org/10.1038/nm.4291>
- [290]. Biswas, R., Bugde, P., He, J., Merien, F., Lu, J., Liu, D. X., Myint, K., Liu, J., McKeage, M., & Li, Y. (2019). **Transport-Mediated Oxaliplatin Resistance Associated with Endogenous Overexpression of MRP2 in Caco-2 and PANC-1 Cells.** *Cancers*, 11(9), 1330. <https://doi.org/10.3390/cancers11091330>
- [291]. Samuel, P., Pink, R. C., Caley, D. P., Currie, J. M., Brooks, S. A., & Carter, D. R. (2016). **Over-expression of miR-31 or loss of KCNMA1 leads to increased cisplatin resistance in ovarian cancer cells.** *Tumour biology: the journal of the International Society for Oncodevelopmental Biology and Medicine*, 37(2), 2565–2573. <https://doi.org/10.1007/s13277-015-4081-z>
- [292]. Ma, H., Cao, W., & Ding, M. (2020). **MicroRNA-31 weakens cisplatin resistance of medulloblastoma cells via NF-κB and PI3K/AKT pathways.** *BioFactors (Oxford, England)*, 46(5), 831–838. <https://doi.org/10.1002/biof.1616>
- [293]. Chen, Y., Zhao, H., Li, H., Feng, X., Tang, H., Qiu, C., Zhang, J., & Fu, B. (2020). **LINC01234/MicroRNA-31-5p/MAGEA3 Axis Mediates the Proliferation and Chemoresistance of Hepatocellular Carcinoma Cells.** *Molecular therapy. Nucleic acids*, 19, 168–178. <https://doi.org/10.1016/j.omtn.2019.10.035>
- [294]. Öman, M., Wettergren, Y., Odin, E., Westermarck, S., Naredi, P., Hemmingsson, O., & Taflin, H. (2021). **Pharmacokinetics of preoperative intraperitoneal 5-FU in patients with pancreatic**

ductal adenocarcinoma. *Cancer chemotherapy and pharmacology*, 88(4), 619–631. <https://doi.org/10.1007/s00280-021-04318-x>

- [295]. Korourian, A., Madjd, Z., Roudi, R., Sharifabrizi, A., & Soleimani, M. (2019). **Induction of miR-31 causes increased sensitivity to 5-FU and decreased migration and cell invasion in gastric adenocarcinoma.** *Bratislavske lekarske listy*, 120(1), 35–39. https://doi.org/10.4149/BLL_2019_005
- [296]. Nakagawa, Y., Kuranaga, Y., Tahara, T., Yamashita, H., Shibata, T., Nagasaka, M., Funasaka, K., Ohmiya, N., & Akao, Y. (2019). **Induced miR-31 by 5-fluorouracil exposure contributes to the resistance in colorectal tumors.** *Cancer science*, 110(8), 2540–2548. <https://doi.org/10.1111/cas.14090>
- [297]. Li, J., Li, X., Cen, C., Ai, X., Lin, C., & Hu, G. (2018). **The long non-coding RNA ENST00000547547 reduces 5-fluorouracil resistance of colorectal cancer cells via competitive binding to microRNA-31.** *Oncology reports*, 39(1), 217–226. <https://doi.org/10.3892/or.2017.6082>
- [298]. Groves, T., Corley, C., Byrum, S. D., & Allen, A. R. (2021). **The Effects of 5-Fluorouracil/Leucovorin Chemotherapy on Cognitive Function in Male Mice.** *Frontiers in molecular biosciences*, 8, 762116. <https://doi.org/10.3389/fmolb.2021.762116>
- [299]. Wen, J., Xiong, K., Aili, A., Wang, H., Zhu, Y., Yu, Z., Yao, X., Jiang, P., Xue, L., & Wang, J. (2020). **PEX5, a novel target of microRNA-31-5p, increases radioresistance in hepatocellular carcinoma by activating Wnt/ β -catenin signaling and homologous recombination.** *Theranostics*, 10(12), 5322–5340. <https://doi.org/10.7150/thno.42371>

- [300]. Körner, C., Keklikoglou, I., Bender, C., Wörner, A., Münstermann, E., & Wiemann, S. (2013). **MicroRNA-31 sensitizes human breast cells to apoptosis by direct targeting of protein kinase C epsilon (PKCepsilon).** *The Journal of biological chemistry*, 288(12), 8750–8761. <https://doi.org/10.1074/jbc.M112.414128>
- [301]. Milenic, D. E., Baidoo, K. E., Shih, J. H., Wong, K. J., & Brechbiel, M. W. (2013). **Evaluation of platinum chemotherapy in combination with HER2-targeted α -particle radiation.** *Cancer biotherapy & radiopharmaceuticals*, 28(6), 441–449. <https://doi.org/10.1089/cbr.2012.1423>
- [302]. Huszno, J., Budryk, M., Kołosza, Z., & Nowara, E. (2015). **The risk factors of toxicity during chemotherapy and radiotherapy in breast cancer patients according to the presence of BRCA gene mutation.** *Contemporary oncology (Poznan, Poland)*, 19(1), 72–76. <https://doi.org/10.5114/wo.2015.50014>
- [303]. Colucci, G., Giuliani, F., Gebbia, V., Biglietto, M., Rabitti, P., Uomo, G., Cigolari, S., Testa, A., Maiello, E., & Lopez, M. (2002). **Gemcitabine alone or with cisplatin for the treatment of patients with locally advanced and/or metastatic pancreatic carcinoma: a prospective, randomized phase III study of the Gruppo Oncologia dell'Italia Meridionale.** *Cancer*, 94(4), 902–910.
- [304]. Chen, S. H., & Chang, J. Y. (2019). **New Insights into Mechanisms of Cisplatin Resistance: From Tumor Cell to Microenvironment.** *International journal of molecular sciences*, 20(17), 4136. <https://doi.org/10.3390/ijms20174136>
- [305]. Martinho, N., Santos, T., Florindo, H. F., & Silva, L. C. (2019). **Cisplatin-Membrane Interactions and Their Influence on Platinum Complexes Activity and Toxicity.** *Frontiers in physiology*, 9, 1898. <https://doi.org/10.3389/fphys.2018.01898>

- [306]. Makovec T. (2019). **Cisplatin and beyond: molecular mechanisms of action and drug resistance development in cancer chemotherapy.** *Radiology and oncology*, 53(2), 148–158.
<https://doi.org/10.2478/raon-2019-0018>
- [307]. Ishida, S., Lee, J., Thiele, D. J., & Herskowitz, I. (2002). **Uptake of the anticancer drug mediated by the copper transporter Ctr1 in yeast and mammals.** *Proceedings of the National Academy of Sciences of the United States of America*, 99(22), 14298–14302.
<https://doi.org/10.1073/pnas.162491399>
- [308]. Kuo, M. T., Fu, S., Savaraj, N., & Chen, H. H. (2012). **Role of the human high-affinity copper transporter in copper homeostasis regulation and cisplatin sensitivity in cancer chemotherapy.** *Cancer research*, 72(18), 4616–4621.
<https://doi.org/10.1158/0008-5472.CAN-12-0888>
- [309]. Ishida, S., McCormick, F., Smith-McCune, K., & Hanahan, D. (2010). **Enhancing tumor-specific uptake of the anticancer drug cisplatin with a copper chelator.** *Cancer cell*, 17(6), 574–583.
<https://doi.org/10.1016/j.ccr.2010.04.011>
- [310]. Sinani, D., Adle, D. J., Kim, H., & Lee, J. (2007). **Distinct mechanisms for Ctr1-mediated copper and cisplatin transport.** *The Journal of biological chemistry*, 282(37), 26775–26785. <https://doi.org/10.1074/jbc.M703973200>
- [311]. Lukanović, D., Herzog, M., Kobal, B., & Černe, K. (2020). **The contribution of copper efflux transporters ATP7A and ATP7B to chemoresistance and personalized medicine in ovarian cancer.** *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, 129, 110401.
<https://doi.org/10.1016/j.biopha.2020.110401>

- [312]. Zhu, S., Shanbhag, V., Wang, Y., Lee, J., & Petris, M. (2017). **A Role for The ATP7A Copper Transporter in Tumorigenesis and Cisplatin Resistance.** *Journal of Cancer*, 8(11), 1952–1958. <https://doi.org/10.7150/jca.19029>
- [313]. Hraběta, J., Belhajová, M., Šubrtová, H., Merlos Rodrigo, M. A., Heger, Z., & Eckschlager, T. (2020). **Drug Sequestration in Lysosomes as One of the Mechanisms of Chemoresistance of Cancer Cells and the Possibilities of Its Inhibition.** *International journal of molecular sciences*, 21(12), 4392. <https://doi.org/10.3390/ijms21124392>
- [314]. Colombo, F., Trombetta, E., Cetrangolo, P., Maggioni, M., Razini, P., De Santis, F., Torrente, Y., Prati, D., Torresani, E., & Porretti, L. (2014). **Giant Lysosomes as a Chemotherapy Resistance Mechanism in Hepatocellular Carcinoma Cells.** *PloS one*, 9(12), e114787. <https://doi.org/10.1371/journal.pone.0114787>
- [315]. Geisslinger, F., Müller, M., Vollmar, A. M., & Bartel, K. (2020). **Targeting Lysosomes in Cancer as Promising Strategy to Overcome Chemoresistance-A Mini Review.** *Frontiers in oncology*, 10, 1156. <https://doi.org/10.3389/fonc.2020.01156>
- [316]. Hou, L., Zhang, X., Jiao, Y., Li, Y., Zhao, Y., Guan, Y., & Liu, Z. (2019). **ATP binding cassette subfamily B member 9 (ABCB9) is a prognostic indicator of overall survival in ovarian cancer.** *Medicine*, 98(19), e15698. <https://doi.org/10.1097/MD.00000000000015698>
- [317]. Brown, A., Kumar, S., & Tchounwou, P. B. (2019). **Cisplatin-Based Chemotherapy of Human Cancers.** *Journal of cancer science & therapy*, 11(4), 97.

- [318]. Wattenberg, M. M., Asch, D., Yu, S., O'Dwyer, P. J., Domchek, S. M., Nathanson, K. L., Rosen, M. A., Beatty, G. L., Siegelman, E. S., & Reiss, K. A. (2020). **Platinum response characteristics of patients with pancreatic ductal adenocarcinoma and a germline BRCA1, BRCA2 or PALB2 mutation.** *British journal of cancer*, 122(3), 333–339. <https://doi.org/10.1038/s41416-019-0582-7>
- [319]. Feng, C., Ma, F., Hu, C., Ma, J. A., Wang, J., Zhang, Y., Wu, F., Hou, T., Jiang, S., Wang, Y., & Feng, Y. (2018). **SOX9/miR-130a/CTR1 axis modulates DDP-resistance of cervical cancer cell.** *Cell cycle (Georgetown, Tex.)*, 17(4), 448–458. <https://doi.org/10.1080/15384101.2017.1395533>
- [320]. Eljack, N. D., Ma, H. Y., Drucker, J., Shen, C., Hambley, T. W., New, E. J., Friedrich, T., & Clarke, R. J. (2014). **Mechanisms of cell uptake and toxicity of the anticancer drug cisplatin.** *Metallomics: integrated biometal science*, 6(11), 2126–2133. <https://doi.org/10.1039/c4mt00238e>
- [321]. Öhrvik, H., & Thiele, D. J. (2015). **The role of Ctr1 and Ctr2 in mammalian copper homeostasis and platinum-based chemotherapy.** *Journal of trace elements in medicine and biology: organ of the Society for Minerals and Trace Elements (GMS)*, 31, 178–182. <https://doi.org/10.1016/j.jtemb.2014.03.006>
- [322]. Lee, Y. Y., Choi, C. H., Do, I. G., Song, S. Y., Lee, W., Park, H. S., Song, T. J., Kim, M. K., Kim, T. J., Lee, J. W., Bae, D. S., & Kim, B. G. (2011). **Prognostic value of the copper transporters, CTR1 and CTR2, in patients with ovarian carcinoma receiving platinum-based chemotherapy.** *Gynecologic oncology*, 122(2), 361–365. <https://doi.org/10.1016/j.ygyno.2011.04.025>

- [323]. Öhrvik, H., Logeman, B., Turk, B., Reinheckel, T., & Thiele, D. J. (2016). **Cathepsin Protease Controls Copper and Cisplatin Accumulation via Cleavage of the Ctr1 Metal-binding Ectodomain.** *The Journal of biological chemistry*, 291(27), 13905–13916. <https://doi.org/10.1074/jbc.M116.731281>
- [324]. Öhrvik, H., Nose, Y., Wood, L. K., Kim, B. E., Gleber, S. C., Ralle, M., & Thiele, D. J. (2013). **Ctr2 regulates biogenesis of a cleaved form of mammalian Ctr1 metal transporter lacking the copper- and cisplatin-binding ecto-domain.** *Proceedings of the National Academy of Sciences of the United States of America*, 110(46), E4279–E4288. <https://doi.org/10.1073/pnas.1311749110>
- [325]. Wang, X., Zhu, W., Zhao, X., & Wang, P. (2016). **miR-133a enhances the sensitivity of Hep-2 cells and vincristine-resistant Hep-2v cells to cisplatin by downregulating ATP7B expression.** *International journal of molecular medicine*, 37(6), 1636–1642. <https://doi.org/10.3892/ijmm.2016.2569>
- [326]. Li, Z., Li, S., Wen, Y., Chen, J., Liu, K., & Jia, J. (2021). **MiR-495 Inhibits Cisplatin Resistance and Angiogenesis in Esophageal Cancer by Targeting ATP7A.** *Technology in cancer research & treatment*, 20, 15330338211039127. <https://doi.org/10.1177/15330338211039127>
- [327]. Lanzi, C., Perego, P., Supino, R., Romanelli, S., Pensa, T., Carenini, N., Viano, I., Colangelo, D., Leone, R., Apostoli, P., Cassinelli, G., Gambetta, R. A., & Zunino, F. (1998). **Decreased drug accumulation and increased tolerance to DNA damage in tumor cells with a low level of cisplatin resistance.** *Biochemical pharmacology*, 55(8), 1247–1254. [https://doi.org/10.1016/s0006-2952\(97\)00599-6](https://doi.org/10.1016/s0006-2952(97)00599-6)

- [328]. Lynam-Lennon, N., Reynolds, J. V., Pidgeon, G. P., Lysaght, J., Marignol, L., & Maher, S. G. (2010). **Alterations in DNA repair efficiency are involved in the radioresistance of esophageal adenocarcinoma.** *Radiation research*, 174(6), 703–711. <https://doi.org/10.1667/RR2295.1>
- [329]. Yumioka, T., Osaki, M., Sasaki, R., Yamaguchi, N., Onuma, K., Iwamoto, H., Morizane, S., Honda, M., Takenaka, A., & Okada, F. (2018). **Lysosome-associated membrane protein 2 (LAMP-2) expression induced by miR-194-5p downregulation contributes to sunitinib resistance in human renal cell carcinoma cells.** *Oncology letters*, 15(1), 893–900. <https://doi.org/10.3892/ol.2017.7423>
- [330]. Dong, Z., Zhong, Z., Yang, L., Wang, S., & Gong, Z. (2014). **MicroRNA-31 inhibits cisplatin-induced apoptosis in non-small cell lung cancer cells by regulating the drug transporter ABCB9.** *Cancer letters*, 343(2), 249–257. <https://doi.org/10.1016/j.canlet.2013.09.034>
- [331]. Nilsson, C., Roberg, K., Grafström, R. C., & Ollinger, K. (2010). **Intrinsic differences in cisplatin sensitivity of head and neck cancer cell lines: Correlation to lysosomal pH.** *Head & neck*, 32(9), 1185–1194. <https://doi.org/10.1002/hed.21317>
- [332]. Chauhan, S. S., Liang, X. J., Su, A. W., Pai-Panandiker, A., Shen, D. W., Hanover, J. A., & Gottesman, M. M. (2003). **Reduced endocytosis and altered lysosome function in cisplatin-resistant cell lines.** *British journal of cancer*, 88(8), 1327–1334. <https://doi.org/10.1038/sj.bjc.6600861>
- [333]. Mauvezin, C., & Neufeld, T. P. (2015). **Bafilomycin A1 disrupts autophagic flux by inhibiting both V-ATPase-dependent acidification and Ca-P60A/SERCA-dependent autophagosome-**

- lysosome fusion. *Autophagy*, 11(8), 1437–1438.
<https://doi.org/10.1080/15548627.2015.1066957>
- [334]. Itoh, S., Kim, H. W., Nakagawa, O., Ozumi, K., Lessner, S. M., Aoki, H., Akram, K., McKinney, R. D., Ushio-Fukai, M., & Fukai, T. (2008). **Novel role of antioxidant-1 (Atox1) as a copper-dependent transcription factor involved in cell proliferation.** *The Journal of biological chemistry*, 283(14), 9157–9167.
<https://doi.org/10.1074/jbc.M709463200>
- [335]. Kodama, H., Fujisawa, C., & Bhadhprasit, W. (2012). **Inherited copper transport disorders: biochemical mechanisms, diagnosis, and treatment.** *Current drug metabolism*, 13(3), 237–250.
<https://doi.org/10.2174/138920012799320455>
- [336]. Collins, J. F., & Klevay, L. M. (2011). Copper. *Advances in nutrition (Bethesda, Md.)*, 2(6), 520–522.
<https://doi.org/10.3945/an.111.001222>
- [337]. Bandmann, O., Weiss, K. H., & Kaler, S. G. (2015). Wilson's disease and other neurological copper disorders. *The Lancet. Neurology*, 14(1), 103–113. [https://doi.org/10.1016/S1474-4422\(14\)70190-5](https://doi.org/10.1016/S1474-4422(14)70190-5)
- [338]. Navarrete, A., González, A., Gómez, M., Contreras, R. A., Díaz, P., Lobos, G., Brown, M. T., Sáez, C. A., & Moenne, A. (2019). **Copper excess detoxification is mediated by a coordinated and complementary induction of glutathione, phytochelatins and metallothioneins in the green seaweed *Ulva compressa*.** *Plant physiology and biochemistry: PPB*, 135, 423–431.
<https://doi.org/10.1016/j.plaphy.2018.11.019>
- [339]. Lelièvre, P., Sancey, L., Coll, J. L., Deniaud, A., & Busser, B. (2020). **The Multifaceted Roles of Copper in Cancer: A Trace Metal**

Element with Dysregulated Metabolism, but Also a Target or a Bullet for Therapy. *Cancers*, 12(12), 3594.
<https://doi.org/10.3390/cancers12123594>

- [340]. Lossow, K., Schwarz, M., & Kipp, A. P. (2021). **Are trace element concentrations suitable biomarkers for the diagnosis of cancer?** *Redox biology*, 42, 101900.
<https://doi.org/10.1016/j.redox.2021.101900>
- [341]. Lener, M. R., Scott, R. J., Wiechowska-Kozłowska, A., Serrano-Fernández, P., Baszuk, P., Jaworska-Bieniek, K., Sukiennicki, G., Marciniak, W., Muszyńska, M., Kładny, J., Gromowski, T., Kaczmarek, K., Jakubowska, A., & Lubiński, J. (2016). **Serum Concentrations of Selenium and Copper in Patients Diagnosed with Pancreatic Cancer.** *Cancer research and treatment*, 48(3), 1056–1064. <https://doi.org/10.4143/crt.2015.282>
- [342]. Hatori, Y., & Lutsenko, S. (2016). **The Role of Copper Chaperone Atox1 in Coupling Redox Homeostasis to Intracellular Copper Distribution.** *Antioxidants (Basel, Switzerland)*, 5(3), 25.
<https://doi.org/10.3390/antiox5030025>
- [343]. Öhrvik, H., & Wittung-Stafshede, P. (2015). Identification of New Potential Interaction Partners for Human Cytoplasmic Copper Chaperone Atox1: Roles in Gene Regulation?. *International journal of molecular sciences*, 16(8), 16728–16739.
<https://doi.org/10.3390/ijms160816728>
- [344]. Kim, D. W., Shin, M. J., Choi, Y. J., Kwon, H. J., Lee, S. H., Lee, S., Park, J., Han, K. H., Eum, W. S., & Choi, S. Y. (2018). **Tat-ATOX1 inhibits inflammatory responses via regulation of MAPK and NF- κ B pathways.** *BMB reports*, 51(12), 654–659.
<https://doi.org/10.5483/BMBRep.2018.51.12.248>

- [345]. Blockhuys, S., Brady, D. C., & Wittung-Stafshede, P. (2020). **Evaluation of copper chaperone ATOX1 as prognostic biomarker in breast cancer.** *Breast cancer (Tokyo, Japan)*, 27(3), 505–509. <https://doi.org/10.1007/s12282-019-01044-4>
- [346]. Galluzzi, L., Vitale, I., Michels, J., Brenner, C., Szabadkai, G., Harel-Bellan, A., Castedo, M., & Kroemer, G. (2014). **Systems biology of cisplatin resistance: past, present and future.** *Cell death & disease*, 5(5), e1257. <https://doi.org/10.1038/cddis.2013.428>
- [347]. Zhang, C., Xu, C., Gao, X., & Yao, Q. (2022). **Platinum-based drugs for cancer therapy and anti-tumor strategies.** *Theranostics*, 12(5), 2115–2132. <https://doi.org/10.7150/thno.69424>
- [348]. Palm, M. E., Weise, C. F., Lundin, C., Wingsle, G., Nygren, Y., Björn, E., Naredi, P., Wolf-Watz, M., & Wittung-Stafshede, P. (2011). **Cisplatin binds human copper chaperone Atox1 and promotes unfolding in vitro.** *Proceedings of the National Academy of Sciences of the United States of America*, 108(17), 6951–6956. <https://doi.org/10.1073/pnas.1012899108>
- [349]. Boal, A. K., & Rosenzweig, A. C. (2009). **Crystal structures of cisplatin bound to a human copper chaperone.** *Journal of the American Chemical Society*, 131(40), 14196–14197. <https://doi.org/10.1021/ja906363t>
- [350]. Beaino, W., Guo, Y., Chang, A. J., & Anderson, C. J. (2014). **Roles of Atox1 and p53 in the trafficking of copper-64 to tumor cell nuclei: implications for cancer therapy.** *Journal of biological inorganic chemistry: JBIC: a publication of the Society of Biological Inorganic Chemistry*, 19(3), 427–438. <https://doi.org/10.1007/s00775-013-1087-0>

- [351]. Kahra, D., Mondol, T., Niemiec, M. S., & Wittung-Stafshede, P. (2015). **Human Copper Chaperone Atox1 Translocates to the Nucleus but does not Bind DNA In Vitro.** *Protein and peptide letters*, 22(6), 532–538.
<https://doi.org/10.2174/0929866522666150506094546>
- [352]. Lameire N. (2014). **Nephrotoxicity of recent anti-cancer agents.** *Clinical kidney journal*, 7(1), 11–22.
<https://doi.org/10.1093/ckj/sft135>
- [353]. Karasawa, T., Sibrian-Vazquez, M., Strongin, R. M., & Steyger, P. S. (2013). **Identification of cisplatin-binding proteins using agarose conjugates of platinum compounds.** *PloS one*, 8(6), e66220.
<https://doi.org/10.1371/journal.pone.0066220>
- [354]. Russo Krauss, I., Ferraro, G., & Merlino, A. (2016). **Cisplatin-Protein Interactions: Unexpected Drug Binding to N-Terminal Amine and Lysine Side Chains.** *Inorganic chemistry*, 55(16), 7814–7816. <https://doi.org/10.1021/acs.inorgchem.6b01234>
- [355]. Mustonen, R., Hemminki, K., Hietanen, P., Leppälä, S., & Takala, M. (1989). **Cisplatin binding to plasma proteins and hemoglobin in cancer patients.** *Archives of toxicology. Supplement. = Archiv fur Toxikologie. Supplement*, 13, 262–264.
https://doi.org/10.1007/978-3-642-74117-3_46
- [356]. Zhang, N., Du, Y., Cui, M., Xing, J., Liu, Z., & Liu, S. (2012). **Probing the interaction of cisplatin with cytochrome C by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry.** *Analytical chemistry*, 84(14), 6206–6212.
<https://doi.org/10.1021/ac301122w>
- [357]. Sze, C. M., Khairallah, G. N., Xiao, Z., Donnelly, P. S., O'Hair, R. A., & Wedd, A. G. (2009). **Interaction of cisplatin and analogues with**

- a Met-rich protein site.** *Journal of biological inorganic chemistry: JBIC : a publication of the Society of Biological Inorganic Chemistry*, 14(2), 163–165. <https://doi.org/10.1007/s00775-008-0452-x>
- [358]. Chen, G. F., Sudhahar, V., Youn, S. W., Das, A., Cho, J., Kamiya, T., Urao, N., McKinney, R. D., Surenkhuu, B., Hamakubo, T., Iwanari, H., Li, S., Christman, J. W., Shantikumar, S., Angelini, G. D., Emanuelli, C., Ushio-Fukai, M., & Fukai, T. (2015). **Copper Transport Protein Antioxidant-1 Promotes Inflammatory Neovascularization via Chaperone and Transcription Factor Function.** *Scientific reports*, 5, 14780. <https://doi.org/10.1038/srep14780>
- [359]. Safaei, R., Maktabi, M. H., Blair, B. G., Larson, C. A., & Howell, S. B. (2009). Effects of the loss of Atox1 on the cellular pharmacology of cisplatin. *Journal of inorganic biochemistry*, 103(3), 333–341. <https://doi.org/10.1016/j.jinorgbio.2008.11.012>
- [360]. Brewer, G. J., Dick, R. D., Johnson, V., Wang, Y., Yuzbasiyan-Gurkan, V., Kluin, K., Fink, J. K., & Aisen, A. (1994). **Treatment of Wilson's disease with ammonium tetrathiomolybdate. I. Initial therapy in 17 neurologically affected patients.** *Archives of neurology*, 51(6), 545–554. <https://doi.org/10.1001/archneur.1994.00540180023009>
- [361]. Carpenter, A., Rassam, A., Jennings, M. H., Robinson-Jackson, S., Alexander, J. S., & Erkuran-Yilmaz, C. (2007). **Effects of ammonium tetrathiomolybdate, an oncolytic/angiolytic drug on the viability and proliferation of endothelial and tumor cells.** *Inflammation research: official journal of the European Histamine Research Society*, 56(12), 515–519. <https://doi.org/10.1007/s00011-007-7025-2>

- [362]. Babak, M. V., & Ahn, D. (2021). **Modulation of Intracellular Copper Levels as the Mechanism of Action of Anticancer Copper Complexes: Clinical Relevance.** *Biomedicines*, 9(8), 852. <https://doi.org/10.3390/biomedicines9080852>
- [363]. Fang, T., Chen, W., Sheng, Y., Yuan, S., Tang, Q., Li, G., Huang, G., Su, J., Zhang, X., Zang, J., & Liu, Y. (2019). **Tetrathiomolybdate induces dimerization of the metal-binding domain of ATPase and inhibits platination of the protein.** *Nature communications*, 10(1), 186. <https://doi.org/10.1038/s41467-018-08102-z>
- [364]. Ryumon, S., Okui, T., Kunisada, Y., Kishimoto, K., Shimo, T., Hasegawa, K., Ibaragi, S., Akiyama, K., Thu Ha, N. T., Monsur Hassan, N. M., & Sasaki, A. (2019). **Ammonium tetrathiomolybdate enhances the antitumor effect of cisplatin via the suppression of ATPase copper transporting beta in head and neck squamous cell carcinoma.** *Oncology reports*, 42(6), 2611–2621. <https://doi.org/10.3892/or.2019.7367>
- [365]. Zhou, J., Kang, Y., Chen, L., Wang, H., Liu, J., Zeng, S., & Yu, L. (2020). **The Drug-Resistance Mechanisms of Five Platinum-Based Antitumor Agents.** *Frontiers in pharmacology*, 11, 343. <https://doi.org/10.3389/fphar.2020.00343>
- [366]. Xi, Z., Guo, W., Tian, C., Wang, F., & Liu, Y. (2013). **Copper binding promotes the interaction of cisplatin with human copper chaperone Atox1.** *Chemical communications (Cambridge, England)*, 49(95), 11197–11199. <https://doi.org/10.1039/c3cc45905e>
- [367]. Hatori, Y., & Lutsenko, S. (2013). **An expanding range of functions for the copper chaperone/antioxidant protein**

- Atox1. Antioxidants & redox signaling**, 19(9), 945–957.
<https://doi.org/10.1089/ars.2012.5086>
- [368]. Eiblmaier, M., Meyer, L. A., & Anderson, C. J. (2008). **The role of p53 in the trafficking of copper-64 to tumor cell nuclei**. *Cancer biology & therapy*, 7(1), 63–69.
<https://doi.org/10.4161/cbt.7.1.5130>
- [369]. Varghese, A. M., Lowery, M. A., Yu, K. H., & O'Reilly, E. M. (2016). **Current management and future directions in metastatic pancreatic adenocarcinoma**. *Cancer*, 122(24), 3765–3775.
<https://doi.org/10.1002/cncr.30342>
- [370]. Yu, J., Yang, X., He, W., & Ye, W. (2021). **Burden of pancreatic cancer along with attributable risk factors in Europe between 1990 and 2019, and projections until 2039**. *International journal of cancer*, 149(5), 993–1001. <https://doi.org/10.1002/ijc.33617>
- [371]. Kikuyama, M., Kamisawa, T., Kuruma, S., Chiba, K., Kawaguchi, S., Terada, S., & Satoh, T. (2018). **Early Diagnosis to Improve the Poor Prognosis of Pancreatic Cancer**. *Cancers*, 10(2), 48.
<https://doi.org/10.3390/cancers10020048>
- [372]. Griffin, J. F., Poruk, K. E., & Wolfgang, C. L. (2015). **Pancreatic cancer surgery: past, present, and future**. *Chinese journal of cancer research = Chung-kuo yen cheng yen chiu*, 27(4), 332–348.
<https://doi.org/10.3978/j.issn.1000-9604.2015.06.07>
- [373]. Yu, S., Zhang, C., & Xie, K. P. (2021). **Therapeutic resistance of pancreatic cancer: Roadmap to its reversal**. *Biochimica et biophysica acta. Reviews on cancer*, 1875(1), 188461.
<https://doi.org/10.1016/j.bbcan.2020.188461>
- [374]. Quiñonero, F., Mesas, C., Doello, K., Cabeza, L., Perazzoli, G., Jimenez-Luna, C., Rama, A. R., Melguizo, C., & Prados, J. (2019).

The challenge of drug resistance in pancreatic ductal adenocarcinoma: a current overview. *Cancer biology & medicine*, 16(4), 688–699. <https://doi.org/10.20892/j.issn.2095-3941.2019.0252>

- [375]. Maity, A., Kao, G. D., Muschel, R. J., & McKenna, W. G. (1997). **Potential molecular targets for manipulating the radiation response.** *International journal of radiation oncology, biology, physics*, 37(3), 639–653. [https://doi.org/10.1016/s0360-3016\(96\)00598-6](https://doi.org/10.1016/s0360-3016(96)00598-6)
- [376]. Lushchak V. I. (2014). **Free radicals, reactive oxygen species, oxidative stress and its classification.** *Chemico-biological interactions*, 224, 164–175. <https://doi.org/10.1016/j.cbi.2014.10.016>
- [377]. Azzam, E. I., Jay-Gerin, J. P., & Pain, D. (2012). **Ionizing radiation-induced metabolic oxidative stress and prolonged cell injury.** *Cancer letters*, 327(1-2), 48–60. <https://doi.org/10.1016/j.canlet.2011.12.012>
- [378]. Cadet, J., & Wagner, J. R. (2013). **DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation.** *Cold Spring Harbor perspectives in biology*, 5(2), a012559. <https://doi.org/10.1101/cshperspect.a012559>
- [379]. Baskar, R., Dai, J., Wenlong, N., Yeo, R., & Yeoh, K. W. (2014). **Biological response of cancer cells to radiation treatment.** *Frontiers in molecular biosciences*, 1, 24. <https://doi.org/10.3389/fmolb.2014.00024>
- [380]. Birben, E., Sahiner, U. M., Sackesen, C., Erzurum, S., & Kalayci, O. (2012). **Oxidative stress and antioxidant defense.** *The World*

Allergy Organization journal, 5(1), 9–19.
<https://doi.org/10.1097/WOX.0b013e3182439613>

- [381]. Sun, J., Chen, Y., Li, M., & Ge, Z. (1998). **Role of antioxidant enzymes on ionizing radiation resistance.** *Free radical biology & medicine*, 24(4), 586–593. [https://doi.org/10.1016/s0891-5849\(97\)00291-8](https://doi.org/10.1016/s0891-5849(97)00291-8)
- [382]. Ivanov, S. V., Goparaju, C. M., Lopez, P., Zavadil, J., Toren-Haritan, G., Rosenwald, S., Hoshen, M., Chajut, A., Cohen, D., & Pass, H. I. (2010). **Pro-tumorigenic effects of miR-31 loss in mesothelioma.** *The Journal of biological chemistry*, 285(30), 22809–22817. <https://doi.org/10.1074/jbc.M110.100354>
- [383]. Chin, V., Nagrial, A., Sjoquist, K., O'Connor, C. A., Chantrill, L., Biankin, A. V., Scholten, R. J., & Yip, D. (2018). **Chemotherapy and radiotherapy for advanced pancreatic cancer.** *The Cochrane database of systematic reviews*, 3(3), CD011044. <https://doi.org/10.1002/14651858.CD011044.pub2>
- [384]. Schieber, M., & Chandel, N. S. (2014). **ROS function in redox signaling and oxidative stress.** *Current biology: CB*, 24(10), R453–R462. <https://doi.org/10.1016/j.cub.2014.03.034>
- [385]. Fruehauf, J. P., & Meyskens, F. L., Jr (2007). **Reactive oxygen species: a breath of life or death?** *Clinical cancer research: an official journal of the American Association for Cancer Research*, 13(3), 789–794. <https://doi.org/10.1158/1078-0432.CCR-06-2082>
- [386]. Kim, W., Lee, S., Seo, D., Kim, D., Kim, K., Kim, E., Kang, J., Seong, K. M., Youn, H., & Youn, B. (2019). **Cellular Stress Responses in Radiotherapy.** *Cells*, 8(9), 1105. <https://doi.org/10.3390/cells8091105>

- [387]. Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). **Free radicals and antioxidants in normal physiological functions and human disease.** *The international journal of biochemistry & cell biology*, 39(1), 44–84. <https://doi.org/10.1016/j.biocel.2006.07.001>
- [388]. Thyagarajan, A., & Sahu, R. P. (2018). **Potential Contributions of Antioxidants to Cancer Therapy: Immunomodulation and Radiosensitization.** *Integrative cancer therapies*, 17(2), 210–216. <https://doi.org/10.1177/1534735416681639>
- [389]. Brigelius-Flohé, R., & Maiorino, M. (2013). **Glutathione peroxidases.** *Biochimica et biophysica acta*, 1830(5), 3289–3303. <https://doi.org/10.1016/j.bbagen.2012.11.020>
- [390]. Kim, U., Kim, C. Y., Lee, J. M., Ryu, B., Kim, J., Bang, J., Ahn, N., & Park, J. H. (2020). **Loss of glutathione peroxidase 3 induces ROS and contributes to prostatic hyperplasia in Nkx3.1 knockout mice.** *Andrology*, 8(5), 1486–1493. <https://doi.org/10.1111/andr.12828>
- [391]. Tian, R., Geng, Y., Yang, Y., Seim, I., & Yang, G. (2021). **Oxidative stress drives divergent evolution of the glutathione peroxidase (GPX) gene family in mammals.** *Integrative zoology*, 16(5), 696–711. <https://doi.org/10.1111/1749-4877.12521>
- [392]. Jiao, Y., Wang, Y., Guo, S., & Wang, G. (2017). **Glutathione peroxidases as oncotargets.** *Oncotarget*, 8(45), 80093–80102. <https://doi.org/10.18632/oncotarget.20278>
- [393]. Ramming, T., Hansen, H. G., Nagata, K., Ellgaard, L., & Appenzeller-Herzog, C. (2014). **GPx8 peroxidase prevents leakage of H₂O₂ from the endoplasmic reticulum.** *Free radical biology &*

- [394]. Zhang, J., Liu, Y., Guo, Y., & Zhao, Q. (2020). **GPX8 promotes migration and invasion by regulating epithelial characteristics in non-small cell lung cancer.** *Thoracic cancer*, 11(11), 3299–3308. <https://doi.org/10.1111/1759-7714.13671>
- [395]. Khatib, A., Solaimuthu, B., Ben Yosef, M., Abu Rmaileh, A., Tanna, M., Oren, G., Schlesinger Frisch, M., Axelrod, J. H., Lichtenstein, M., & Shaul, Y. D. (2020). **The glutathione peroxidase 8 (GPX8)/IL-6/STAT3 axis is essential in maintaining an aggressive breast cancer phenotype.** *Proceedings of the National Academy of Sciences of the United States of America*, 117(35), 21420–21431. <https://doi.org/10.1073/pnas.2010275117>
- [396]. Cellini, F., Morganti, A. G., Genovesi, D., Silvestris, N., & Valentini, V. (2014). **Role of microRNA in response to ionizing radiations: evidences and potential impact on clinical practice for radiotherapy.** *Molecules (Basel, Switzerland)*, 19(4), 5379–5401. <https://doi.org/10.3390/molecules19045379>
- [397]. Peng, H., Wang, L., Su, Q., Yi, K., Du, J., & Wang, Z. (2019). **MiR-31-5p promotes the cell growth, migration and invasion of colorectal cancer cells by targeting NUMB.** *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie*, 109, 208–216. <https://doi.org/10.1016/j.biopha.2018.10.048>
- [398]. Luo, L. J., Yang, F., Ding, J. J., Yan, D. L., Wang, D. D., Yang, S. J., Ding, L., Li, J., Chen, D., Ma, R., Wu, J. Z., & Tang, J. H. (2016). **MiR-31 inhibits migration and invasion by targeting SATB2 in triple negative breast cancer.** *Gene*, 594(1), 47–58. <https://doi.org/10.1016/j.gene.2016.08.057>

- [399]. Estrela, J. M., Ortega, A., & Obrador, E. (2006). **Glutathione in cancer biology and therapy**. *Critical reviews in clinical laboratory sciences*, 43(2), 143–181.
<https://doi.org/10.1080/10408360500523878>
- [400]. Pajic, M., Froio, D., Daly, S., Doculara, L., Millar, E., Graham, P. H., Drury, A., Steinmann, A., de Bock, C. E., Boulghourjian, A., Zaratzian, A., Carroll, S., Toohey, J., O'Toole, S. A., Harris, A. L., Buffa, F. M., Gee, H. E., Hollway, G. E., & Molloy, T. J. (2018). miR-139-5p Modulates Radiotherapy Resistance in Breast Cancer by Repressing Multiple Gene Networks of DNA Repair and ROS Defense. *Cancer research*, 78(2), 501–515.
<https://doi.org/10.1158/0008-5472.CAN-16-3105>
- [401]. Xu, Z., Zhang, Y., Ding, J., Hu, W., Tan, C., Wang, M., Tang, J., & Xu, Y. (2018). **miR-17-3p Downregulates Mitochondrial Antioxidant Enzymes and Enhances the Radiosensitivity of Prostate Cancer Cells**. *Molecular therapy. Nucleic acids*, 13, 64–77.
<https://doi.org/10.1016/j.omtn.2018.08.009>
- [402]. Yang, W., Shen, Y., Wei, J., & Liu, F. (2015). **MicroRNA-153/Nrf-2/GPx1 pathway regulates radiosensitivity and stemness of glioma stem cells via reactive oxygen species**. *Oncotarget*, 6(26), 22006–22027. <https://doi.org/10.18632/oncotarget.4292>
- [403]. Pennati, M., Lopergolo, A., Profumo, V., De Cesare, M., Sbarra, S., Valdagni, R., Zaffaroni, N., Gandellini, P., & Folini, M. (2014). **miR-205 impairs the autophagic flux and enhances cisplatin cytotoxicity in castration-resistant prostate cancer cells**. *Biochemical pharmacology*, 87(4), 579–597.
<https://doi.org/10.1016/j.bcp.2013.12.009>
- [404]. Drayton, R. M., Dudziec, E., Peter, S., Bertz, S., Hartmann, A., Bryant, H. E., & Catto, J. W. (2014). **Reduced expression of**

- miRNA-27a modulates cisplatin resistance in bladder cancer by targeting the cystine/glutamate exchanger SLC7A11.** *Clinical cancer research: an official journal of the American Association for Cancer Research*, 20(7), 1990–2000. <https://doi.org/10.1158/1078-0432.CCR-13-2805>
- [405]. Ko, S. H., Apple, E. C., Liu, Z., & Chen, L. (2020). **Age-dependent autophagy induction after injury promotes axon regeneration by limiting NOTCH.** *Autophagy*, 16(11), 2052–2068. <https://doi.org/10.1080/15548627.2020.1713645>
- [406]. Gravina, G. L., Senapedis, W., McCauley, D., Baloglu, E., Shacham, S., & Festuccia, C. (2014). **Nucleo-cytoplasmic transport as a therapeutic target of cancer.** *Journal of hematology & oncology*, 7, 85. <https://doi.org/10.1186/s13045-014-0085-1>
- [407]. Wang, H., Fang, L., Jiang, J., Kuang, Y., Wang, B., Shang, X., Han, P., Li, Y., Liu, M., Zhang, Z., & Li, P. (2018). **The cisplatin-induced lncRNA PANDAR dictates the chemoresistance of ovarian cancer via regulating SFRS2-mediated p53 phosphorylation.** *Cell death & disease*, 9(11), 1103. <https://doi.org/10.1038/s41419-018-1148-y>

Appendices

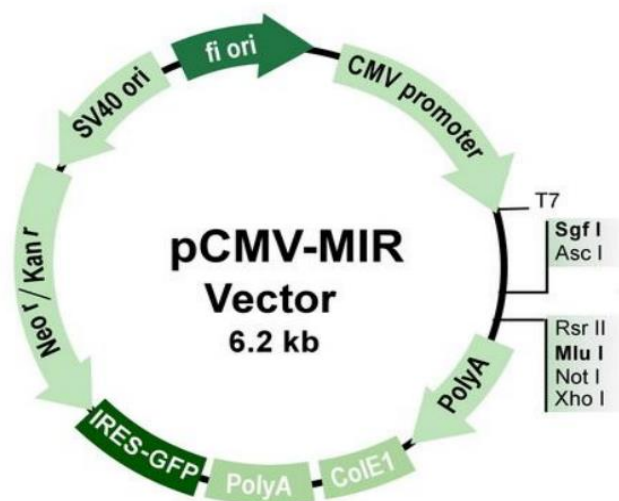


Figure A1.1. Plasmid map for miR-31 overexpression/reintroduction.

Under the control of a CMV promoter, miR-31 was produced from the plasmid. A GFP reported was encoded on the plasmid for detection. A kanamycin resistance gene was encoded for bacterial selection; a geneticin (also known as neomycin) resistance gene was encoded for mammalian selection. The plasmid was purchased from Origene.

Image sourced at: <http://www.origene.com/MicroRNA/pCMV-MIR-Vector.aspx>

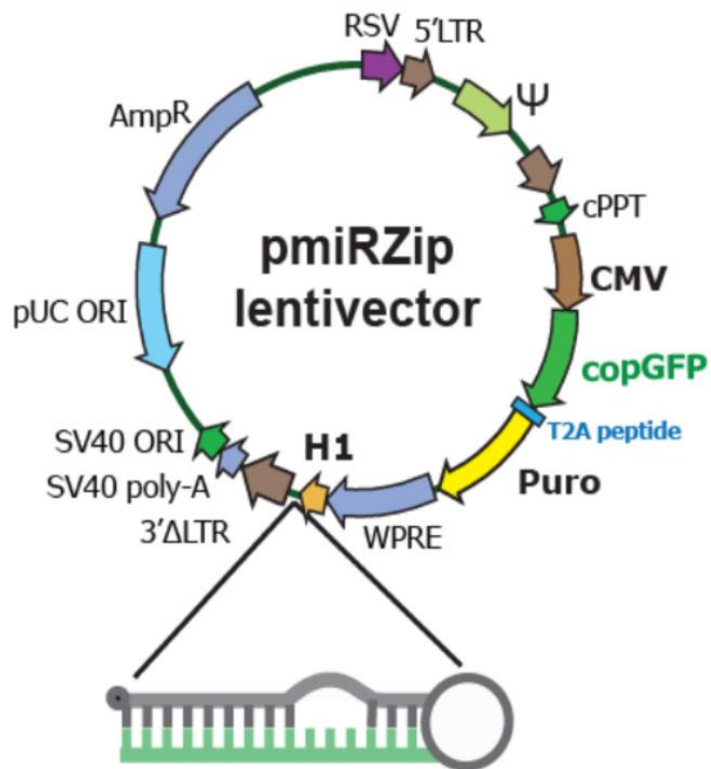


Figure A1.2. Plasmid map for Zip-miR-31 suppression. Under the control of a CMV promoter, an antisense-miR-31 (anti-miR) was produced from the plasmid bound to endogenous miR-31, effectively inhibiting its functionality. A GFP reporter was encoded on the plasmid for detection. An ampicillin resistance gene was encoded for bacterial selection; a puromycin resistance gene was encoded for mammalian selection. The plasmid was purchased from SBI.

Image sourced at: <https://www.systembio.com/microrna-research/microRNA-knockdown/mirzip/technical-details>.

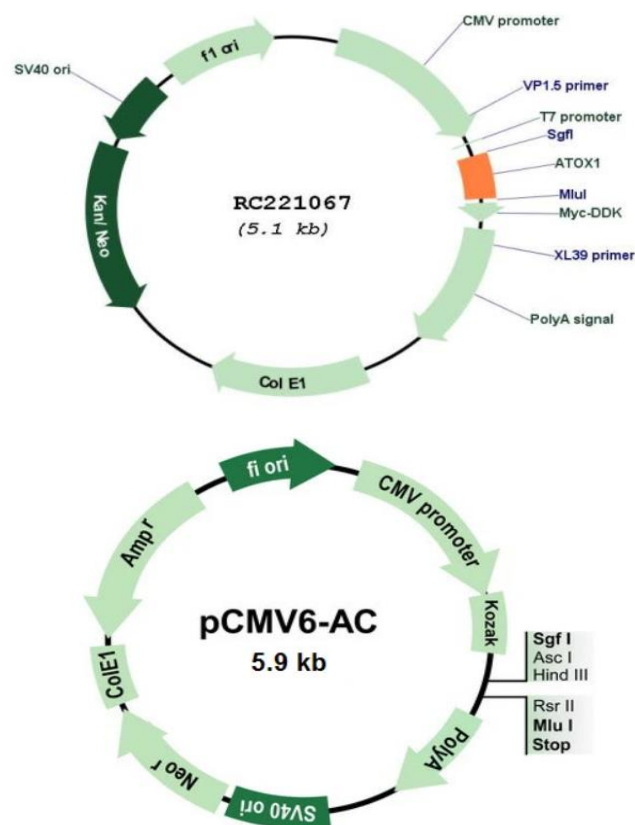


Figure A1.3. Plasmid map for ATOX1 overexpression plasmid. The ATOX1 overexpressing plasmid was designed by adding the ORF subclone of RC221067 into the control untagged pCMV6-AC vector PS100020. An ampicillin resistance gene was encoded for bacterial selection; a neomycin n resistance gene was encoded for mammalian selection. The plasmid was purchased from Origene.

Table A1. Optimised Compact Hough and Radial Map (CHARM) settings for PDAC cell lines. The algorithm was optimised to ensure that sufficient colonies were analysed, without considering anomalies.

Panel	Function	Setting
F1 Pre-Processed	Smoothing	BxPC-3: 4 Panc-1: 2
F2 Edge Detection	Contrast	BxPC-3: 40/100 Panc-1: 30/100
F3 Centre Detection	Detection mode	Dark on light
	Centre Detection Sensitivity	BxPC-3: 75/100 Panc-1 85/100
	Indicative Colony Diameter Ranger	BxPC-3: Lower 50 μ M / Upper 2000 μ M Panc-1: Lower 100 μ M / Upper 2500 μ M
	Min Centre to Centre Separation	55 μ M
	Auto-select	Yes
	Smoothing	3
F4 Shape Control	Circularity Factor	50/100
	Edge Distance Threshold	75/100
	No. of Spokes	32
	Shape Filtering	Fast Gaussian, Filter size 3
	Shape Processing	Best Fit Circle
F5 Filtering Controls	Colony Diameter Filter	BxPC-3: Min 50 μ M / Max 2000 μ M Panc-1: Lower 100 μ M / Upper 2500 μ M
	Colony Intensity	Min 0.1 Max 2.50
	Good Edge Factor	40/50
	Borders from Centroids	Yes
F6 Overlap Controls	Merge Overlapping Objects	100
	Overlap Circulation	Area
	Retain the	Most Intense
	Calc new Cluster Boundaries	Yes

Table A2. Optimising cell seeding densities for the clonogenic assay. The number of cells seeded per well of a six six-welled plate was optimised to ensure a minimum of ~200 colonies after fixing and staining. The mean colony number was taken from all wells for all experiments.

Cell Line	Treatment	Cells Seeded
Cells Seeded		
BxPC-3 Parental/miR-VC/miR-31	Ctrl	1500
	IC ₅₀ drug	4000
Panc-1 Parental/Zip-miR-VC/Zip-miR-31	4 Gy	4000
BxPC-3 Parental/miR-VC/miR-31	Ctrl	1500
	2 Gy	3000
	4 Gy	4000
Panc-1 Parental/Zip-miR-VC/Zip-miR-31	6 Gy	5000
	8 Gy	6000
BxPC-3 si-Scramble/si-GPx8	Ctrl	1500
	4 Gy	5000
Panc-1 Vector Ctrl/ Panc-1 ATOX1	Ctrl	1000
	IC ₅₀ Cisplatin	3000

Definitions / Units

3'	3 prime
5'	5 prime
5-FU	5-Fluorouracil
ABCB9	ATP binding cassette subfamily B member 9
Ago	Argonaut protein
ATOX1	Antioxidant 1
Amp	Ampicillin
ANOVA	Analysis of variance
APS	Ammonium persulfate
ATP	Adenosine triphosphate
ATP7A	ATPase copper transporting alpha
ATP7B	ATPase copper transporting beta
BA1	Bafilomycin A1
BCA	Bicinchoninic acid
BER	Base excision repair
BSA	Bovine serum albumin
cDNA	Complimentary DNA
CHARM	Compact Hough and radial map
Cis	Cisplatin
CTR1	Copper transporter receptor 1
DDR	DNA damage response
DGCR	DiGeorge syndrome chromosomal region
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic Acid
EMT	Epithelial-mesenchymal transition
FBS	Foetal bovine serum

G418	Geneticin antibiotic
GFP	Green fluorescent protein
GSH	Glutathione
GSSG	Glutathione disulfide
GPx(8)	Glutathione peroxidase (8)
HRP	Horseradishj peroxidise
IC₅₀	Concentration of a drug where the response is reduced by half
IHC	Immunohistochemistry
LAMP1	Lysosomal-associated membrane protein 1
MDR	Multi-drug resistance
miR-	MicroRNA-
miRNA	MicroRNA
miR-Zip	MicroRNA suppression plasmid
mRNA	Messenger RNA
<i>n</i>	Number of replicates
ns	Non-significant
<i>p</i>	Probability
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDAC	Pancreatic ductal adenocarcinoma
pH_{Lys}	Lysosomal pH
Pt₁₉₅	Platinum
Pre-miRNA	Pre-miRNA
Pri-miRNA	Primary-microRNA
Puro	Puromycin
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
RIPA	Radioimmunoprecipitation assay buffer

RISC	RNA-induced silencing complex
RNA	Ribose nucleic acid
<i>RNase</i>	Ribonuclease
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute cell culture medium
RT	Reverse transcription
SEM	Standard error of mean
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TBST	Tris buffered saline with tween 20
TEMED	Tetramethylenediamine
β	Beta
°C	Degrees Celsius
Gy	Gray
g	Grams
h	Hours
kDa	Kilo Dalton
μL	Microlitres
μG	Micrograms
μM	Micromolar
M	Molar
mg	Milligrams
mL	Millilitres
mM	Millimolar
min	Minutes
nM	Nanomolar
ppm	Part per million

ppb	Part per billion
s	Seconds
v/v	Volume per volume
w/v	Weight per volume

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