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Subtle Controllers: MicroRNAs Drive Pancreatic Tumorigenesis and Progression: A Dissertation

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SUBTLE CONTROLLERS: MICRORNAS DRIVE PANCREATIC TUMORIGENESIS AND PROGRESSION

A Dissertation Presented

By

Brian Joseph Quattrochi

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Medical Scientist Training Program (MSTP)

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Medical Scientist Training Program (MSTP)
April 13, 2015

To Jenn:

Without you, this thesis would not have been possible.

You are here, in these pages, a silent partner in every experiment, the supporting shoulder of every late night.

I can think of no other self-aware assemblage of stardust with whom I'd rather spend my life exploring the cosmos.

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal malignancies in the United States, with an average five-year survival rate of just 6.7%. One unifying aspect of PDAC is mutational activation of the KRAS oncogene, which occurs in over 90% of PDAC. Therefore, inhibiting KRAS function is likely an effective therapeutic strategy for this disease, and current research in our lab and others is focused on identifying downstream effectors of KRAS signaling that may be therapeutic targets.

miRNAs are powerful regulators of gene expression that can behave as oncogenes or tumor suppressors. Dysregulation of miRNA expression is commonly observed in human tumors, including PDAC. The *mir-17~92* cluster of miRNAs is an established oncogene in a variety of tumor contexts, and members of the *mir-17~92* cluster are upregulated in PDAC, but their role has not been explored *in vivo*.

This dissertation encompasses two studies exploring the role of miRNAs in pancreatic tumorigenesis. In Chapter II, I demonstrate that deletion of the *mir-17~92* cluster impairs PDAC precursor lesion formation and maintenance, and correlates with reduced ERK signaling in these lesions. *mir-17~92* deficient tumors and cell lines are also less invasive, which I attribute to the loss of the miR-19 family of miRNAs. In Chapter III, I find that *Dicer* heterozygosity inhibits PDAC metastasis, and that this phenotype is attributable to an increased sensitivity to anoikis. Ongoing experiments will determine whether shifts in particular miRNA signatures between cell lines can be attributed to this

phenotype. Together these findings illustrate the importance of miRNA biogenesis, and the *mir-17~92* cluster in particular, in supporting PDAC development and progression.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF THIRD-PARTY COPYRIGHTED MATERIAL

Figure	Publisher	License
Figure 1.1	American Society for Clinical Investigation	3598600600679

LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
AAV	Adeno-associated virus
αSMA	α-smooth muscle actin
CCK	Cholecystokinin
CLASH	Crosslinking, ligation, and sequencing of miRNA-mRNA hybrids
CTC	Circulating tumor cell
DCIS	Ductal carcinoma *in situ*
DUSP	Dual-specificity phosphatase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FAMMM	Familial atypical multiple mole and melanoma
GAP	GTPase activating protein
GEF	Guanidine nucleotide exchange factor
HPM-T	Hydroxypropylmethylcellulose with 0.2% Tween-80
IGF	Insulin-like growth factor
IGF1R	Insulin-like growth factor 1 receptor
KSRP	KH-type splicing regulatory protein
MAPK	Mitogen-activated protein kinase
MET	Mesenchymal-to-epithelial transition
miRISC	miRNA-induced silencing complex
OIS	Oncogene-induced senescence
PACT	Protein activator of PKR
PanIN	Pancreatic intraepithelial neoplasia
PBS	Phosphate-buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PEG	Polyethylene glycol
PI3K	Phosphoinositide 3-kinase
PJS	Peutz-Jeghers Syndrome
PyMT	Polyoma middle-T antigen
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RCAS	Replication competent avian sarcoma-leukosis virus long terminal repeat with a splice acceptor
SILAC	Stable isotope labeling of amino acids in cell culture
TARBP	trans-activation response RNA-binding protein
TBS-T	Tris-buffered saline with 0.1% Tween20
TGF-β	Transforming growth factor beta
TUT4	Terminal uridylyl transferase
UTR	Untranslated region

PREFACE

Almost all of the work described in this thesis was performed by myself at the University of Massachusetts in the lab of Dr. Brian Lewis. Invadopodia and gelatin degradation assays described in Figures 2.12 and 2.13 were performed by Anushree Gulvady in the lab of Dr. Chris Turner at SUNY Upstate. Leanne Ahronian and David Driscoll shared responsibility in drug preparation and health checks during the preclinical toxicity study. Leanne Ahronian, David Driscoll, and Brian Lewis assisted in the euthanasia and tissue harvest at the end of the toxicity study.

CHAPTER I

Introduction

"Good men, the last wave by, crying how bright
Their frail deeds might have danced in a green bay,
Rage, rage against the dying of the light.

[…]

Grave men, near death, who see with blinding sight
Blind eyes could blaze like meteors and be gay,
Rage, rage against the dying of the light."
- Dylan Thomas

Modern science has brought us to an age of incredible momentum and progress in the fight against human disease. Thanks to research in fields like cancer biology, immunology, genetics, structural biology and metabolism, many previously incomprehensible illnesses can now be understood and effectively fought using therapies targeted with molecular and even atomic precision.

Sadly, our achievements are still far outweighed by our failures, and our understanding of most disease pathophysiology is incomplete. Within human disease, cancer presents one of the most significant hurdles to understanding because of the incredible diversity of aberrations in normal biology that occurs during tumor evolution. For decades, scientists and clinicians have fought to gain a detailed understanding of its molecular underpinnings, and we have consequently amassed a tremendous literature on the intricacies of cancer evolution. Indeed, we have even occasionally been able to achieve radical breakthroughs for one malignancy or another, saving hundreds of thousands of lives with a single drug. These successes often arise from the discovery of a

proverbial Achilles' heel, but the discovery of such an exploitable weakness is historically a very rare event. Moreover, most of the wonder drugs do not work for everyone or forever. Far more often, what we obtain through research is a fractured understanding of the many contributing aspects of cancer, without meaningful integration. Most distressingly, findings that have been robustly characterized and repeated in animal models commonly fail to translate across species into humans, due to a mistaken understanding of the context of the original data or simply due to differences in human and mouse biology.

This view of our progress, highlighting the difficulties and setbacks of cancer biology, is certainly bleak, but I think we are now at the beginning of a new age of discovery. Innovations in the accumulation and curation of large biological datasets have allowed us to begin gathering information at an unprecedented rate. The diverse paths of tumor evolution guarantee the continuing diversity of tumor appearance, behavior and response to treatment, and to date, generalizing from this spectrum has been our greatest hurdle to forward progress. But with continuing advancement in genome, transcriptome, and proteome profiling technologies, there is hope that this diversity can be built into a more complex and adaptable understanding in the near future.

I offer this as a bit of hope, because the subject of this dissertation, pancreatic cancer, is truly one of the most devastating and intractable cancers of modern times. Frustration, it is probably fair to say, is ubiquitous among researchers and clinicians, but the pace of progress is objectively accelerating.

Indeed, optimism and hope are among the most impressive attributes of the patients, families, clinicians and researchers who battle this disease. Speaking for scientists and clinicians, we learn hope from patients and their families, who are, on the whole, unaccountably hopeful. They care deeply about progress in research, but not in the way one might expect. They and their families and friends go to Capitol Hill, they speak at symposia, they raise funds and awareness, but they aren't doing it for themselves. They have hope for their own survival, true, but they *work* for everyone else's future. With likely less than a year to live, they know that no research can help them; for them, there will be no last-minute wonder drug, no last-resort surgery. There is time only to live, to help build a better future for those who will come after, and to hope. It is for them that we work so hard, and it is because of the hope that they teach us, that we can manage to do it at all. They and their families deserve all that we, as scientists and clinicians, can achieve. We are the source of their hope, and they are the cause of our determination.

Annual deaths from pancreatic cancer in the United States have remained unchanged at approximately 10.9 per 100,000 individuals since 1975 (Howlader et al. 2014). This disturbing statistic derives from our inability to detect early lesions or to treat advanced stages of disease. Reasons for these failures originate from both the complexity of malignant and premalignant disease and the particular challenges posed by normal pancreas anatomy. In this section, we will explore what is known about the origins and evolution of pancreatic cancer, as well as the modern tools that facilitate our growing understanding and where the field hopes to make progress in the coming years. Following that will be a foundational discussion of the particular class of molecules, called microRNAs, that constitutes the focus of my research.

Normal Pancreas

The pancreas is an organ containing two distinct compartments with disparate functions: the endocrine and the exocrine. The endocrine pancreas comprises 1-2% of the mass of the organ and is distributed throughout in the form of the islets of Langerhans, of which an adult human has approximately one million (Weir and Bonner-Weir 2013). These spheres of tissue contain distinct regions of specialized cells that secrete various hormones into the circulation. The major cell types and their products include α-, β-, δ-, ε-, and PP-cells, which secrete glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide, respectively (Jain and Lammert 2009). Largely, these molecules exert complementary effects in the regulation of blood glucose levels, and they are coordinated by direct cellular monitoring of blood chemistry as well as paracrine signaling between

cells within the islet. Interestingly, the pancreas possesses a portal circulation between the endocrine and exocrine compartments, such that a portion of the hormones secreted by some of the islets pass in relatively high concentration through the capillary beds of the exocrine tissue prior to circulating into the venous system (Weaver and Sorenson 1989; Gorczyca et al. 2010). The physiological and pathological ramifications of this 'insulo-acinar' circulation remain largely unexplored, although there is an appreciation that the effects of insulin and somatostatin play major roles in exocrine pancreas signaling (Barreto et al. 2010). The lymphatic system of the pancreas, although not histologically prominent, has been well-described and plays a critical role in the clearance of interstitial fluid for the prevention of pancreatitis (Mastracci and Sussel 2012).

The exocrine pancreas comprises the vast majority of the organ (>95%) and consists predominantly of acinar cells organized around a ductal network. The pancreatic acinar cell is highly active in secretion and is responsible for the production of large quantities of digestive enzymes, including trypsinogen, chymotrypsinogen, pancreatic lipase, and pancreatic amylase. Trypsinogen and chymotrypsinogen are secreted as inactive precursors categorically referred to as zymogens, which are only normally activated upon exposure to enteropeptidase in the intestinal lumen. This assures that proteolytic activity is targeted to the digestive compartment of the gut and that the pancreatic parenchyma is protected from enzymatic damage (Leung and Ip 2006).

Acinar cells possess a striking histological appearance in standard hematoxylin and eosin stains: their extensive reservoirs of zymogen granules are eosinophilic and concentrated apically, whereas their extensive rough endoplasmic reticula are basally

located, creating a high-contrast dichromatic cell. They are organized into small clusters called acini (hence their name), which facilitate the efficient secretion of fluid and enzymes into a shared cul-du-sac of the ductal system (Figure 1.1) (Low et al. 2010; Reichert and Rustgi 2011).

The ductal network begins here, in the center of the acinus, with the suitably named centroacinar cell. These cells begin the job of modifying the acinar secretion by alkalizing it with the addition of bicarbonate (Lee et al. 2012b). Alkalization of pancreatic secretions is continued throughout the rest of the ductal network as the centroacinar cells give way to the low cuboidal cells of the intercalated ducts, and thence to the columnar cells of the lobular and main pancreatic ducts. Alkalization and hydration of pancreatic juice in these ducts is dependent on the cystic fibrosis (CF) transmembrane receptor, which is why individuals with CF experience pancreatic insufficiency and pancreatitis (Ishiguro et al. 2012). The main pancreatic duct combines with the common bile duct, which passes through the head of the pancreas on its journey from the liver, and this common duct then travels a very short distance and empties into the duodenum through the Sphincter of Oddi.

When nutrients are detected by enterocytes in the small intestine, it stimulates enterocytes to secrete cholecystokinin-releasing peptide onto nearby I-cells in a paracrine fashion. I-cells then secrete cholecystokinin (CCK) into the circulation, resulting in the coordinated relaxation of the Sphincter of Oddi, stimulation of acinar cell secretion, and constriction of the gall bladder (Niederau et al. 1994; Rehfeld 2011). The activity of CCK is regulated by exocrine and endocrine feedback loops. Its activity is hormonally

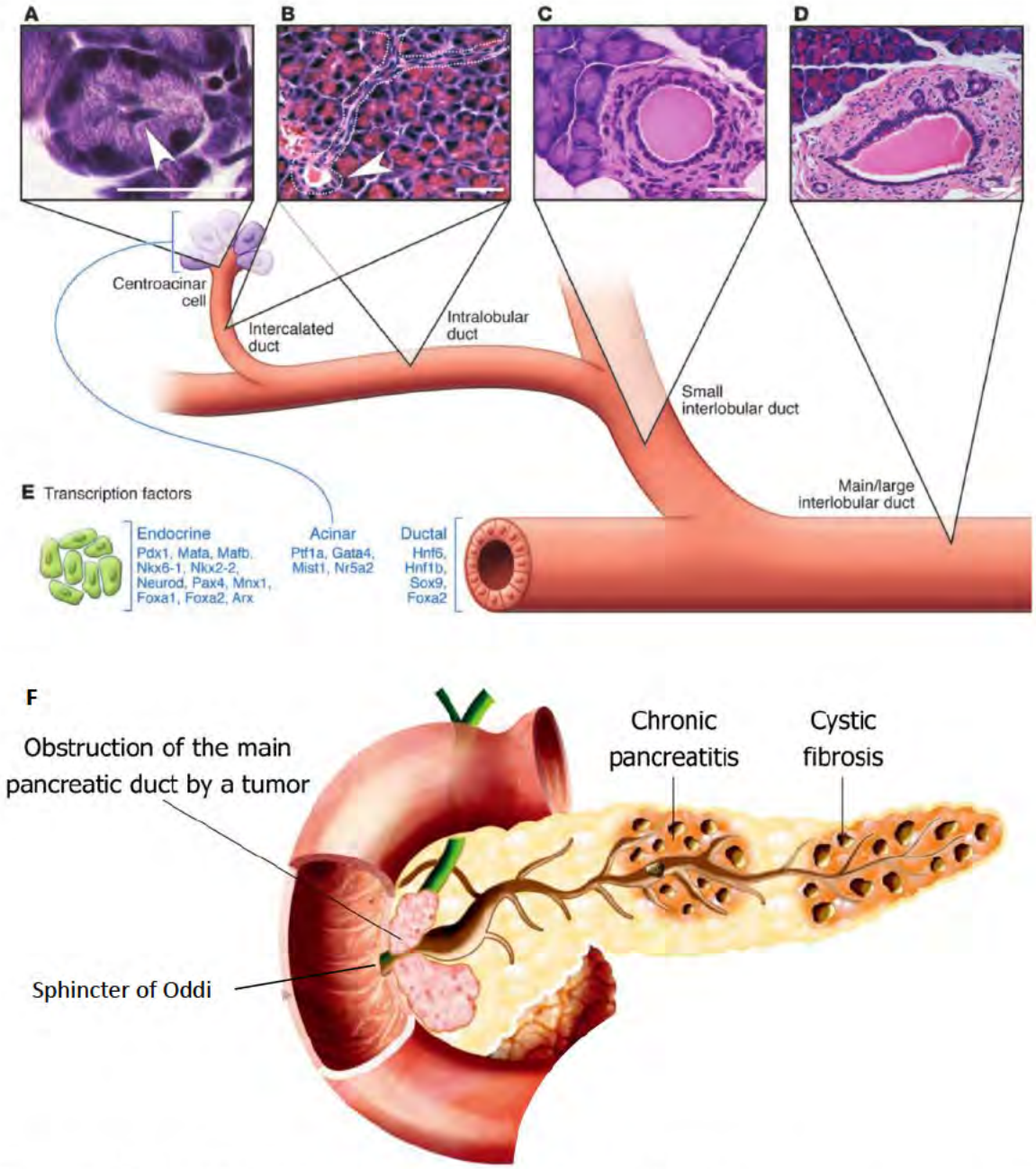


Figure 1.1. Normal histology and architecture of the exocrine pancreas.
(**A,B**) Acinar cells possess a characteristic dichromatism in standard hematoxylin and eosin stains and exist in intimate relationship with the ductal network via the centroacinar cells (arrowhead in **A**). (**C,D**) Larger ducts conduct and modify acinar secretions on their way to the duodenum. (**E**) Some major transcription factors involved in the development of the pancreas are listed. (**F**) Duct blockage is a source of pancreatitis and jaundice. Top figure reproduced with permission from Reichert and Rustgi, 2011, panel **F** is adapted from Lindkvist, 2013.

antagonized by the action of pancreatic peptide and somatostatin, secreted by PP- and δ-cells. CCK release from I-cells is also inhibited through trypsin-mediated digestion of CCK-releasing peptide in the intestinal lumen (Miyasaka et al. 1989). This arrangement allows the carefully coordinated storage and release of secretions from the pancreas and gall bladder, ensuring optimal digestion and assisting in glucose homeostasis.

This anatomical arrangement also has ramifications for disease. In the event of ductal obstruction, backup of pancreatic enzymes or bile can occur. Stasis of pancreatic secretions causes pancreatitis from the action of digestive enzymes on the pancreatic parenchyma, and bile stasis causes jaundice, which is the backup of the yellow waste pigment bilirubin into systemic tissues. These obstructive effects can occur due to primary idiopathic or hereditary pancreatitis, gall stones, cystic fibrosis, and pancreatic cancer (Figure 1.1) (Lee et al. 2012b).

Neoplasia

Demographics

'Pancreatic cancer' as a categorical term encompasses a range of distinct pathologies with disparate origins and prognoses, including adenocarcinoma, acinar carcinoma, cystadenocarcinoma, endocrine carcinomas, and rarer forms such as poorly differentiated or undifferentiated carcinoma (Lack 1989). However, neoplasms of the exocrine pancreas are dominated by pancreatic ductal adenocarcinoma (PDAC), which accounts for approximately 85% of all cases (Alexakis et al. 2004). Accordingly, the term 'pancreatic cancer' in general usage typically refers to PDAC, which is not only the most

common form of pancreatic cancer, but also the most deadly, with an average five-year survival rate of 6.7% (Howlader et al. 2014). It is typically discovered in patients over 40 years old, with a median age at diagnosis of 71 (Howlader et al. 2014). Its prevalence in the US makes it the fourth-leading cause of cancer-related death in the country (Jemal et al. 2007).

Environmental and behavioral risk factors for pancreatic cancer are varied, and between 30 and 40% of all sporadic pancreatic cancer cases can be attributable to a known risk factor (Yeo 2015). Smoking (Maisonneuve and Lowenfels 2015), obesity (Eheman et al. 2012), diabetes (Liu et al. 2015), and pancreatitis (Lowenfels and Maisonneuve 2004) each significantly increase one's risk of developing pancreatic cancer. Due to the general population prevalence of tobacco use, obesity and diabetes, more pancreatic cancer cases are attributable to these risk factors than to pancreatitis, even though pancreatitis, particularly when chronic, contributes far greater risk (Maisonneuve and Lowenfels 2015).

Most pancreatic cancers are sporadic, but between 5 and 10% of all cases can be attributed to an inheritable predisposition (Murphy et al. 2002; Permuth-Wey and Egan 2009). The most dangerous heritable conditions are Peutz-Jehgers syndrome and hereditary pancreatitis, which carry a 132-fold and 87-fold increased risk of pancreatic cancer, respectively (Rebours et al. 2008; Weiss 2014; Bruenderman and Martin 2015). Other heritable syndromes, including familial adenomatous multiple mole and melanoma, hereditary non-polyposis colon cancer, and hereditary breast and ovarian cancer syndromes, also carry significantly increased risk of pancreatic cancer over that of the

general population. Generally, the degree of additional risk is less than that for Peutz-Jehgers and chronic pancreatitis (Becker et al. 2014; Bruenderman and Martin 2015). The genetic bases of many other familial pancreatic cancer syndromes, which do not track with known mutations, have yet to be elucidated (Rustgi 2014). For now, high-risk individuals with familial pancreatic cancer are the only ones receiving active screening for disease (Pandharipande et al. 2014; Urayama 2015), but efforts are being made to define high-risk populations without a family history of pancreatic cancer who would benefit from screening (Bruenderman and Martin 2015). Screening methods currently focus on endoscopic ultrasound as the diagnostic tool with the highest sensitivity and specificity, which may be supplemented by mutational profiling of cellular material from pancreatic secretions (Urayama 2015).

Initiation and Progression

Early neoplastic lesions of the pancreas are called pancreatic intraepithelial neoplasias (PanINs) because they exhibit clear evidence of neoplastic change but they are restricted to the epithelial layer of the duct system (Distler et al. 2014). In oncology and cancer biology, it is the ability to invade across the basement membrane and into other tissues that defines malignant disease (Hanahan and Weinberg 2011).

PanINs are graded based on cellular and histological criteria (Hruban et al. 2001). PanIN-1 lesions possess a tall columnar morphology with prominent supranuclear mucin, but their nuclei are normal in appearance and remain basally located (Figure 1.2) (Maitra and Hruban 2008). PanIN-2 lesions are defined by the presence of mild nuclear

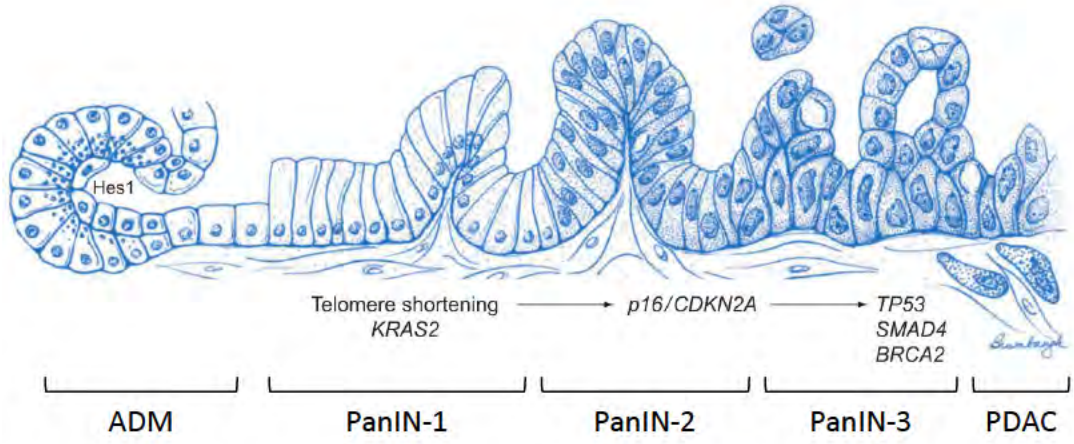


Figure 1.2. PanINs progress through stages of increasing dysplasia to PDAC.
PanINs can originate from ductal, acinar, or centroacinar cells, and the precise cell of origin is a matter of debate. Early metaplastic lesions are characterized by Hes1 positivity. Established PanIN-1 lesions exhibit high rates of mutated KRAS and EGFR overexpression. Genetic instability and the inactivation of tumor suppressors contribute to histological atypia and neoplastic progression. Figure is adapted from Maitra and Hruban, 2008.

abnormalities including loss of polarity, crowding, and hyperchromatism, and they may take on papillary or micropapillary architecture. PanIN-3 lesions, which can be considered ductal carcinoma *in situ* (DCIS), are characterized by elaboration of the epithelial layer into papillary or micropapillary architecture that may be stratified or exhibit cribriforming (the appearance of many small holes piercing a stratified epithelium, suggestive of ductal lumens). Cells in PanIN-3 lesions also have more advanced nuclear atypia than what is observed in PanIN-2, including aberrant mitoses, significant nuclear morphology defects, and prominent nucleoli. Luminal necrosis may occur as aggressive proliferation *in situ* forces cells away from the sustaining vascular beds of the surrounding stroma, and dystrophic goblet cells may also be present (Maitra and Hruban 2008). In the strictest usage, 'PanIN' refers to human lesions, whereas the separate term 'mPanIN' refers to lesions described in mice, although their pathological criteria are equivalent.

PDAC is classically thought to arise from PanIN-3 lesions, but it can also evolve from other exocrine neoplasias, notably intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs) (Distler et al. 2014). IPMNs are typically solitary lesions of the larger pancreatic ducts in the pancreatic head, which can be visualized radiographically and are considered benign in the absence of an associated adenocarcinoma (Castellano-Megias et al. 2014). MCNs are also benign solitary lesions, but in contrast to IPMN are typically found in women and are localized to the pancreatic tail with an ovarian-type stroma (Fukushima and Zamboni 2014). Although initiating mutations can differ among PanIN, IPMN, and MCN (Saiki and Horii 2014), progression

to PDAC from any of these precursors typically involves mutations in *TP53* and *SMAD4* (Yonezawa et al. 2008). Molecular biology and progression-related events in PDAC will be discussed in greater detail later on in this chapter.

Detection, Staging, and Treatment

It is known that PanINs occur in the general population at much higher frequencies than pancreatic cancer (Singh and Maitra 2007). The implications of this are profound, since it suggests that although these lesions histologically anticipate the development of malignancy, there are many individuals who never progress to cancer despite having low-grade PanINs. This is very similar to the current situation with prostate cancer: most older men exhibit some degree of benign prostatic hyperplasia (BPH), which is the precursor to prostate cancer, but most of those men will not progress to invasive disease, and a biomarker commonly used to stage prostate cancer is also produced by BPH, further complicating diagnosis (Canto et al. 2003). Similarly, any effective screening modality for PanINs would have to be sensitive and highly specific for the rarer PanIN-2 and PanIN-3 lesions, rather than the highly prevalent PanIN-1 lesions. Thus far, very few studies have identified candidate gene signatures of advanced PanINs with potential as biomarkers (Pan et al. 2009; Yi et al. 2013). This is partially due to the difficult and expensive nature of proteomics, and recent advances in RNA biology have resulted in a shift towards a search for microRNA biomarkers, which can be easily profiled in a relatively inexpensive and high-throughput manner (Xue et al. 2013).

Considering the difficulties involved in detecting and discriminating PanIN lesions, the detection of mature pancreatic cancer in its earliest non-invasive stages is the highest priority. Currently the only potentially curative treatment for pancreatic cancer is surgical removal of localized disease. 'Localized' describes the fact that the tumor has not extended beyond the pancreas and has not metastasized to regional lymph nodes or more distant sites. Just 9% of all patients diagnosed with pancreatic cancer are localized at the time of diagnosis (Howlader et al. 2014). These patients are candidates for a radical surgery: a pancreaticoduodenectomy, or 'Whipple,' as it is often called in the US, memorializing the American surgeon who refined it in the 1930's. The surgery is quite drastic, removing the distal portion of the stomach, most of the duodenum, the head of the pancreas, the common bile duct and the gall bladder. This subsequently requires re-anastomosing of the remaining third of the duodenum to the proximal stomach, the tail of the pancreas, and the remaining portion of the bile duct. The procedure involves the dissection of numerous blood vessels which can be the source of life-threatening blood loss. If patients survive the surgery, then they experience the best possible prognosis for one who has been diagnosed with pancreatic cancer: a 25.8% chance at a five-year survival (Howlader et al. 2014). They spend this time adapting to insulin-dependent diabetes and chronic maldigestion, requiring exogenous insulin and supplementary pancreatic enzymes (Lindkvist 2013). Once their surgical wounds have healed, patients may undergo adjuvant chemotherapy to improve their chances of long-term survival. Treatment with the chemotherapeutic drug gemcitabine in this setting can extend median survival by approximately six months (Oettle et al. 2013).

Most patients with radiographically localized disease will undergo surgery, but in many cases, open surgery will reveal that the tumor has invaded adjacent structures, or pathological analysis of regional lymph nodes obtained during the resection will reveal disseminated disease. These patients with 'regional' disease comprise 28% of all pancreas cancer patients, and their five-year survival rate is 10% (Howlader et al. 2014). If staging is properly upgraded in the operating room prior to the full commencement of the Whipple, then the surgery may be aborted. However, due to the complex anatomical nature of the pancreas' location in the abdominal cavity, and the aggressive potential of the tumors to progress in the short time between imaging and surgery, surgeons may not visualize or anticipate regionally advanced disease until the procedure is well under way. In my personal experience observing a Whipple here at the University, this occurred approximately eleven hours into surgery when the stomach and duodenum had been bisected and most major vessels to the pancreas had been ligated. It then became apparent that the tumor was invasive to the aorta, ruling out the possibility of clean surgical margins. Recent efforts have pursued pre-surgical treatments to downstage regional disease or preserve localized status until surgery can be performed. Such 'neoadjuvant' therapies show great promise and are improving outcomes (Rajagopalan et al. 2013; Ansari et al. 2014; Mellon et al. 2015).

For the 53% of pancreas cancer patients who present with widely metastatic disease at diagnosis, surgery is not an option, and these patients have an appalling 2.3% chance of surviving for five years (Siegel et al. 2015). This is simultaneously the most dismal and the most common occurrence. Standard of care for these patients is a

chemotherapeutic regimen that has evolved from the original anti-pancreatic-cancer drugs, 5-fluorouracil (5-FU) and gemcitabine.

5-FU and gemcitabine are nucleoside analogs that incorporate into the RNA and DNA of dividing cells, respectively, disrupting protein translation and arresting DNA synthesis, leading to cell death (Wilkinson et al. 1975; Plunkett et al. 1995). 5-FU was used for several decades as the standard of care for patients prior to the creation of gemcitabine (Arbuck 1990). Initial studies evaluating the benefit of gemcitabine versus 5-FU found only a one-month improvement in median survival, but a higher percentage of patients on gemcitabine reported improved pain symptoms compared to those receiving 5-FU (23.8% vs. 4.8%) (Burris et al. 1997). Since this study, oncologists have attempted to develop improved drug regimens using gemcitabine as the core therapeutic and standard of care. The best currently available treatment is a cocktail of oxaliplatin, irinotecan, leucovorin, and 5-FU in combination with gemcitabine. This regimen, called FOLFIRINOX, provides a median survival benefit of 4.3 months over gemcitabine alone, typically with three months of progression-free survival (Conroy et al. 2011). However, this regimen carries increased toxicity compared to gemcitabine, with higher rates of severe adverse events including neutropenia, thrombocytopenia, diarrhea, and neuropathy (Conroy et al. 2011). Ongoing work is being done to tailor the dosing of FOLFIRINOX to find efficacious schedules that will reduce toxicity, and thereby increase the number of patients who can tolerate this treatment (Gunturu et al. 2013), and for now FOLFIRINOX is considered standard of care for patients with advanced pancreatic cancer. Therefore, in the two decades since we began using gemcitabine to treat pancreatic cancer, we've been

able to provide the average patient with an additional four months of life. This stalemate has spurred basic biologists to explore the molecular biology of PDAC in the hopes of identifying druggable weaknesses in this nearly untreatable disease.

Molecular Biology

The greatest unifying pattern of pancreatic cancer is the mutational activation of *KRAS* (Almoguera et al. 1988; Smit et al. 1988). It is observed in the earliest PanINs (Shi et al. 2009; Kanda et al. 2012) and its expression is maintained late in disease progression (Biankin et al. 2012). Pancreatic cancers with activated *KRAS* exhibit oncogene addiction, whereby suppression of *KRAS* activity induces tumor cell death and regression of PanINs back to normal tissue (Collins et al. 2012a; Collins et al. 2012b; Ying et al. 2012). Understanding KRAS is an essential prerequisite to an understanding of pancreatic cancer.

KRAS is a member of a larger family of RAS proteins, including HRAS and NRAS, which are responsible for relaying and amplifying signals from growth factor receptors at the cell surface (Cox and Der 2010). Signaling through RAS begins when growth factors, such as EGF, bind to their transmembrane receptors, triggering their dimerization and the phosphorylation of their cytoplasmic tails. These phosphorylated sites can then recruit growth-factor-receptor-bound protein 2 (GRB2) to the plasma membrane by interacting with its SH2 domain. GRB2 in turn recruits Son-of-Sevenless (SOS), which activates RAS by facilitating RAS binding to GTP.

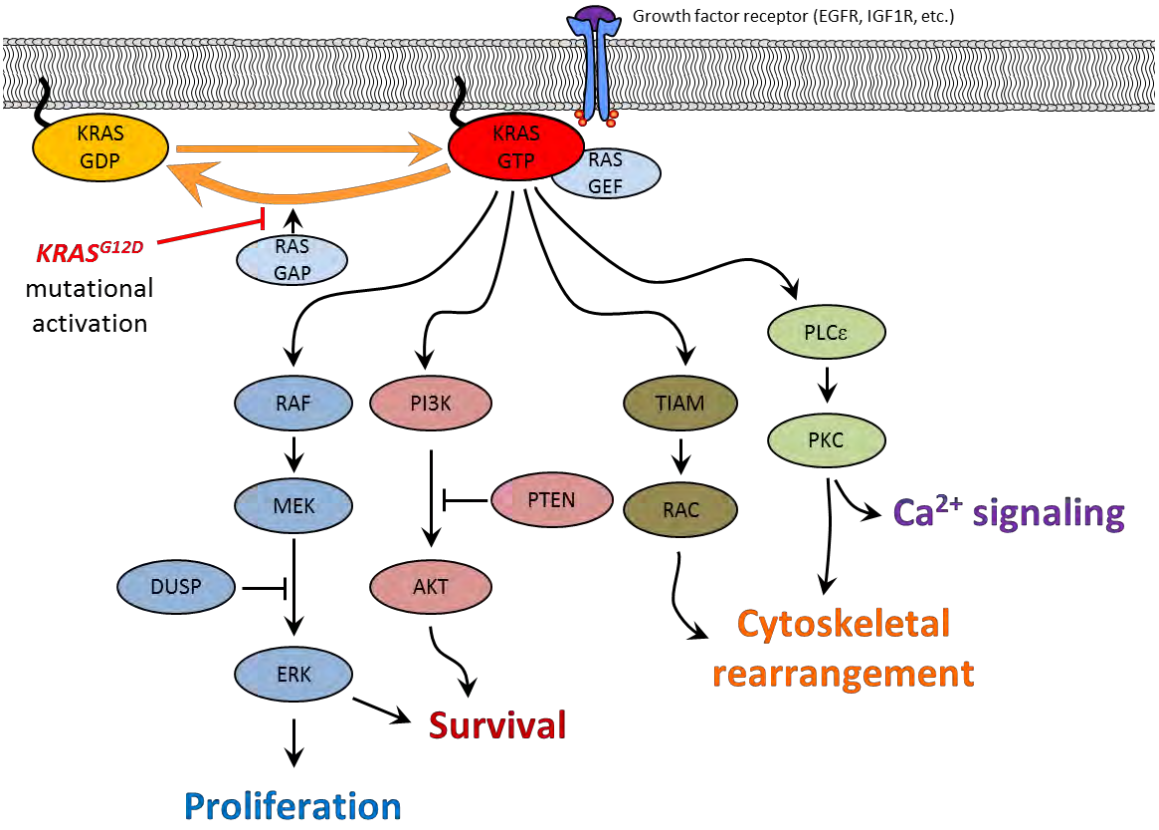


Figure 1.3. RAS signaling is controlled by GAPs and GEFs.
This abbreviated schematic shows some of the pathways activated by RAS proteins and is not exhaustive. Mutations commonly associated with pancreatic cancer (e.g. $KRAS^{G12D}$) affect RAS' association with GAPs. All downstream signaling from RAS occurs only in the GTP-bound 'on' state. Downstream pathways generate overlapping and distinct effects in pancreatic cancer, and are subject to regulation at multiple steps. DUSP: dual-specificity phosphatase.

RAS proteins cycle through active and inactive conformational states by their association with GTP and GDP, respectively (Figure 1.3) (Scolnick et al. 1979; Schlichting et al. 1990; Cox and Der 2010). This cycling is governed by the hydrolysis of RAS-bound GTP to GDP, and the exchange of GDP for fresh GTP. The hydrolysis of GTP to GDP is catalyzed by RAS itself and is accelerated by the binding of GTPase activating proteins (GAPs) (Vigil et al. 2010). The exchange of GDP for GTP is facilitated by a family of proteins that includes SOS, called guanidine nucleotide exchange factors (GEFs). GDP-bound RAS is unable to recruit and activate downstream effectors, and in the course of normal cellular signaling, RAS proteins spend most of their time in a GDP-bound 'off' state with only intermittent pulses of GTP-induced activity. Importantly, the auto-inactivating GTPase reaction of an isolated RAS molecule is very slow (Chung et al. 1993), and cells therefore rely heavily on the activity of GAPs for the proper inactivation of RAS signaling.

Relevant to the balance in RAS' interaction with GAPs and GEFs is the fact that RAS is normally restricted to endomembranes by farnesylation of its C-terminus (Casey et al. 1989; Fehrenbacher et al. 2009). This localization brings RAS in close proximity to its activating GEFs and is required for RAS activity (Kohl et al. 1993). *KRAS* can generate two protein products through alternative splicing: KRAS4A and KRAS4B. KRAS4A, like the other members of the RAS family, HRAS and NRAS, is additionally modified for membrane association by palmitoylation (Laude and Prior 2008). KRAS4B, however, lacks the upstream amino acid signals required for palmitoylation, and therefore has a more dynamic interaction with the plasma membrane (Silvius et al. 2006). There is

evidence from studies of HRAS and NRAS that depalmitoylation can occur and drive rapid redistribution away from membranes, thereby inactivating RAS signaling, but the relevance of this regulation to the activity of KRAS4A remains unclear (Ahearn et al. 2012). RAS proteins can also be post-translationally modified by phosphorylation, mono- and di-ubiquitination, and S-nitrosylation, although the specific impact of these alterations are largely uncharacterized (Ahearn et al. 2012).

There are many downstream pathways that are activated by KRAS in a GTP-dependent manner (Figure 1.3) (Cox and Der 2010). One of the first identified downstream effectors of KRAS signaling was the RAF/MEK/ERK pathway (Dickson et al. 1992; Moodie et al. 1993). This signaling cascade results in the activation of transcription factors including c-JUN, FOS, and ELK1 to drive cell cycle progression (Downward 2003). KRAS also stimulates PI3K to activate AKT (Marte and Downward 1997) and PDK1 (Eser et al. 2013; Ferro and Falasca 2014). These pathways relay pro-survival and proliferative signals through yet other effectors including mammalian target of rapamycin (mTOR), Cyclin D1, and HIF-1α (Bauer et al. 2015). Independent of PI3K, RAS proteins can signal through TIAM to drive RAC1 activation and promote cytoskeletal rearrangement (Lambert et al. 2002). Another major pathway known to be activated by RAS is the phospholipase C-epsilon (PLCε) signaling pathway, which activates protein kinase C (PKC) to modulate cytoskeletal activity (Kelley et al. 2001)

Mutational activation of KRAS in cancer typically entails a missense mutation at codon 12 or 13, substituting wild-type glycine residues for a more bulky aspartate or valine (e.g. $KRAS^{G12D}$ or $KRAS^{G12V}$) (Capon et al. 1983). These mutations render KRAS

incapable of effectively binding to the GAPs that normally arrest KRAS signaling, resulting in aberrantly stable activation of downstream pathways (Scheffzek et al. 1997). The far less common mutation at codon 61 of a glutamine (e.g. *KRAS*^{Q61L} or *KRAS*^{Q61H}; Prior et al, Cancer Res 2012) achieves a similar end by destroying a critical residue in the active site of KRAS' GTPase domain (Figure 1.3). Mouse models indicate, however, that mutational activation of KRAS is not sufficient to drive carcinogenesis, and some component of inflammation must be present to drive progression (Guerra et al. 2007; Guerra et al. 2011; Daniluk et al. 2012; Zhang et al. 2013). Moreover, EGFR signaling is required for the full oncogenic effect of KRAS^{G12D} (Ardito et al. 2012; Navas et al. 2012), as is signaling downstream of the IGF1 receptor (Appleman et al. 2012), emphasizing that although mutational activation of KRAS is a very early event, its primary function appears to be to amplify or deregulate cellular responses to extracellular stimuli. This broadens the possibilities of therapeutic intervention against *KRAS*-mutant cancers to include upstream signaling components.

Excessive activation of growth factor signaling in normal cells induces oncogene-induced senescence (OIS), which is a physiological response that forms one of the first lines of defense against neoplasia. Senescence is characterized by an irreversible exit from the cell cycle, and in the particular case of OIS, is driven by upregulation of the *CDKN1A* and *CDKN2A* genes (Larsson 2011). The tumor suppressor gene *CDKN2A* encodes two protein products by an alternative splicing mechanism: the cell cycle inhibitor P16^{INK4A} and the MDM2 antagonist P14^{ARF} (Quelle et al. 1995). INK4A is a cyclin-dependent kinase inhibitor that blocks the G1-S transition, and ARF drives

proteolysis of the TP53 antagonist MDM2, thereby driving TP53 accumulation and cell cycle arrest in G1 and G2 (Zhang et al. 1998). Senescence is observable in PanIN-1 lesions, where activation of *KRAS* is the primary driver, but telomere shortening and accrual of DNA damage are also present and contribute to the induction of senescence in a TP53-P21-dependent manner (Hruban et al. 2008). This occurs via the ATM/ATR-TP53-$P21^{WAF1}$ cascade that detects DNA damage (Larsson 2011). Thus progression beyond PanIN-1 requires the inactivation of key tumor suppressors in order to bypass OIS. Indeed, disruption of *CDKN2A* function is commonly observed in PanIN-2 lesions (Hruban et al. 2008), and in over 95% of PDAC tumors, where INK4A and ARF function can both be lost (Caldas et al. 1994; Bardeesy et al. 2006a). This single mutation therefore attenuates two tumor suppressive responses to OIS by simultaneously deleting INK4A to allow cell cycle progression and deleting ARF to desensitize cells to DNA damage.

TP53 disruption is also observed in late PanINs and over 75% of PDAC cases, and is thought to contribute greatly to the genomic instability of PDAC by promoting DNA damage (Halazonetis et al. 2008; Jones et al. 2008; Koorstra et al. 2009). Interestingly, *TP53* is not normally completely lost in PDAC, as one allele may be deleted, but the remaining allele is typically disrupted by missense mutation (Redston et al. 1994), suggesting a gain-of-function activity for TP53 in these tumors.

Genetic disruptions of the TGF-β effector *SMAD4* are seen in over 50% of PDAC cases, which allows bypass of TGF-β-induced growth arrest (Hahn et al. 1996; Iacobuzio-Donahue et al. 2004). Mouse modeling has demonstrated that loss of *SMAD4* definitively

enhances PanIN progression to PDAC; but in a subset of tumors, intact *SMAD4* can promote growth, supporting the clinical evidence that *SMAD4* loss is not universally required for tumor progression (Bardeesy et al. 2006b). In the absence of SMAD4, TGF-β signaling plays a role in the activation of RAS to drive tumor progression (Xia et al. 2015).

microRNAs

Nature, Pathway, and Mechanism

microRNAs (miRNAs) are a class of short RNA molecules initially discovered and described as the non-coding RNA product of the *C. elegans* gene *lin-4* (Lee et al. 1993; Wightman et al. 1993). Since then, miRNAs have come to be appreciated as highly diversified molecules that are broadly conserved across species (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001). They play roles in development, normal physiology and disease (Schickel et al. 2008; Sayed and Abdellatif 2011; Vidigal and Ventura 2015), and are predicted to control the expression of over half of the human transcriptome (Bartel 2009; Friedman et al. 2009). Our evolving understanding of miRNA processing and activity has been extensively reviewed (Filipowicz et al. 2008; Davis and Hata 2009; Newman and Hammond 2010; Huntzinger and Izaurralde 2011; Braun et al. 2012; Blahna and Hata 2013; Olive et al. 2015). Graphical representation of the miRNA biogenesis pathway about to be discussed is summarized in Figure 1.4 (Hata and Lieberman 2015).

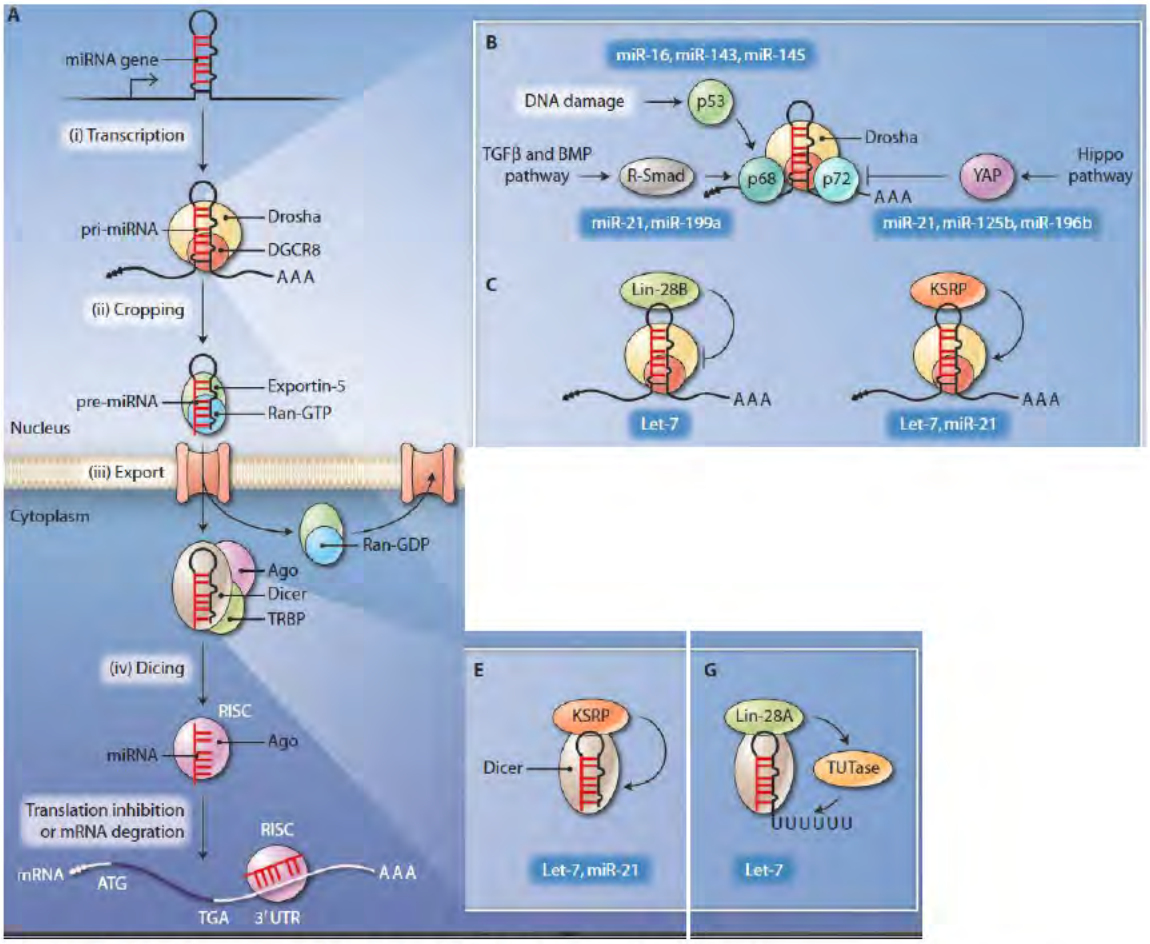


Figure 1.4. Schematic of the core miRNA biogenesis pathway.
This figure summarizes the core miRNA biogenesis pathway and highlights a few miRNAs that are particularly influenced by the activity of certain factors. Figure is excerpted from: A. Hata and J. Lieberman, Dysregulation of microRNA biogenesis and gene silencing in cancer. *Sci. Signal.* **8**, re3 (2015), reprinted with permission from AAAS.

miRNAs are initially transcribed as primary, or 'pri-miRNAs,' which possess a stereotypical stem-loop structure (aka hairpin) within the context of a longer, single-stranded RNA transcript (Lee et al. 2002). This hairpin is recognized by the microprocessor complex, key components of which are the RNase III enzyme DROSHA (Lee et al. 2003) and its partner DiGeorge syndrome critical region 8 (DGCR8) (Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). The microprocessor cleaves the pri-miRNA near the base of the stem to release a free hairpin, now a 'pre-miRNA.' This is then exported from the nucleus by Exportin 5 (XPO5) for further processing in the cytosol (Yi et al. 2003). Notably, some miRNAs can arise from within introns through splicing and debranching reactions that bypass the need for microprocessor cleavage (Okamura et al. 2007; Ruby et al. 2007; Yang and Lai 2011).

Once in the cytosol, pre-miRNAs are bound by another RNase III enzyme, DICER. DICER, in association with trans-activation response RNA-binding protein (TRBP) (Chendrimada et al. 2005; Haase et al. 2005) or protein activator of PKR (PACT) (Lee et al. 2006), cleaves off the terminal loop of the pre-miRNA to generate a double-stranded product around 22 nucleotides in length with small 3' overhangs (Bernstein et al. 2001; Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001). TRBP and PACT are not strictly necessary for pre-miRNA processing by DICER, but they do play a role in modulating the position of DICER cleavage within the pre-miRNA, which alters the activity of the mature miRNA (Fukunaga et al. 2012; Lee et al. 2013).

The DICER-bound double-stranded RNA intermediate (dsRNA) now consists of the mature miRNA and its complementary strand (miRNA and miRNA*, respectively).

The transfer of one strand to Argonaute family proteins (AGO) to act as the mature miRNA is influenced by a number of factors including the thermodynamic stability of the RNA strands and Dicer complex association with TRBP or PACT, select one strand (Khvorova et al. 2003; Matranga et al. 2005). The complementary strand is then degraded by AGO's RNase activity. Mammals possess four AGO family proteins (AGO1-4) but catalytic activity appears to be restricted to AGO2 (Hutvagner and Simard 2008). The AGO2-miRNA complex is called the miRNA-induced silencing complex (miRISC) and is now capable of screening mRNA transcripts for complementary sequences and catalyzing post-transcriptional gene suppression (herein 'translational repression'), through mechanisms that may or may not involve mRNA degradation.

The precise mechanisms of miRISC-induced translational repression, and the cofactors involved, remain an area of intense investigation. The earliest descriptions of miRNA function demonstrated that mRNA decay was not required for translational repression (Olsen and Ambros 1999; Seggerson et al. 2002). Since then, it has been shown that miRNAs can repress translation in active polysomes by inhibiting translational initiation or promoting ribosomal drop-off during peptide elongation (Humphreys et al. 2005; Pillai et al. 2005; Maroney et al. 2006; Nottrott et al. 2006; Petersen et al. 2006; Kiriakidou et al. 2007). Although not required for translational repression, mRNA degradation does also occur through decapping and deadenylation of the targeted mRNA (Behm-Ansmant et al. 2006; Giraldez et al. 2006; Wu et al. 2006; Wu et al. 2010; Chekulaeva et al. 2011; Fabian et al. 2011) via the recruitment of additional factors to the miRISC complex (Huntzinger and Izaurralde 2011).

AGO2 is the critical component of miRISC (Hammond et al. 2001), and it possesses the ability to cleave mRNA transcripts (Pillai et al. 2005). Direct mRNA cleavage in mammals by AGO2's 'slicer' activity typically requires complete sequence complementarity, as in the maturation and activity of small interfering and short hairpin RNAs[1] (siRNAs and shRNAs) (Wang et al. 2009). While mRNA cleavage has been well-described in plants and flies (Carbonell et al. 2012), it is less prominent in mammals, possibly due to differences in the kinetics of mammalian AGO2 catalytic activity (Wee et al. 2012). Slicer-independent translational repression involving partial sequence complementarity is vastly more common in the normal activity of mammalian miRNAs, and it is responsible for much of the off-target effects of engineered siRNAs and shRNAs (Jackson et al. 2003; Jackson et al. 2006). Translational repression in the context of partial complementarity depends greatly on the miRNA seed sequence, which consists of nucleotides 2-7 in the 5' end of the mature miRNA (Lewis et al. 2003b). The contribution of miRNA-mRNA binding events to target selection will be discussed in greater in a later section.

There are two intriguing findings that bear mention for their inconsistency with the bulk of the published literature regarding miRNAs. One study found that target gene *up*regulation occurred as a result of miRNA binding to the mRNA transcript, but this activity appeared restricted to quiescent cells (Vasudevan et al. 2007). A more recent

[1] siRNAs are double-stranded RNAs delivered transiently into cells that are capable of gene suppression (Fire et al. 1998; Hammond et al. 2000) through their ability to be recognized by DICER and loaded into miRISC. shRNAs are single-stranded precursors with the conserved stem-loop secondary structure common to pre-miRNAs, which makes them more efficient substrates for DICER processing (Siolas et al. 2005). They can be synthesized and introduced directly for short-term processing and activity, or they may be expressed from a transgene for long-term gene suppression (Paddison et al. 2002)

supporting observation has demonstrated miRNA-dependent gene upregulation in a cancer context, where miR-483 bound to the 5'UTR of actively transcribing IGF2 to recruit factors and promote transcription (Liu et al. 2013). Another interesting observation that expands the possibilities of normal miRNA activity is that miR-451 can evidently be processed from pre-miRNA to mature and functional miR-451 through a DICER-independent pathway that requires the slicer activity of AGO2 (Cheloufi et al. 2010; Cifuentes et al. 2010). To date, no other miRNAs have yet been identified that can be processed in this manner.

Regulation of microRNA Activity

As pervasive regulators of gene expression, miRNAs are themselves subject to many layers of regulation across all levels of their biogenesis and activity. At this early stage in our understanding, the data suggests a daunting diversity and complexity of regulatory mechanisms, but even a superficial understanding of normal miRNA regulation greatly informs our understanding of how it can be deregulated in cancer. Much of what is discussed below is graphically depicted in Figures 1.4 and 1.5 (Hata and Lieberman 2015; Olive et al. 2015).

The microprocessor complex is a major hub of regulatory activity in the biogenesis of miRNAs. The association of DEAD-box RNA helicase p68 (DDX5 or p68) and DEAD-box RNA helicase p72 (DDX17 or p72) with the microprocessor creates the 'super microprocessor,' and their binding regulates the expression of different subsets of miRNAs (Slezak-Prochazka et al. 2010; Fuller-Pace and Moore 2011). For example, it is

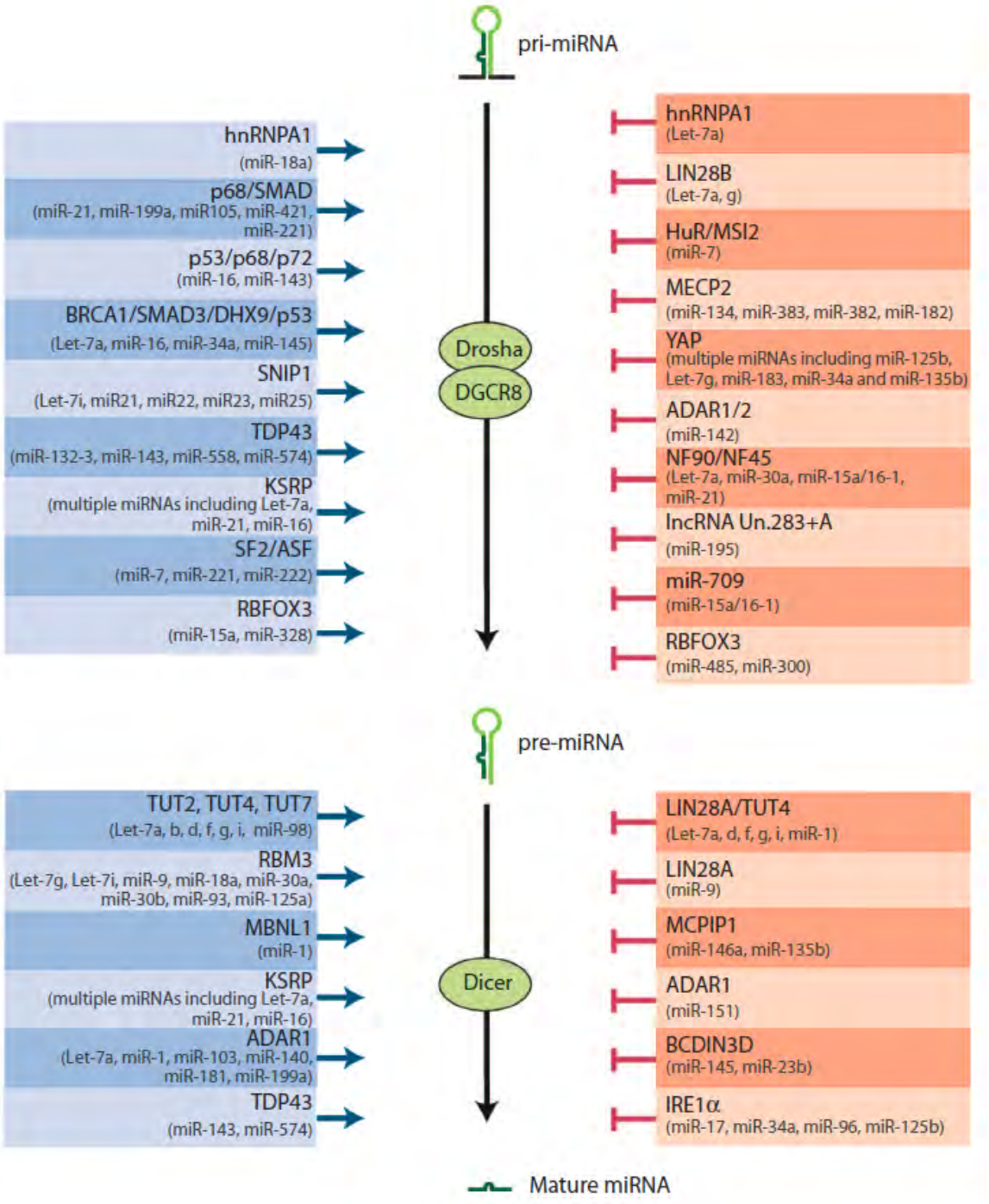


Figure 1.5. Mechanisms regulating miRNA biogenesis.
A more exhaustive representation of the modulatory mechanisms of miRNA biogenesis is presented. Blue boxes are stimulatory effects, whereas red boxes are inhibitory. Listed miRNAs are those that are known to be particularly affected by each factor. Figure is reproduced from: V. Olive, A. C. Minella, and L. He, Outside the coding genome, mammalian microRNAs confer structural and functional complexity. *Sci. Signal.* **8**, re2 (2015), reprinted with permission from AAAS.

known that ligand-specific SMADs can bind to DDX5 and enhance the processing of some miRNAs (Davis et al. 2008). Similar DDX5 binding activity has been observed for TP53 in its promotion of several tumor-suppressive miRNAs upon DNA damage (Suzuki et al. 2009). These studies suggest that transcription factors may play transcription-independent roles in regulating miRNA biogenesis. Furthermore, it has been shown that BRCA1 can recognize the secondary structure of some pri-miRNAs directly and recruit the microprocessor through its association with TP53, SMAD3, and DDX5 (Kawai and Amano 2012). In a conceptually similar model, nuclear YAP dissociates DDX17 from the microprocessor to impair the expression of DDX17-facilitated miRNAs, thereby linking cellular confluency to miRNA expression (Mori et al. 2014).

Regulation of microprocessor activity also occurs through binding of other factors to pri-miRNAs, which can enhance or suppress processing. Enhancement can occur through protein binding to conserved regions in the terminal loops of pri-miRNAs, resulting in relaxation of the pri-miRNA stem to provide more favorable DROSHA cleavage (Michlewski et al. 2008). This has been described for HNRNPA1, which binds to the terminal loop of pri-miR-18a in a sequence-dependent manner and is required for the processing of this miRNA (Guil and Caceres 2007). KH-type splicing regulatory protein (KSRP) also promotes the processing of a subset of miRNAs, including let-7, miR-20a and miR-21, by binding to their pri- and pre-miRNA terminal loops and recruiting DROSHA and DICER complexes (Trabucchi et al. 2009). Suppression of processing can also occur, as in the case of let-7's regulation by LIN28, where terminal loop binding of LIN28 inhibits microprocessor recruitment (Newman et al. 2008;

Viswanathan et al. 2008). Nuclear Factor-90 (NF90) and NF-45 also bind the terminal loops of pri-miRNAs and are negative regulators of processing (Sakamoto et al. 2009). Furthermore, HNRNPA1, previously described as a processing enhancer, has been shown to suppress processing of pri-let-7 by displacing KSRP from the terminal loop (Michlewski and Caceres 2010). This study illustrates the importance of cellular context and balance for the net activity of these factors.

Cytosolic regulation of miRNA processing can occur in a miRNA-specific manner. A particularly well-studied example of this is the relationship between the miRNA let-7 and its regulator LIN28. In addition to its role in the nucleus regulating pri-miRNA processing, LIN28 also binds to the terminal loop of pre-let-7 in the cytoplasm and inhibits its processing by DICER (Heo et al. 2008; Rybak et al. 2008). This occurs via the recruitment of terminal uridylyl transferase 4 (TUT4), which uridylates the 3' end of the pre-miRNA, thereby preventing DICER binding and targeting the pre-miRNA for degradation (Heo et al. 2009). Uridylation has since been observed to occur across a broad range of pre-miRNAs (Ple et al. 2012; Westholm et al. 2012). TUT4 has been implicated in the regulation of other pre-miRNAs besides let-7 (Jones et al. 2012), and the related protein TUT7 has been shown to regulate a similar subset of pre-miRNAs (Thornton et al. 2014), but other protein factors involved in pre-miRNA uridylation have yet to be described. Opposing uridylation, adenylation of the 3' end of mature miRNAs by poly-A polymerases promotes stability (Katoh et al. 2009), but the mechanisms of poly-A polymerase recruitment to miRNAs remains unknown.

AGO2 and DICER activity can also be modulated by cellular pathways to create broad effects on miRNA processing. AGO2 and DICER levels can be suppressed through selective autophagy (Gibbings et al. 2012), and DICER is itself regulated by miRNAs (Martello et al. 2010), including the tumor-suppressive miRNA let-7 (Jakymiw et al. 2010). Numerous phosphorylation events integrate growth and stress signals to exert additional control over the output of the miRNA pathway. It is known that AGO2 is phosphorylated downstream of RAS signaling and that this is a reversible process critical to the gene silencing that occurs in OIS (Yang et al. 2014). AGO2 phosphorylation has been shown to occur in a MAPK- (Zeng et al. 2008) and AKT3-dependent manner (Horman et al. 2013), although additional effectors downstream of RAS likely contribute. Phosphorylation of AGO2 on serine^{387} is required for translocation of AGO2-bound mRNAs to processing bodies (P Bodies) (Zeng et al. 2008), where translational suppression occurs via inhibition of translational initiation and eventual mRNA destabilization. Also, phosphorylation of TRBP via the ERK pathway promotes DICER-mediated processing of many miRNAs as an effector of RAS signaling (Paroo et al. 2009), although in this study let-7 was specifically suppressed by activated TRBP via an unknown mechanism.

Very recently, a class of RNA molecules has been identified, called competing endogenous RNA (ceRNA). These RNAs encode miRNA binding sites that allow them to act as decoy transcripts and alleviate miRNA suppression of a particular mRNAs (Tan and Marques 2014). A key determinant of whether an RNA will behave as a ceRNA appears to be the relative expression ratios of the miRNA, the target mRNA, and the

ceRNA (Bosson et al. 2014), such that very highly expressed mRNAs or miRNAs are not likely to be subject to significant regulation by ceRNAs. One prominent example of ceRNA biology that has garnered a lot of attention is the recently discovered activity of the PTEN pseudogene, PTENP1. PTENP1 acts as a ceRNA for PTEN, such that expression of PTENP1 increases expression of PTEN by alleviating miR-21-mediated suppression of the coding transcript (Yu et al. 2014). This is a strikingly elegant example of a previously unappreciated role for pseudogene transcripts in miRNA-mediated gene regulation. However, ceRNAs have since grown as a concept to include protein-coding transcripts as well, such that now the field is faced with the prospect of interpreting the interrelationships of all mRNAs within a miRNA targetome to define mRNA-ceRNA relationships (Poliseno and Pandolfi 2015). The feasibility of such a daunting analysis remains to be seen.

Methods of Target Prediction and Validation

Because miRNAs exert their effects in a sequence-specific manner, significant effort has been put to devising methods of predicting functional miRNA targets. Predictive algorithms have predominantly evolved from the idea that canonical miRNA binding sites (binding to positions 2-7, or '6mer' sites) have a stronger effect on mRNA expression than binding to other regions of the miRNA (Lim et al. 2005). Target prediction methods also typically take into account that binding to position 8 in the miRNA (7mer-m8), or the presence of an adenine nucleotide in the mRNA across from

position 1 in the miRNA (7mer-A1), or both of these conditions together (8mer) enhance translational repression (Grimson et al. 2007; Nielsen et al. 2007).

One of the first available tools for predicting miRNA targets was TargetScan (Friedman et al. 2009). This platform leveraged 3'UTR conservation across more than 20 species to develop probability scores for predicted miRNA targets: a strong predicted miRNA target is one with a conserved canonical miRNA binding site within a poorly conserved 3'UTR, whereas highly conserved 3'UTRs may contain conserved miRNA sites, but these may be conserved for reasons unrelated to miRNA activity and therefore receive a lower score. Friedman and colleagues also confirmed that binding sites for the 'offset 6mer' (binding to miRNA positions 3-8) were significantly conserved in mammalian 3'UTRs, although the overall descending order of conservation proceeded as 8mer > 7mer-m8 > 7mer-A1 > 6mer > offset 6mer (Friedman et al. 2009). Furthermore, their model included estimates of conservation allowing for G:U wobble in the 5' end of the miRNA (Doench and Sharp 2004), as well as bulges and compensatory binding in the 3' end of the miRNA at nucleotides 13-16 (Brennecke et al. 2005). Thus, a TargetScan context score for a particular mRNA incorporates miRNA binding site conservation, 3'UTR conservation, predicted suppression activity based on the type of seed match, the background conservation for that type of seed match, and the number of seed matches in the transcriptome for that particular miRNA (Friedman et al. 2009).

Numerous other tools have been developed to predict miRNA targets in addition to TargetScan (John et al. 2004; Krek et al. 2005; Miranda et al. 2006; Maragkakis et al. 2009), but they produce widely different target sets for the same miRNA, depending on

how various factors are weighed in each algorithm (Min and Yoon 2010; Thomson et al. 2011). Furthermore, not enough is known about the biology of unusual miRNA binding sites to allow accurate predictions, including the predominantly 3' pairing seen between miR-92 and its targets (Helwak Cell 2013), and so-called 'centered pairing' (Shin et al. 2010). Moreover, although miRNA target sites preferentially occur at the start and end of long 3'UTRs (Gaidatzis et al. 2007), it has been shown that miRNAs can also bind to coding regions of mRNA transcripts (Tay et al. 2008; Rigoutsos 2009). Such sites are effective in translational suppression but are less effective than 3'UTR sites at mRNA destabilization (Fang and Rajewsky 2011; Reczko et al. 2012; Hausser et al. 2013). Further complicating efficacy prediction, local A/U content of the 3'UTR is known to promote destabilization of the transcript (Jing et al. 2005; Hausser et al. 2009).

More definitive strategies for the identification of miRNA targets rely on the experimental validation of binding events, although even these strategies are limited by fixation steps that cannot capture the dynamic nature of binding reactions. High-throughput sequencing of RNA that has been cross-linked to AGO2 protein (AGO2 HITS-CLIP) offers the ability of knowing RNA sequences bound to AGO2 at the time of fixation, but the true miRNA-mRNA binding relationships can be difficult to determine and must be partly inferred with bioinformatics (Chi et al. 2009). A more direct approach involves the crosslinking, ligation, and sequencing of miRNA-mRNA hybrids (CLASH), which preserves the miRNA and its cognate binding site in one coherent read (Helwak et al. 2013), but it reports large numbers of noncanonical binding sites and has poor predictive value of the strength of miRNA function at an observed site. A more recent

advance is the use of biotinylated, psoralen-containing pre-miRNA mimics that purportedly improves capture frequency of binding sites (Imig and Brunschweiger 2015), although it still cannot predict the repressive strength of a given interaction.

Proteomics ultimately offers the most powerful means of determining true relationships between miRNAs and net gene expression. Stable isotope labeling with amino acids in cell culture (SILAC) combined with mass spectroscopy offers a means to measuring acute changes in protein expression caused by miRNAs (Baek et al. 2008; Selbach et al. 2008). Combining SILAC with target prediction algorithms or HITS-CLIP approaches presents the greatest chance of experimentally identifying true miRNA targets in an unbiased way.

The microRNA Pathway in Cancer

Soon after their discovery, miRNAs gained attention as regulators of cancer biology, and the earliest miRNAs to be studied in this context were let-7, miR-21, and the miRNAs of the *mir-17~92* cluster (Takamizawa et al. 2004; Chan et al. 2005; Hayashita et al. 2005). Since then, an explosion of work has begun to characterize the many context-specific activities of miRNAs in cancer. Due to the evolutionary complexity of cancer, there exist near limitless possibilities in the ways a tumor may alter miRNA biogenesis in its favor, and just a few of those mechanisms will be discussed here, along with some of the more well-described oncogenic and tumor-suppressive miRNAs.

Oncogenes and tumor suppressors involved in cancer initiation and progression are known to alter miRNA expression. It has been shown that AGO2 phosphorylation by

EGFR causes deficits in the processing of a particular subset of miRNAs (Shen et al. 2013). This can occur downstream of RAS signaling in a MAPK and AKT-dependent manner, which has profound implications in the cancer context where these pathways are aberrantly activated (Zeng et al. 2008; Horman et al. 2013). EGF has been shown to upregulate a pro-metastatic subset of miRNAs in mammary cells , and ERK activation can also increase TRBP phosphorylation to promote DICER-mediated processing of many miRNAs, which again places miRNA biogenesis downstream of RAS signaling (Paroo et al. 2009). Much of the work regarding oncogene control of miRNA processing is fairly recent and has not received as much attention as the characterization of individual miRNAs that themselves play the part of tumor-suppressors or oncogenes.

Let-7 is now a well-established tumor suppressor whose expression is reduced in many cancers (Takamizawa et al. 2004; Zhang et al. 2014b). Overexpression of let-7 in cancer cells can revert EMT phenotypes and trigger cell cycle arrest and cell death (Kumar et al. 2008; Li et al. 2009). Reduced let-7 expression is thought to be selected for during tumor progression because of its ability to suppress key oncogenes, including RAS family proteins, MYC, and high-mobility group A protein 2 (HMGA2) (Johnson et al. 2005; Lee and Dutta 2007; Mayr et al. 2007; Sampson et al. 2007). HMGA2 is a non-histone chromatin factor whose expression is often upregulated in cancer, leading to an invasive phenotype (Watanabe et al. 2009). HMGA2 is normally regulated by let-7, and overexpression of HMGA2 can occur as a result of a shortened 3'UTR that omits these let-7 binding sites (Klemke et al. 2010; He et al. 2014b).

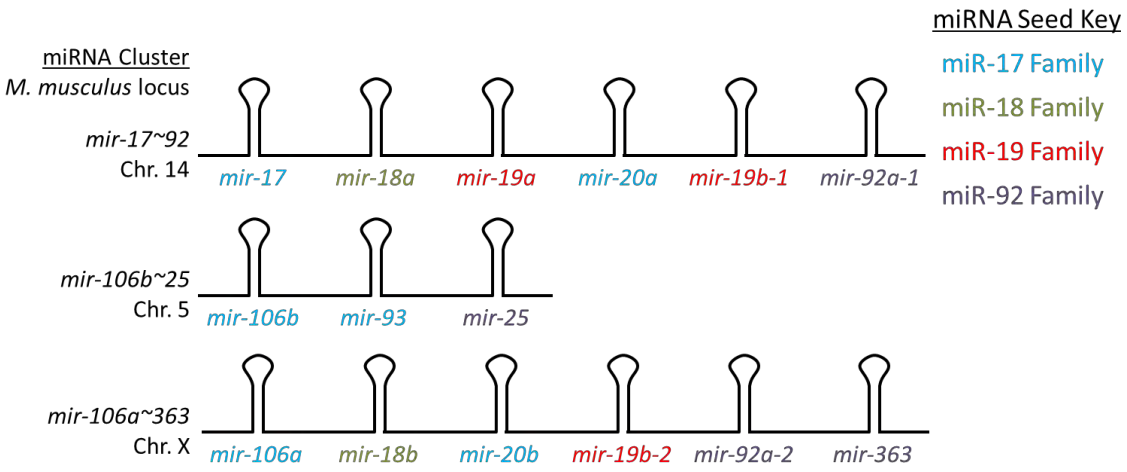


Figure 1.6. The *mir-17~92* cluster and its related paralogs encode members of four miRNA families.
miRNAs sharing the same seed sequence are grouped by color. Not all clusters contain miRNAs from all four families: notably, *mir-106b~25* encodes only miR-17 and miR-92 families.

miR-21 is an equally well-established oncogene that is overexpressed in most tumor types (Chan et al. 2005; Volinia et al. 2006; Si et al. 2007; Schetter et al. 2008). It has been shown to suppress the expression of several key tumor suppressors, including PTEN, PDCD4, SPRY2, TP63, and KIP2, among others (Meng et al. 2007; Frankel et al. 2008; Bhatti et al. 2011; Kwak et al. 2011; Quintavalle et al. 2013; Mishra et al. 2014). Fascinatingly, inhibition of miR-21 can cause robust cancer regression in a mouse model of lymphoma, demonstrating that tumors can exhibit oncomir addiction (Medina et al. 2010).

The first oncomir ever characterized was the *mir-17~92* cluster, so-called 'oncomir-1' (He et al. 2005; Kort et al. 2008). This miRNA cluster encodes four distinct miRNA families and has two paralogs in the genome that most likely arose from gene duplication events (Figure 1.6) (Tanzer and Stadler 2004). Its various miRNA families have been powerfully implicated across many tumor contexts (Olive et al. 2010), and inhibition of cluster components can successfully combat tumor growth and survival (Olive et al. 2009; Murphy et al. 2013). Key pathways that are validated targets of *mir-17~92* include TGF-β signaling, PTEN, $p21^{WAF1}$, E2F transcription factors, and the pro-apoptotic protein BIM, (Novotny et al. 2007; Petrocca et al. 2008a; Ventura et al. 2008; Kim et al. 2009; Olive et al. 2009; Grillari et al. 2010; Hong et al. 2010).

Although particular miRNAs may be characterized as predominantly tumor-suppressive or oncogenic, miRNAs are on balance thought to exert generally tumor-suppressive effects in cancer. This thinking arises from the observations that 3'UTR shortening occurs in rapidly proliferating (Sandberg et al. 2008) and neoplastic cells

(Mayr and Bartel 2009), which would allow mRNAs to escape miRNA regulation. Moreover, *DICER* expression is reduced in lung cancer and this loss is associated with poor prognosis (Karube et al. 2005). Indeed, *DICER* loss or mutation is associated with tumor progression in several cancer contexts, and knockdown of miRNA processing machinery has been shown to drive tumor progression (Kumar et al. 2007; Hill et al. 2009; Kumar et al. 2009; Lambertz et al. 2010). Also, transcription factors that are known positive regulators of miRNA expression are frequently mutated or functionally impaired in cancer, including SMAD proteins and TP53 (Davis et al. 2008; Maitra and Hruban 2008; Suzuki et al. 2009). It has additionally been shown that defects in XPO5 (Melo et al. 2010) or TRBP (Melo et al. 2009) can severely impact global miRNA maturation in some cancers.

Counter to these observations, mutations in *DICER* or other components of the miRNA biogenesis pathway are not seen in PDAC, and miRNA *up*regulation is more common than downregulation in this disease (Bloomston et al. 2007; Zhang et al. 2009; Frampton et al. 2014). This suggests that miRNAs may play an oncogenic role in the promotion of pancreatic tumorigenesis and that mutations in the miRNA processing machinery are selected against during tumor evolution.

Scope of Thesis

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal malignancies in the United States, with an average five-year survival rate of just 6.7%. Advancements in the understanding of PDAC molecular biology are desperately needed in order to

improve patient survival and quality of life. miRNAs are powerful regulators of gene expression that can behave as oncogenes or tumor suppressors, and understanding their role in disease processes will inform our understanding and potentially lead to new therapeutic approaches.

This dissertation encompasses two studies exploring the role of miRNAs in pancreatic tumorigenesis. In Chapter II, I test the hypothesis that the *mir-17~92* cluster of miRNAs promotes $KRAS^{G12D}$-driven pancreatic tumorigenesis. I present evidence that loss of this miRNA cluster inhibits PanIN development and also promotes PanIN loss and acinar regeneration in the context of continuing $KRAS^{G12D}$ signaling. This phenotype is associated with reduced ERK activity in PanIN lesions. Moreover, *mir-17~92*-deficient tumors are less invasive *in vivo*, and cell lines derived from these tumors are less invasive *in vitro*. I demonstrate that antagonizing the activity of miR-19 family miRNAs is sufficient to suppress the invasiveness of *mir-17~92*-competent PDAC cell lines from mice and humans. Thus *mir-17~92* regulates pancreatic tumorigenesis and progression.

Chapter III presents my exploration of the hypothesis that miRNA biogenesis broadly promotes pancreatic tumorigenesis. I find that *Dicer* heterozygosity inhibits PDAC metastasis, but it does not appear to alter other aspects of tumor biology. Phenotypic characterization of cell lines derived from tumors in this study suggests that the failure of *Dicer*-heterozygous tumors to metastasize is attributable to an increased sensitivity of these cancer cells to anoikis. Ongoing experiments will determine whether shifts in miRNA signatures between cell lines can be attributed to this phenotype.

Unrelated work regarding the preclinical testing of a triple-drug therapeutic regimen to treat advanced pancreatic cancer is presented in Appendix A.

CHAPTER II

mir-17~92 Promotes Pancreatic Tumorigenesis and Progression

Introduction

The *mir-17~92* cluster, expressing miRNAs from the miR-17, -18, -19, and -92 families, is upregulated in numerous cancers, including pancreatic cancer (Olive et al. 2010). Profiling of human pancreatic tumors and pancreatic cancer cell lines has shown that miRNAs from the *mir-17~92* cluster and its paralogs, *mir-106b~25* and *mir-106a~363*, are upregulated compared to samples of normal pancreatic tissue or chronic pancreatitis (Volinia et al. 2006; Szafranska et al. 2007; Ohuchida et al. 2012). mir-17 expression in pancreatic tumors has been associated with reduced survival in humans, and its overexpression in human pancreatic cancer cell lines accelerates growth and promotes invasion (Yu et al. 2010). miR-17 levels in the serum of patients with pancreatic cancer also correlates with tumor stage and the presence of metastasis (Que et al. 2013). Other studies have found elevated levels of mir-18 (Morimura et al. 2011) and miR-25 (Ren et al. 2012) in the blood of patients with pancreatic cancer. A fairly recent study using laser capture microdissection of PanIN lesions found that mir-18, -17*, and -93 were upregulated in PanINs compared to normal tissue, and miR-18 and -93 were further elevated in PanIN-3 lesions compared to earlier PanIN grades (Yu et al. 2012a). Together these data provide strong correlative evidence for the oncogenic role of *mir-17~92* family miRNAs in pancreatic carcinogenesis.

Functional studies of the role of *mir-17~92* in pancreatic cancer are rare. One study found that simultaneous inhibition of all six miRNAs from the *mir-17~92* cluster with pooled antagomirs impaired soft agar growth in some human pancreatic cancer cell lines (Kent et al. 2009). Another study identified upregulation of miR-106a in pancreatic cancer cell lines and linked its pro-invasive role to the suppression of TIMP2 (Li et al. 2014). No *in vivo* studies of the cluster in mouse models of pancreatic tumorigenesis have been published. Therefore, based on the abundance of clinical data linking the *mir-17~92* cluster to pancreatic cancer progression and the clear need for a better understanding of the functional role these miRNAs play in this context, I sought to experimentally test the role of these miRNAs in a mouse model of pancreatic cancer.

The *mir-17~92* cluster is essential for proper development in mice and humans (Ventura et al. 2008; de Pontual et al. 2011; Hemmat et al. 2014) and germline deletion of *mir-17~92* is lethal due to ventricular septal defects and lung hypoplasia (Ventura et al. 2008). Therefore in the following experiments, I make use of conditional alleles to delete the cluster specifically in the pancreas, thereby allowing me to determine the role of these miRNAs in pancreatic tumorigenesis and progression. The study itself integrates two model systems: the first measures effects of *mir-17~92* loss on precursor lesion development in the presence of oncogenic $KRAS^{G12D}$, and the second model incorporates tumor suppressor deficiency to accelerate progression and tumor formation, thereby allowing me to study the role of the cluster in later stages of disease.

Results

To determine the effect of *mir-17~92* loss on pancreatic development, I induced pancreas-specific cluster deletion using the conditional allele *mir-17~92*^{flox} and the recombination driver *Ptf1a-Cre* (Figure 2.1a). Importantly, *mir-17~92*$^{flox/flox}$*, Ptf1a-Cre* mice robustly lose expression of the cluster, as measured by qRT-PCR on whole pancreas RNA (Figure 2.1b), yet they experience normal organ development, with typical exocrine and endocrine architecture and composition (Figure 2.1c). Thus the *mir-17~92* cluster is dispensable for normal pancreas development.

To assess the impact of *mir-17~92* deletion on PanIN development, I crossed conditional cluster mice onto the *LSL-Kras*^{G12D} background (for the *LSL-Kras*^{G12D} allele schematic, see Figure 2.1a). Breeding pairs were designed with *mir-17~92*$^{flox/wt}$*, LSL-Kras*^{G12D} and *mir-17~92*$^{flox/wt}$*, Ptf1a-Cre* individuals to generate littermate *mir-17~92*$^{wt/wt}$*, LSL-Kras*^{G12D}*, Ptf1a-Cre* ('KC') and *mir-17~92*$^{flox/flox}$*, LSL-Kras*^{G12D}*, Ptf1a-Cre* ('17KC') mice. Littermate KC and 17KC animals were aged to four or nine months and subsequently sacrificed to obtain pancreata for histological analysis. At both ages, I observed greater amounts of healthy acinar tissue in 17KC animals than KC animals (Figure 2.2a-i). I also observed that 17KC pancreata contained less PanIN tissue by area than KC pancreata at nine months. 17KC animals gained healthy acinar tissue with age, whereas KC animals completely lost their acinar tissue by nine months. However, the distribution of PanIN grades shows no difference between genotypes (Figure 2.2j). These data suggest that loss of *mir-17~92* impacts PanIN development and maintenance but not PanIN progression.

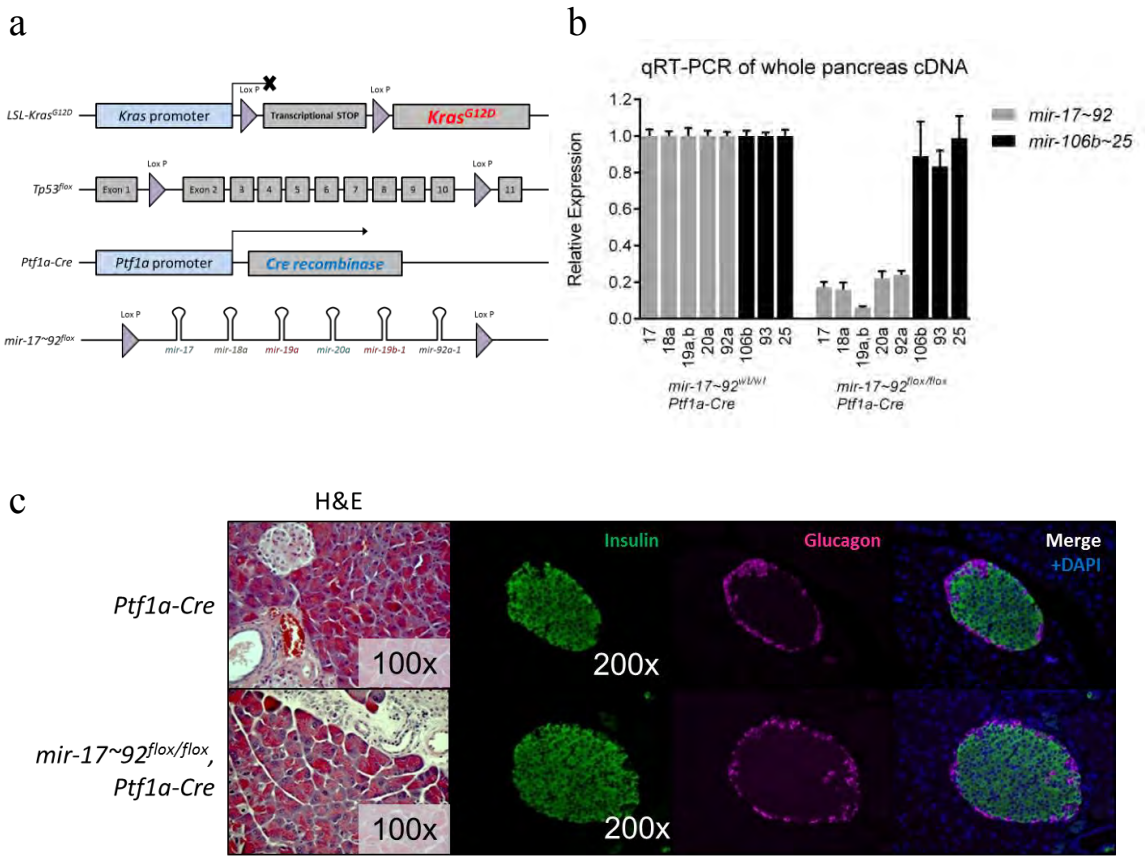


Figure 2.1. Homozygous loss of *mir-17~92* in the pancreas is developmentally tolerated. (**A**) Deletion of conditional alleles in the pancreas is achieved by driving Cre recombinase from the endogenous *Ptf1a* promoter. Activated Kras^{G12D} is expressed upon deletion of an upstream stop cassette flanked by lox-p sites. The majority of the *Tp53*^{flox} and the entirety of the *mir-17~92*^{flox} alleles are deleted in a similar fashion. (**B**) qRT-PCR of whole pancreas RNA shows robust suppression of *mir-17~92* miRNAs in *mir-17~92*$^{flox/flox}$, *Ptf1a-Cre* animals; residual expression likely derives from endocrine and stromal components not targeted by *Ptf1a-Cre.* Expression of the related cluster *mir-106b~25* is unaffected. (**C**) *mir-17~92*$^{flox/flox}$, *Ptf1a-Cre* pancreata are histologically normal by hematoxylin and eosin stain ('H+E') with appropriate differentiation and bulk in both exocrine and endocrine compartments.

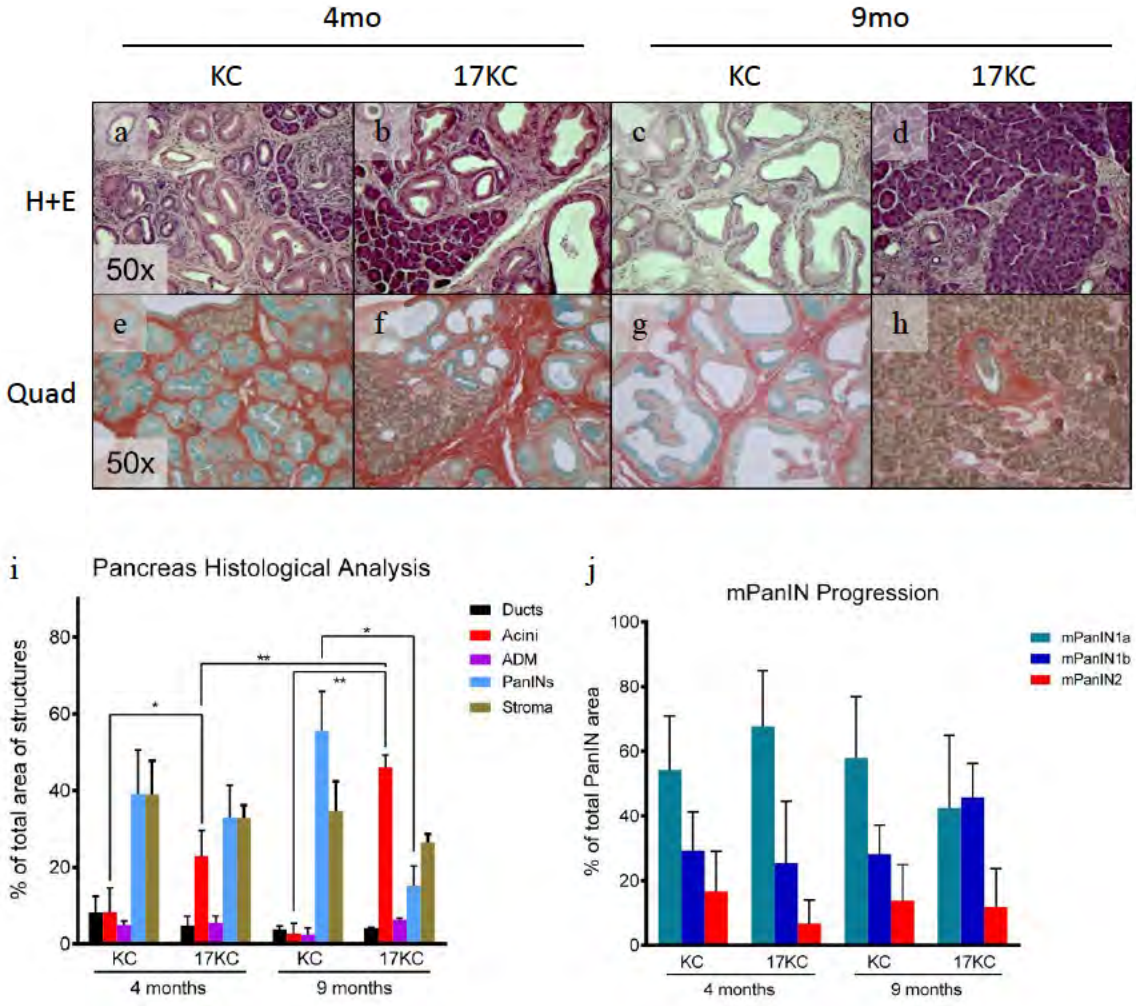


Figure 2.2. 17KC PanINs regress with age and are replaced by healthy acinar tissue. Hematoxylin and eosin staining ('H+E') of pancreata from four- and nine-month-old KC and 17KC animals was performed (**A**-**D**). Quadchrome staining ('Quad') was also performed, marking collagen red, mucin blue, cytoplasm yellow-brown, and nucleic acids black (**E**-**H**). Quantification of tissue prevalence as a percentage of slide area (**I**), and PanIN grade prevalence as a percentage of total PanIN area (**J**), were performed by a licensed pathologist. Note that nine-month-old 17KC mice have fewer PanIN lesions and more acinar tissue than age-matched KC animals. p values: * < 0.05, ** < 0.01

Immunohistochemical staining of these pancreata demonstrate no measureable differences in PanIN proliferative or apoptotic markers (Figure 2.3). Other measures of PanIN biology, including the stromal activation marker α-smooth muscle actin (α-SMA), the Notch pathway effector HES1, and the TGF-β effector phosphorylated SMAD3 (pSMAD3), also show no difference in the PanINs of KC and 17KC animals (Figure 2.4). ADM lesions in 17KC pancreata stain more strongly with pSMAD3 compared to the ADM lesions of KC animals at equivalents ages, but early PanINs in both groups are negative for this marker (Figure 2.4i-l). However, 17KC PanINs do display marked reduction in MAPK signaling, as determined by staining for phosphorylated extracellular signal-regulated kinase (pERK) (Figure 2.5). These data suggest that *mir-17~92* regulates PanIN maintenance by influencing ERK pathway activity downstream of $KRAS^{G12D}$.

To look beyond PanIN formation and examine the effect of *mir-17~92* on carcinoma development, I accelerated the KC model by including conditional loss of one copy of *Tp53* (*LSL-*$Kras^{G12D}$*,* $Tp53^{flox/wt}$*, Ptf1a-Cre*; hereafter "KPC" and "17KPC" mice; for $Tp53^{flox}$ allele schematic, see Figure 2.1a). I observed that KPC and 17KPC mice display similar overall survival, tumor size, and rates of metastatic disease (Figure 2.6a-c). Carcinoma subtypes were diagnosed by a licensed pathologist and the distributions of both groups were found to consist mostly of adenocarcinoma with less frequent poorly differentiated forms (Figure 2.6d-f). Histological evidence of invasion was also equally prevalent in both groups, and variously involved the stomach, liver, duodenum, colon, and spleen (Figure 2.7). Moreover, tumors across both groups exhibited similar rates of

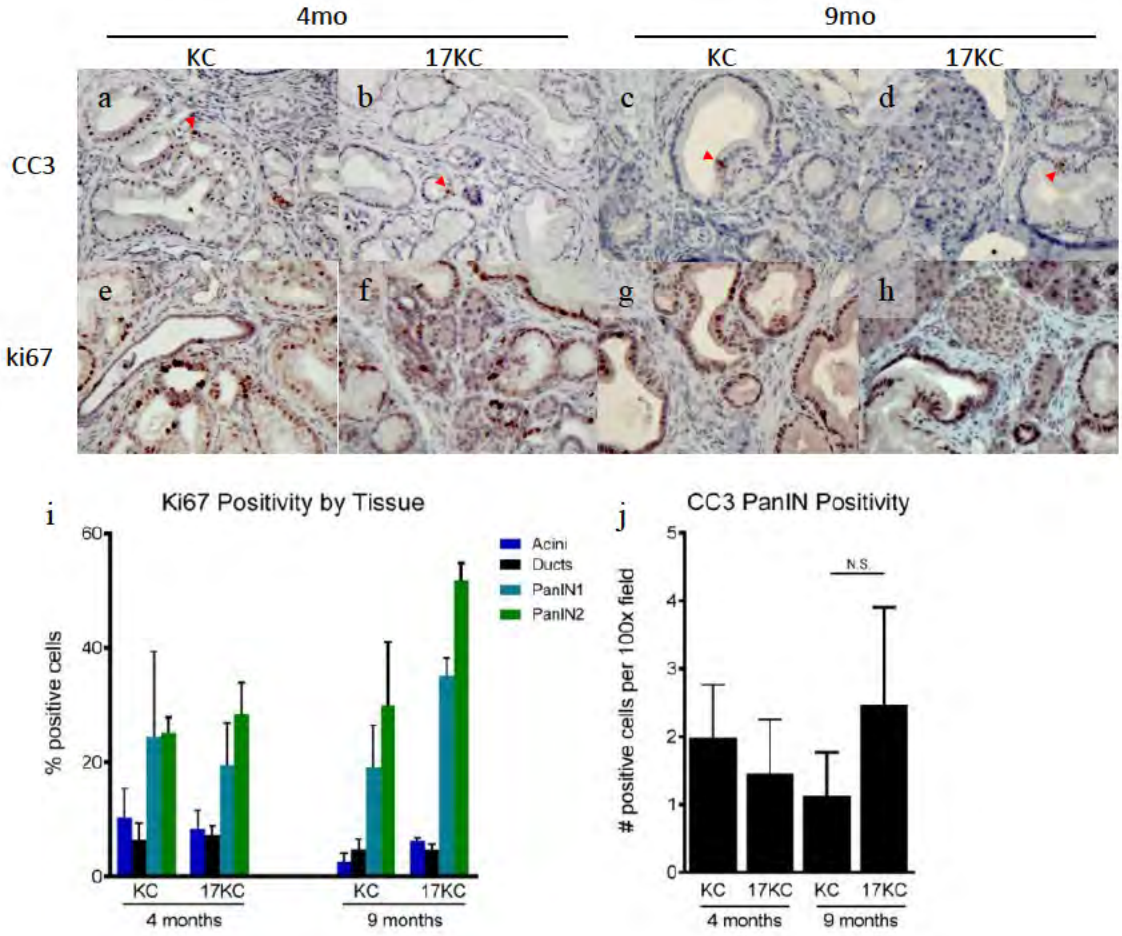


Figure 2.3. 17KC PanINs are equally proliferative and apoptotic compared to KC PanINs. Immunohistochemistry for the apoptosis marker cleaved caspase 3 ('CC3'; **A-D, J**) and the proliferative marker ki67 (**E-I**) demonstrates equal posititivty between KC and 17KC pancreata at four and nine months of age. Arrowheads indicate CC3-positive cells.

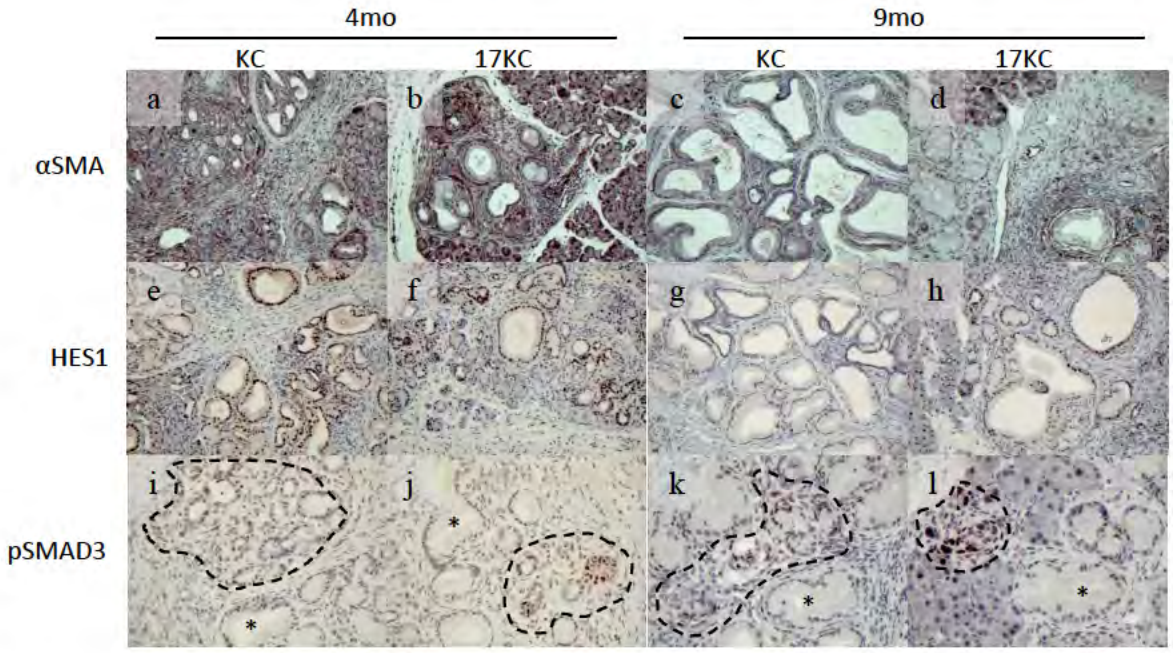


Figure 2.4. 17KC PanINs are immunohistochemically indistinguishable from KC PanINs. Immunohistochemistry for the stromal marker α-smooth muscle actin ('αSMA';**A-D**), the progenitor marker HES1 (**E-H**), and the progression-related marker phospho-SMAD3 ('pSMAD3'; **I-L**) demonstrate no differences in the PanINs of KC and 17KC animals. pSMAD3 staining is generally higher in 17KC ADM lesions than KC ADM lesions (outlines), but is absent in early PanINs of both genotypes (*). pSMAD3 staining was performed separately for each time point, and therefore comparisons should be limited to differences between genotypes within each time point.

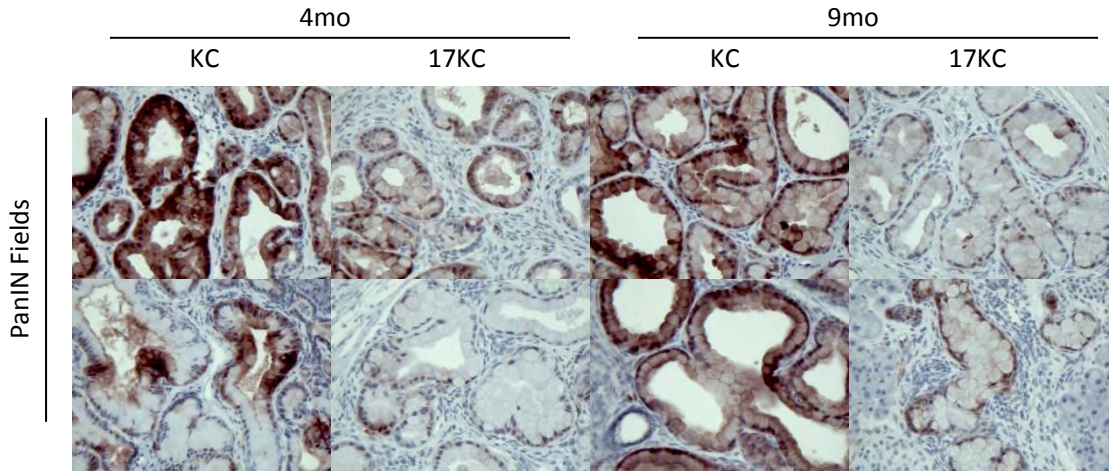


Figure 2.5. 17KC PanINs display reduced phospho-ERK staining compared to KC PanINs. Immunohistochemical staining of phosphorylated ERK demonstrates substantially reduced positivity in 17KC PanINs compared to KC PanINs at both time points. Two examples are provided for each to demonstrate representative ranges in positivity.

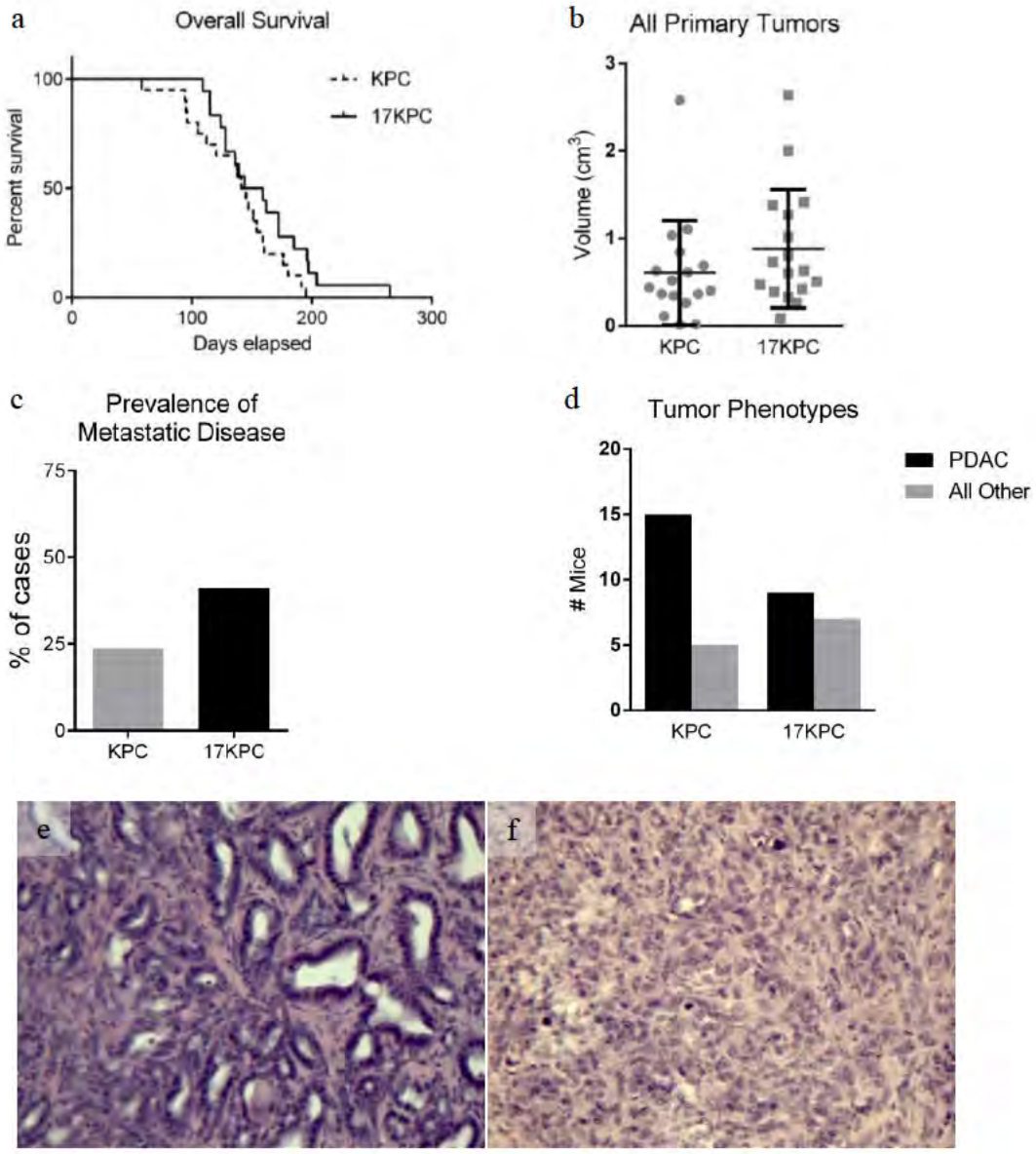


Figure 2.6. Loss of *mir-17~92* does not cause differences in disease progression among unstratified individuals.

Overall survival (**A**), tumor burden (**B**), incidence of metastatic disease (**C**), and the distribution of carcinoma subtypes (**D**) do not differ significantly between KPC and 17KPC animals. Examples of ductal adenocarcinoma (**E**) and poorly differentiated carcinoma (**F**) are provided.

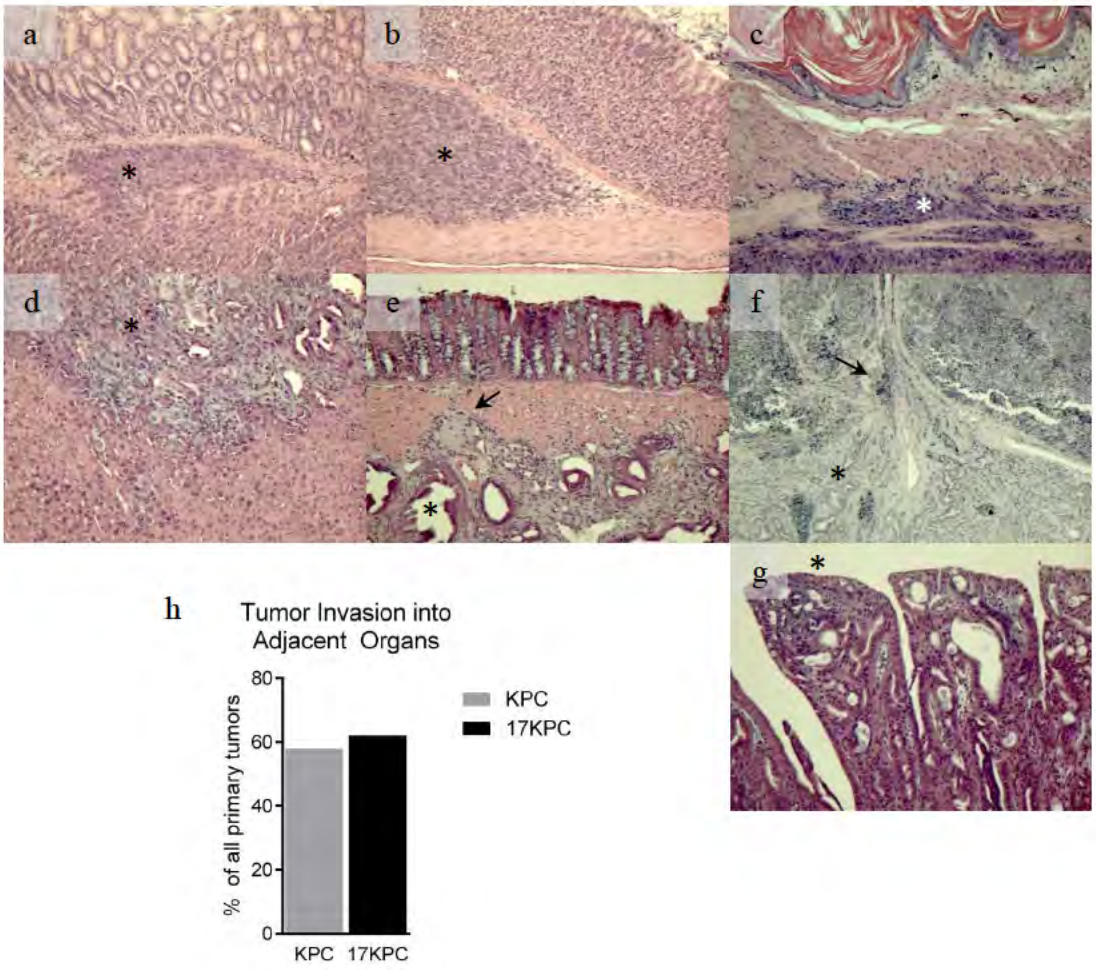


Figure 2.7. 17KPC primary tumors are invasion-competent at time of sacrifice.
(**A**-**F**) Histological evidence of invasion was sought for each primary tumor in the survival study. Serial step sectioning was performed at 200um steps until invasion was confirmed or the entire tumor was sectioned. Tumors were capable of invading all portions of the stomach, including the pylorus (**A**), fundus (**B**), and forestomach (**C**), as well as the liver (**D**), colon (**E**), and spleen (**F**). More aggressive invasion causing widespread mucosal destruction or cancerization also occurred (**G**). Many tumors invaded along the myenteric plexus, dissecting the internal and external muscularis layers (**A**-**C**). In these cases, coincident destruction of the muscularis was common (**A,C**). (**H**) Quantification of tumors as invasive or noninvasive based on histological findings shows no difference between KPC and 17KPC tumors. * denotes adenocarcinoma; arrows highlight invasion along collagen tracks.

proliferation and apoptosis as demonstrated by ki67 and cleaved caspase 3 (CC3) staining (Figure 2.8).

17KPC mice sacrificed for illness arising from localized disease ('localized' 17KPC mice) exhibited significantly prolonged survival compared to localized KPC mice (Figure 2.9a). Counterintuitively, these mice also had larger tumors than localized KPC mice (Figure 2.9b). Due to anatomical complexity around the pancreatic head, localized pancreatic cancer in mice can manifest with jaundice (evident in the ears, footpads, or pancreas) or GI obstruction, as seen by GI lumen distension upstream of an adhesion with the absence of downstream luminal contents (Figure 2.10a,b). 17KPC mice never presented with any form of GI or biliary obstruction (Figure 2.10c,d). Together, these *in vivo* findings suggest that loss of the *mir-17~92* cluster generates a less invasive form of advanced pancreatic cancer, supporting an oncogenic role for this miRNA cluster in disease progression.

To better understand the biology of *mir-17~92* deficient pancreatic cancer cells, I generated a collection of cell lines from KPC and 17KPC tumors to measure cancer-related phenotypes *in vitro*. I measured proliferative capacity by direct counting of adherent cell numbers over time and found that KPC and 17KPC cell lines display a spectrum of proliferative rates within each group, but there is no significant difference by genotype (Figure 2.11a). Similarly, cell lines from both groups display a spectrum of proficiency in their ability to grow in a suspended agarose matrix, which measures dependence on adhesion signaling for growth and survival (Figure 2.11b). More direct measures of cellular survival by trypan blue exclusion assay demonstrate that apoptotic

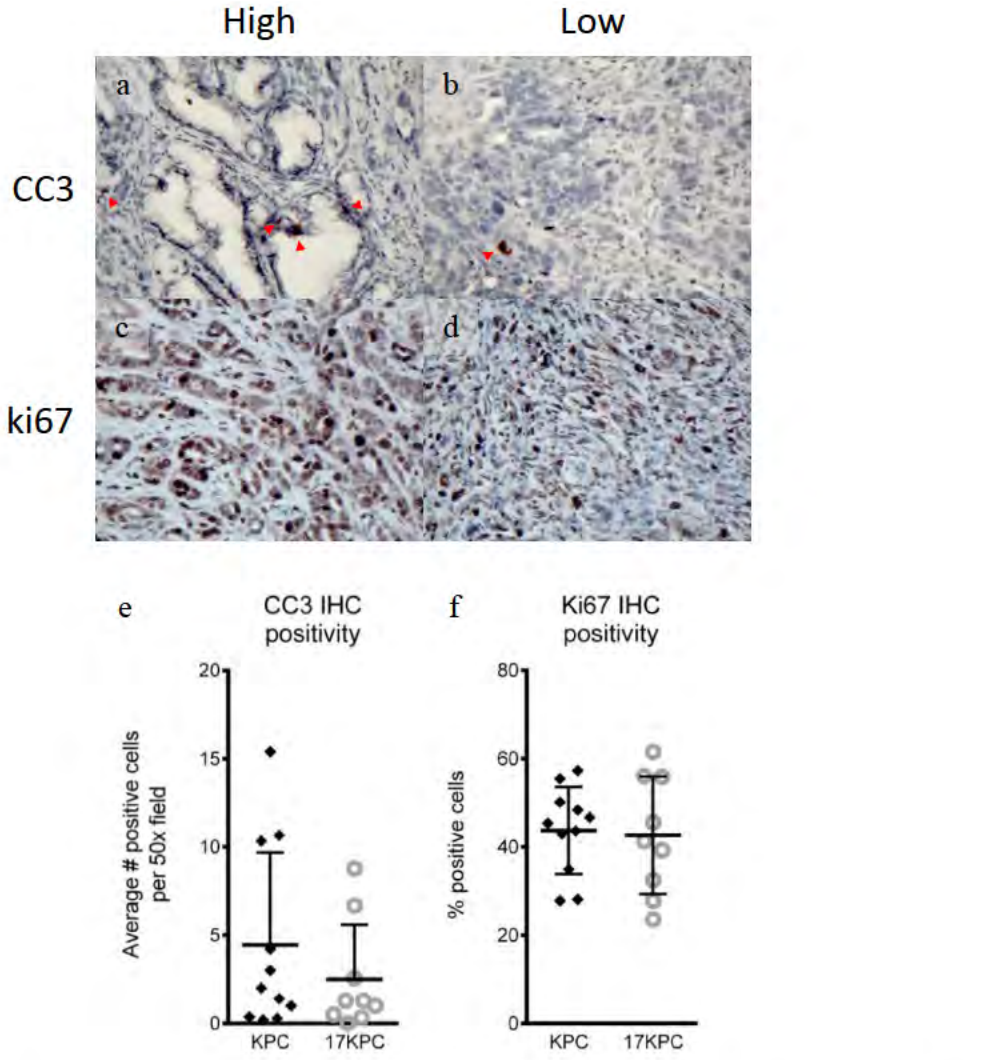


Figure 2.8. Immunohistochemical staining of tumors demonstrates equivalent spectra of proliferative and apoptotic rates.
Immunohistochemical staining of the apoptosis marker CC3 and the proliferative marker ki67 were performed on KPC and 17KPC tumors (**A**-**D**). Quantification of CC3 staining (**E**) and ki67 staining (**F**) shows no difference between KPC and 17KPC tumors. CC3-positive cells were counted if they possessed appreciable attachment to the epithelium; sloughed cells in ductal lumens were not counted. Arrowheads indicate CC3-positive cells. Images are representative of high (**A,C**) and low (**B,D**) staining for both markers.

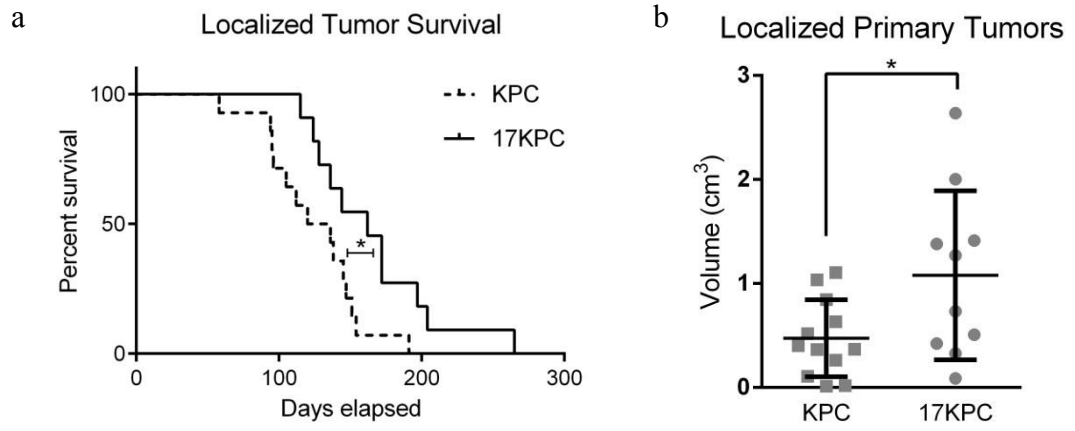


Figure 2.9. Loss of *mir-17~92* prolongs survival in the absence of metastatic disease.
(**A**) 17KPC survival is significantly longer after mice with metastatic disease are removed from the analysis. (**B**) Localized 17KPC tumors are larger than localized KPC tumors.
Log Rank and Student's t test p values: * < 0.05

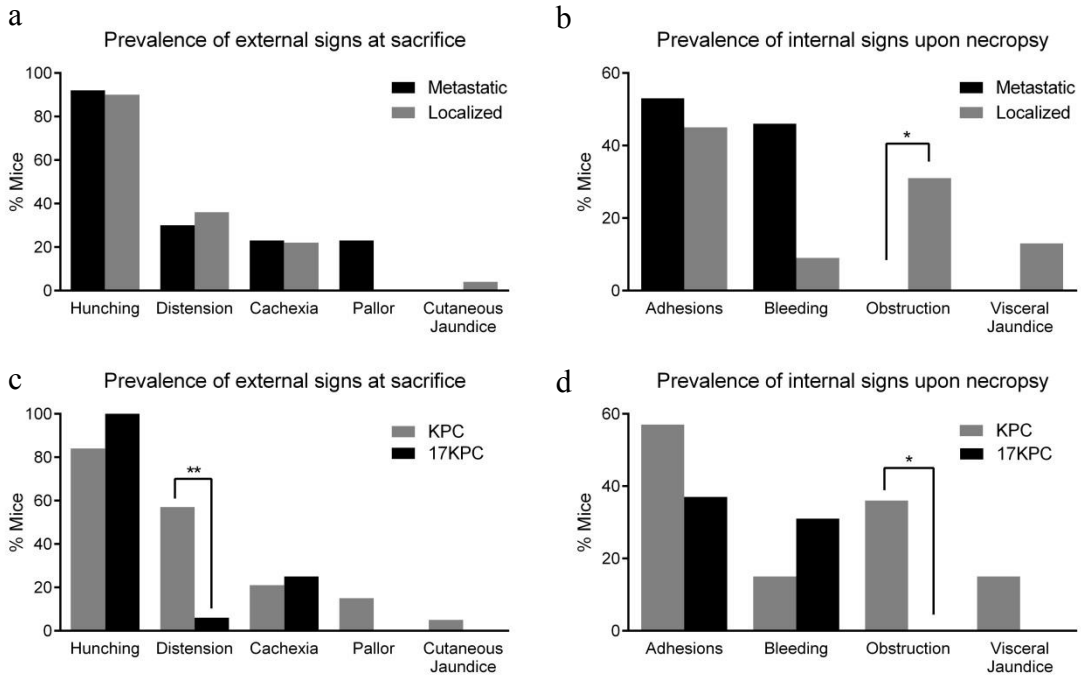


Figure 2.10. Obstruction, a common feature of localized KPC disease, is never observed in 17KPC mice.
(**A,B**) Metastatic disease is more likely to cause internal bleeding and less likely to present with obstruction. (**C**) 17KPC mice were significantly less likely to present with abdominal distension. (**D**) 17KPC tumors were never observed to cause obstruction of the biliary or gastrointestinal systems. Fisher's exact p values: * < 0.05, ** < 0.01

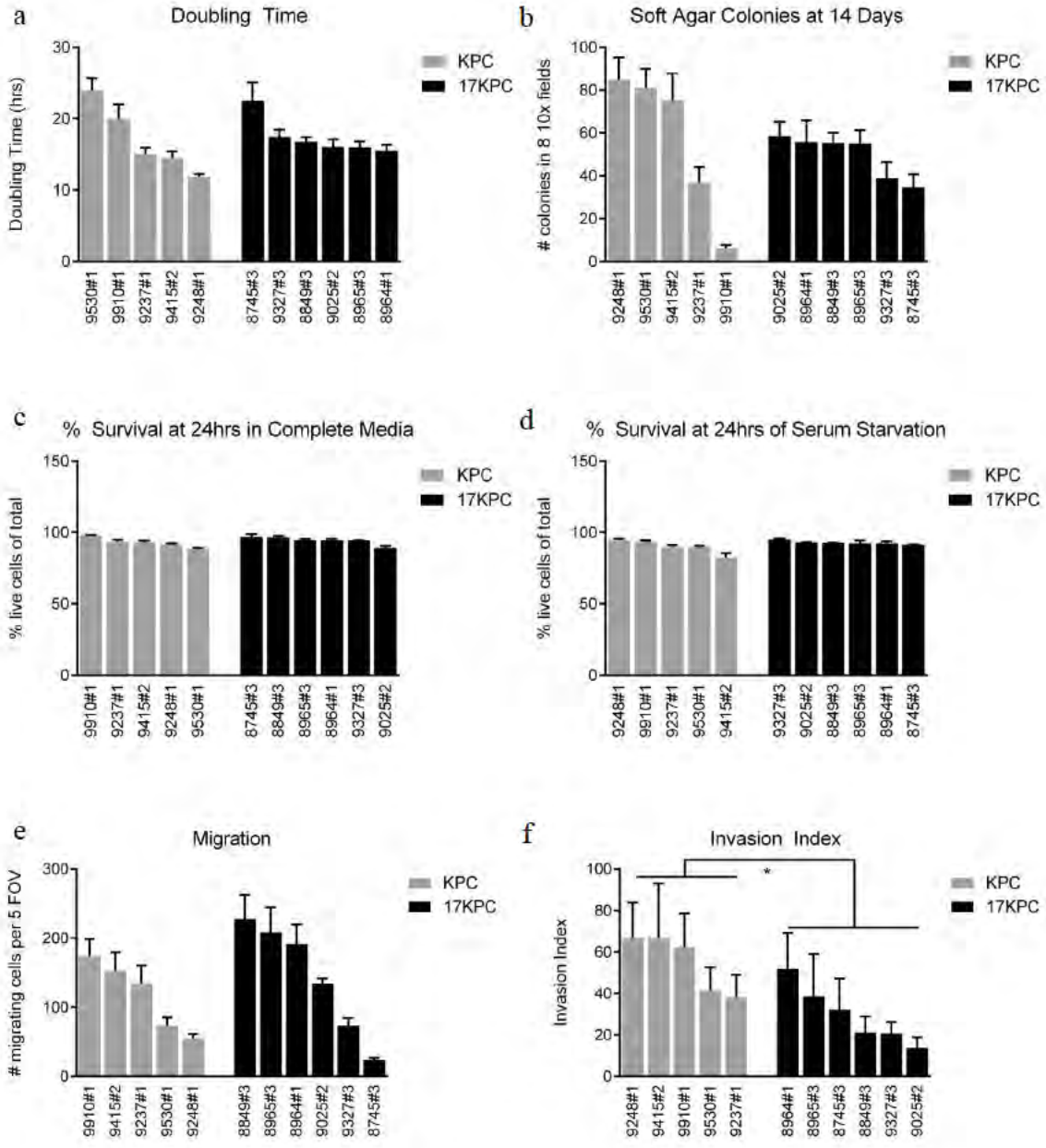


Figure 2.11. 17KPC cell lines are deficient in their invasive capacity *in vitro*.
Proliferative rate (**A**), soft agar colony formation (**B**), baseline apoptotic rate (**C**), sensitivity to serum starvation (**D**), and migration (**E**) show no differences between KPC and 17KPC cell lines.
(**F**) Invasion capacity is significantly reduced in 17KPC cell lines.
Cell line nomenclature is cage #animal (e.g. 9025#2 is the cell line derived from the primary tumor of mouse #2 from cage 9025). FOV = fields of view; p value: * < 0.05

rates in normal growth conditions and in the absence of serum are also not significantly different between KPC and 17KPC cell lines (Figure 2.11c,d). I also performed transwell migration assays to measure each cell line's capacity to move through a porous membrane in response to a serum gradient. A variation of this assay, called an invasion assay, includes a layer of Matrigel coating the membrane, the degradation of which is required prior to movement through the membrane. I find that 17KPC cell lines as a group are significantly disadvantaged in their invasive capacity compared to KPC cell lines (Figure 2.11f). However, no differences exist by genotype in their migration across uncoated membranes (Figure 2.11e). These data agree with my results *in vivo* that 17KPC tumors are less invasive, and they suggest that this is due to a specific defect in 17KPC cancer cells' ability to manipulate extracellular matrix rather than more general defects in cellular locomotion.

Invasion through basement membranes requires two major activities: degradation of the extracellular matrix and attachment-mediated movement through the mobilized substrate, which are coordinated in cancer cells by cellular protrusions called invadopodia (Chen et al. 1985). Invadopodia can be identified by the colocalization of their core protein components actin, cortactin, and paxillin into punctate or rosette-shaped structures (Chen 1989; Linder and Aepfelbacher 2003). To better understand the nature of the invasive defect seen in 17KPC cell lines, we collaborated with Chris Turner's lab at SUNY Upstate to analyze invadopodia formation by immunofluorescence. They found that invadopodia in murine PDAC cell lines take the form of rosettes and that KPC cells lines exhibit higher rates of rosette formation than 17KPC lines (Figure 2.12). They

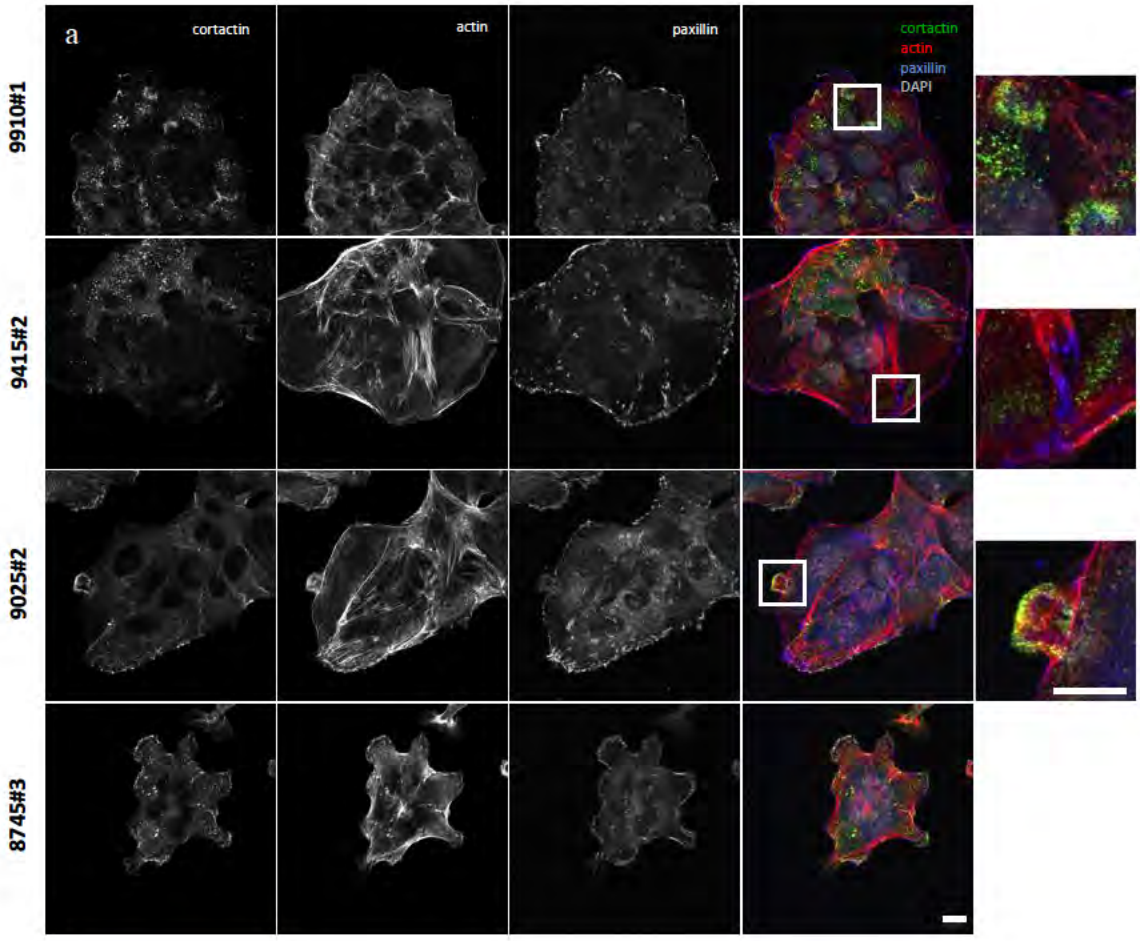
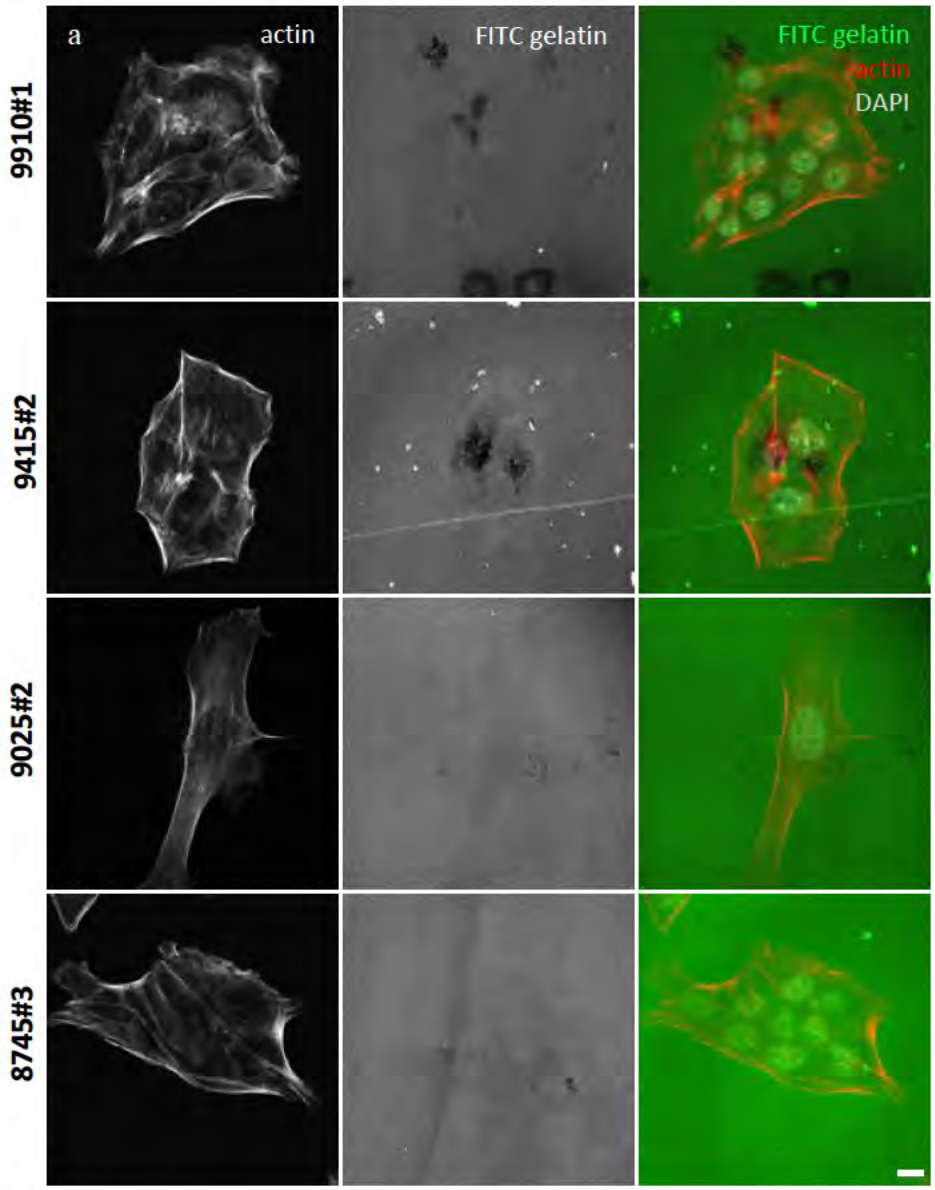


Figure 2.12. 17KPC cell lines form fewer invadopodia rosettes than KPC lines.
Invadopodia were visualized by the colocalization of cortactin, actin and paxillin (**A**). Classic punctate invadopodia are largely absent, and instead invadopodia took the form of rosettes (insets). An extreme example of rosette formation in the 9910#1 cell line is provided (**B**). Invadopodia formation is significantly higher in KPC cell lines than in 17KPC cell lines (**C**). p values: * < 0.05, scale bar = 10um

also performed FITC-conjugated gelatin degradation assays, which demonstrate that KPC lines degrade more substrate than 17KPC lines (Figure 2.12), which aligns with the higher prevalence of invadopodia in these KPC cell lines. These data suggest that loss of *mir-17~92* reduces invadopodia formation.

The *mir-17~92* cluster encodes six miRNAs encompassing four miRNA seed families (Figures 1.4, 2.14), implicating thousands of predicted mRNA targets as downstream effectors of the cluster's invasive program. To aid in my determination of which miRNA families may be most important in the invasive phenotype, I evaluated nine KPC and nine 17KPC cell lines for the expression of miR-17, -18, -19, and -92 family miRNAs from the three cluster paralogs, *mir-17~92*, *mir-106b~25*, and *mir-106a~363*. Quantitative RT-PCR demonstrates that 17KPC cell lines are indeed null for miRNAs from *mir-17~92*, however they retain robust expression from *mir-106b~25* (Figure 2.14a,b). In fact, this locus is sufficient to drive expression of miRNAs for the miR-17 and miR-92 families to levels close to that observed in KPC lines (Figure 2.14d,e). This suggests that differences in the expression of miR-17 and -92 families may not be responsible for the invasive defect of 17KPC cell lines. However, miR-19 family miRNAs are only expressed from the *mir-17~92* and *mir-106a~363* clusters, and 17KPC lines completely lack expression of this miRNA family (Figure 2.14g). Based on the partial residual expression of the miR-17 and miR-92 families, and the generally very low expression of the miR-18 family (Figure 2.14c,f), I hypothesized that loss of the miR-19 family was responsible for the defective invasion of 17KPC cell lines.



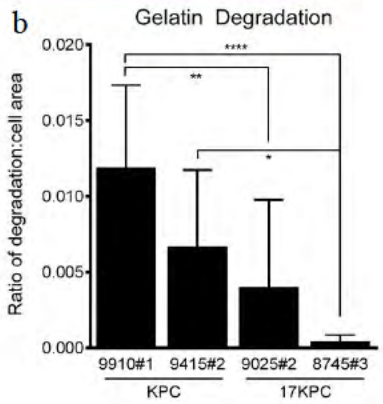


Figure 2.13. Gelatin degradation occurs near invadopodia and is higher in KPC cell lines.
Cells were seeded on FITC-gelatin-coated coverslips for 24 hours, fixed with 4 % paraformaldehyde and stained for actin and cortactin. One pre-selected field along with 8 surrounding random fields were analyzed. Scale bar =10um

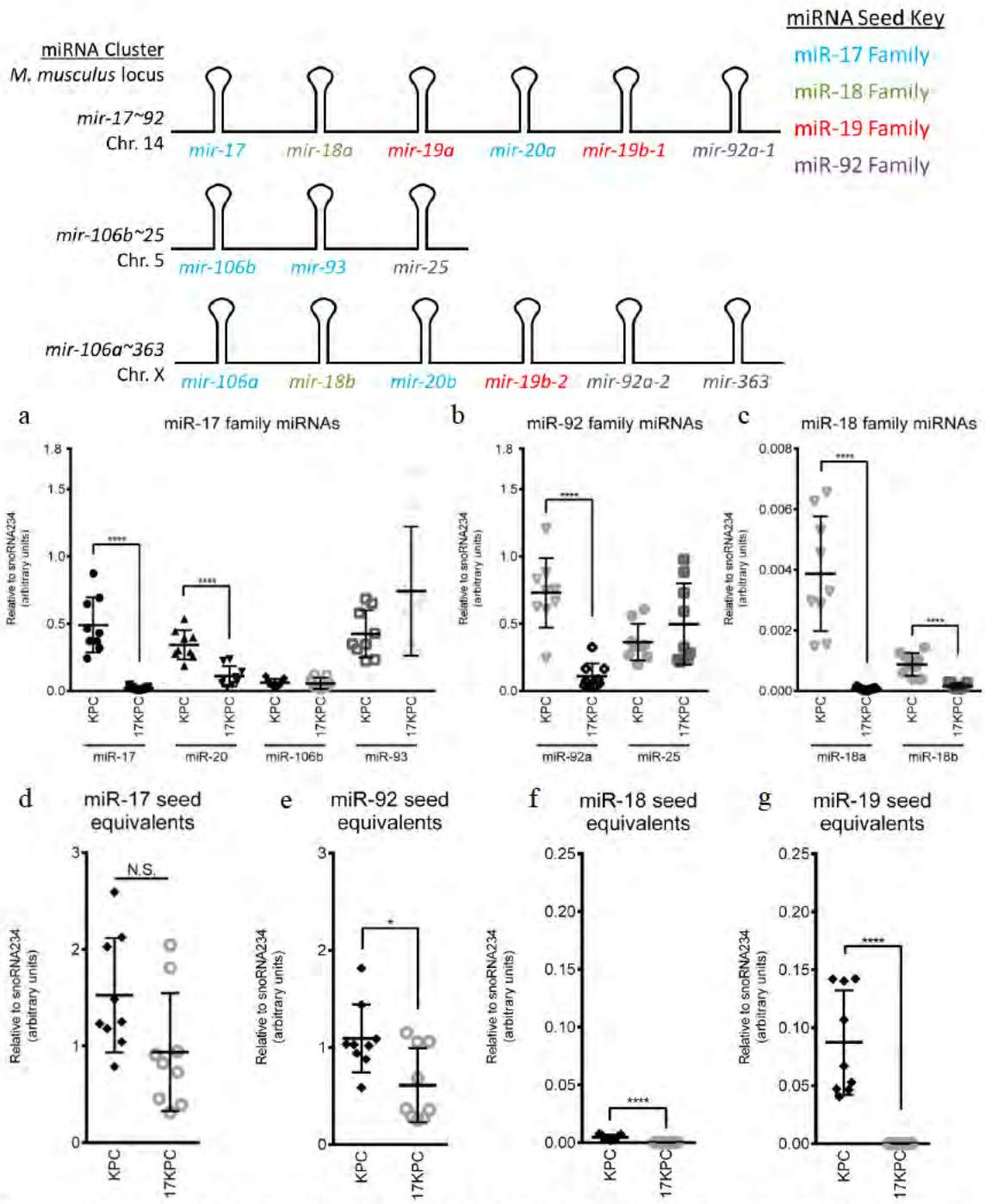


Figure 2.14. miR-19 is robustly expressed in KPC cell lines, but absent in 17KPC cell lines. (**A**-**C**,**G**) Quantitative RT-PCR was performed to measure mature miRNA expression across eighteen cell lines derived from the tumor study. Some miRNAs share sufficient sequence similarity that standard oligonucleotides amplify both species equally (e.g. miR-19a and miR-19b), and therefore not all miRNAs are individually plotted. (**D**-**G**) miRNAs were binned according to family as total molecular equivalents controlled to snoRNA234. p values: * < 0.05, **** < 0.0001

To validate the role for miR-19 family miRNAs in invasion, I utilized antagomirs – short oligonucleotides that bind and inactivate miRNAs – to specifically knock down miR-19 function in KPC lines with high invasive capacity and varying levels of miR-19 expression. I first confirmed miR-19 antagomir activity using a β-galactosidase (β-Gal) reporter assay. The reporter construct contained the β-Gal coding sequence followed by a 3'UTR with multiple miR-19 binding sites that stimulate translational suppression in the presence of miR-19. In a cotransfection experiment, pooled antagomirs against miR-19a and miR-19b enhance reporter activity in the KPC line 9248#1 (Figure 2.15b). However, miR-19 antagomirs cannot enhance reporter activity in 8849#3 cells, which have no endogenous miR-19 to antagonize (Figure 2.15a,b).

Transfected antagomirs against miR-19 effectively suppress KPC cell line invasion in a Matrigel-coated transwell assay (Figure 2.16a). This response inversely correlates to the endogenous expression of miR-19 family miRNAs (Figure 2.16e), suggesting a dosage response. Indeed, the cell line with the highest expression of miR-19, 9415#2, is resistant to antagomirs at a concentration of 50nM, but responds when treated with antagomirs at 100nM (Figure 2.16a). Notably, this effect is specific to invasive capacity, and antagomir treatment does not significantly affect migration ability across uncoated membranes (Figure 2.16c). The human pancreatic cancer cell lines MIA Paca-2 and PANC-1 are also invasive and express very high levels of miR-19 (Figure 2.16f). Treating these human cell lines with miR-19 antagomirs reduces their invasive capacity without affecting baseline migration (Figure 2.16b,d).

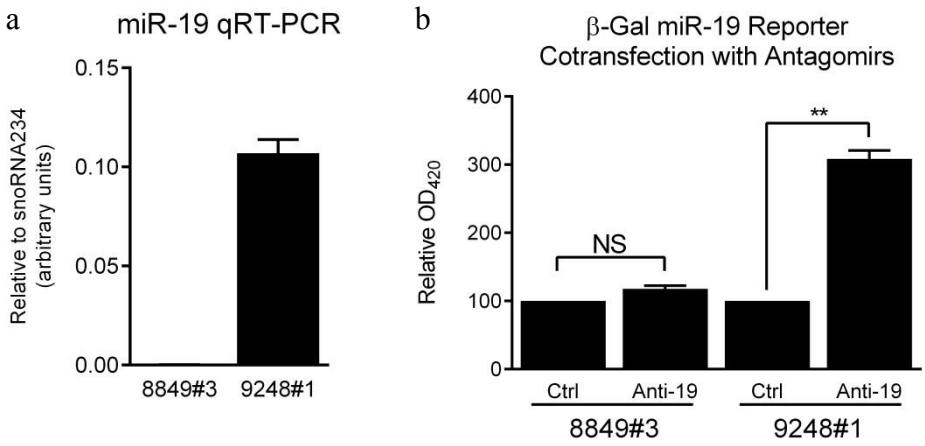


Figure 2.15. A β-galactosidase reporter assay confirms that miR-19 antagomirs inhibit the activity of endogenous miR-19.
A β-galactosidase reporter plasmid encoding a specially designed 3'UTR with miR-19 binding sites was cotransfected into cells with either control or miR-19 antagomirs at 50nM. The 8849#3 cell line completely lacks miR-19 but the 9248#1 cell line expresses high levels of miR-19 (**A**). Relative density of the colorimetric reaction is plotted with control antagomir-treated samples set to 100 (**B**). p values: ** < 0.01

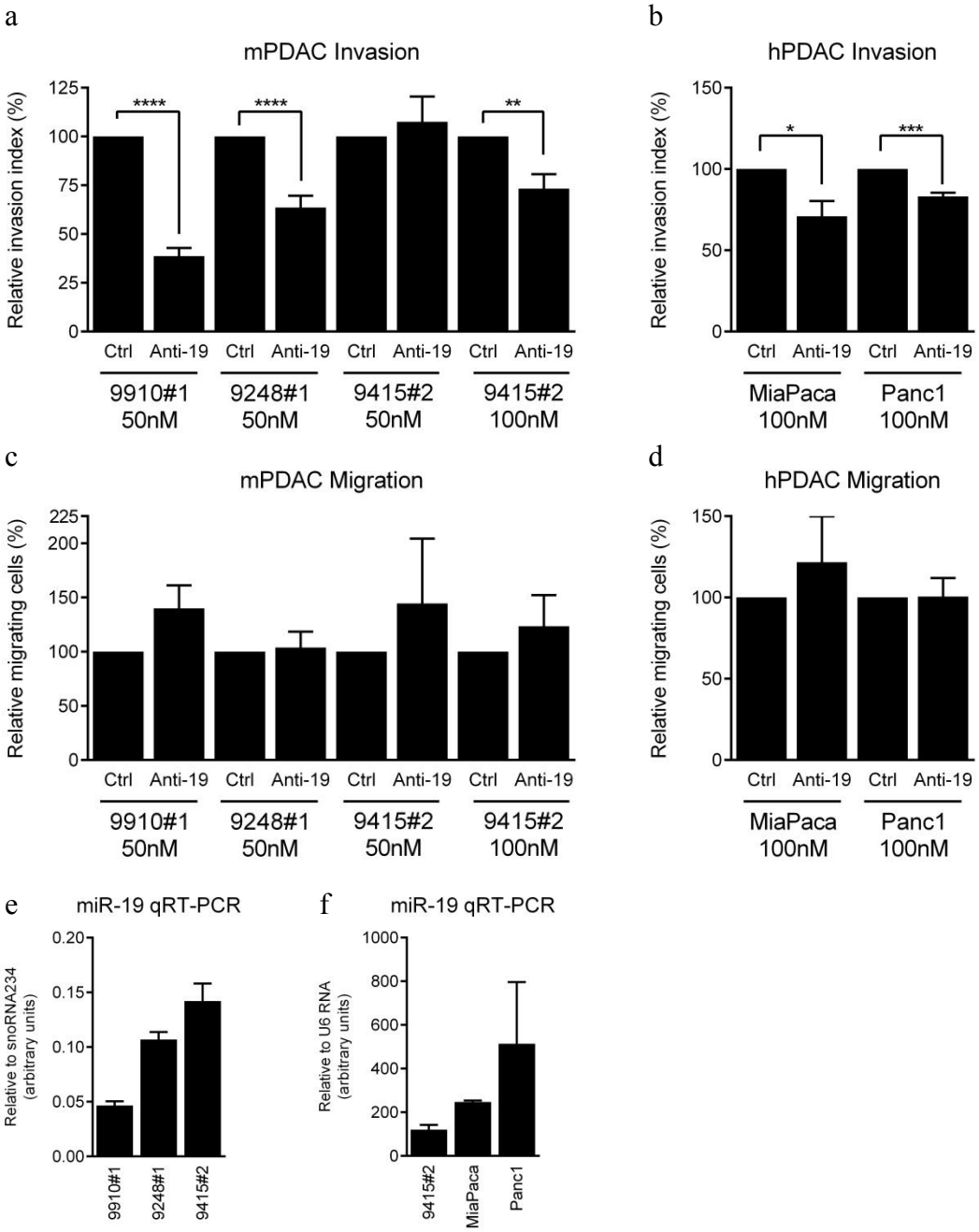


Figure 2.16. PDAC cell line invasiveness is suppressed by miR-19 antagomirs.
(**A,B**) Transwell invasion was measured for cell lines transfected with control or miR-19 antagomirs. (**C,D**) Baseline migration across uncoated membranes served as an internal control. miR-19 expression levels are shown relative to the murine-specific control snoRNA234 (**E**) or the cross-species compatible U6 RNA (**F**). p values: * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001

Discussion

Loss of mir-17~92 causes PanIN loss and acinar regeneration

I demonstrate that deletion of the *mir-17~92* cluster in the KC mouse model results in a significant reduction in the PanIN burden of aged animals. The distribution of PanIN grades in 17KC mice remains consistent with that of aged KC animals, suggesting that progression is unaffected by loss of the *mir-17~92* cluster. Moreover, the difference in PanIN prevalence between KC and 17KC mice is less for younger animals than for older animals, suggesting that although PanIN formation may be reduced in 17KC animals, the predominant effect of *mir-17~92* loss is to promote PanIN regression over time. Only a single study has shown that *mir-17~92*-related miRNAs are upregulated in human PanINs (Yu et al. 2012a), and in that study these miRNAs did not have the strongest association with PanIN grade among the many miRNAs profiled. Therefore the absence of progression-related differences between KC and 17KC mice agrees with the published human data and does not support a direct role for the *mir-17~92* cluster in PanIN progression.

Based on the published role of *mir-17~92* in other tumor contexts as a promoter of cell cycle progression and as an antagonist of apoptosis, I initially hypothesized that the gradual loss of PanINs in 17KC animals was due to a shift in the balance of apoptosis and proliferation within these lesions. IHC staining for ki67 and CC3 clearly show no differences between KC and 17KC PanINs at either age, suggesting that the loss of PanINs in 17KC mice is not due to enhanced cell death and must be occurring by some other means. Moreover, aged 17KC pancreata not only lose PanIN content, but also gain

acinar content, suggesting that a regenerative process is occurring in parallel with lesion regression.

Acinar-to-ductal metaplasia is a transformative event that converts the normal tissue of the pancreas into PanINs and contributes significantly to the neoplastic process in mouse models of pancreatic cancer (Kopp et al. 2012). Recent evidence suggests that this process is reversible in the pancreas, and mechanisms exist by which PanINs can regress back into healthy acinar tissue. Using an inducible-$KRAS^{G12D}$ model, wherein $KRAS^{G12D}$ expression is controlled by doxycycline dosing, one research group has demonstrated that withdrawal of KRAS signaling in established PanINs results in the regression of these lesions and the recovery of healthy acinar tissue (Collins et al. 2012a). The authors of that study also observe the remodeling and loss of the desmoplastic stroma around PanINs upon $KRAS^{G12D}$ inactivation, and these changes are preceded by a loss of phosphorylated ERK in the PanIN lesions. In a critical follow-up study, the authors demonstrate that small molecule inhibition of ERK in the setting of continued $KRAS^{G12D}$ signaling is sufficient to cause PanIN regression and acinar regeneration (Collins et al. 2014). These data not only provide proof-of-principle that mature PanIN lesions can regress and be replaced by healthy acinar tissue, but they also demonstrate that this can occur in the continuing presence of $KRAS^{G12D}$ signaling.

Within my own genetic model, $KRAS^{G12D}$ remains active in the pancreas throughout the lifetime of the animal. Critically, this suggests that whatever molecular differences exist between KC and 17KC PanINs are sufficient to overcome continuing $KRAS^{G12D}$ signaling to promote PanIN loss over time. I am able to demonstrate that loss

of *mir-17~92* results in reduced phosphorylated ERK in the PanIN lesions of 17KC animals, but does not increase cell death, as measured by CC3 staining. This finding aligns well with the published work of Collins *et al*, who also showed that inhibition of ERK signaling does not stimulate apoptosis, and instead appears to promote the redifferentiation of PanINs into acinar cells (Collins et al. 2014). Together, these data suggest that the loss of PanINs in 17KC animals may indeed be occurring by direct regression back into acinar tissue, thereby simultaneously reducing PanIN burden while increasing acinar content within 17KC pancreata. Importantly, phosphorylated ERK is not completely lost in 17KC PanINs, which could explain why PanIN regression was incomplete at nine months in my study, whereas small molecule inhibition of ERK or withdrawal of $KRAS^{G12D}$ signaling are capable of much more rapid changes (Collins et al. 2014).

The results of my *in vivo* study of PanIN formation clearly demonstrate that loss of the *mir-17~92* cluster promotes PanIN loss over time, yet in the tumor study, where I additionally delete one copy of *Tp53*, tumor formation and overall survival are not impacted. Moreover, pancreas tissue adjacent to tumors harvested in this study show qualitatively similar and very high rates of PanIN formation (data not shown). This suggests that the tumor suppressive effect of reduced *mir-17~92* expression is overcome by the pro-tumorigenic effect of *Tp53* loss in this model. A recent paper showed that the TP53 effector protein CDKN1A (aka $p21^{WAF1}$) is highly upregulated in acinar cells downstream of TP53 activation during acute pancreatitis, but it is strongly downregulated in ADM lesions (Grabliauskaite et al. 2015). In their mouse model, complete loss of

CDKN1A accelerated cerulean-induced ADM, suggesting that CDKN1A protects the acinar differentiation state in the setting of pancreatitis. Interestingly, absence of CDKN1A did not impair pancreas regeneration after withdrawal of the inflammatory stimulus (Grabliauskaite et al. 2015). Together, these data suggest that an intact TP53 response helps to maintain the acinar differentiation state and that its downregulation promotes ADM. Thus, 17KPC animals likely experience enhanced progression into ADM compared to 17KC animals due to the genetic impairment of *Tp53* function and a subsequent reduction in CDKN1A activity. Given the long timeline of PanIN loss and acinar recovery observed in 17KC mice, it is conceivable that the increased exuberance of ADM inherent to the 17KPC model simply overbalances the regenerative effect of *mir-17~92* loss, resulting in no appreciable difference in the PanIN or tumor formation of 17KPC mice compared to KPC mice. Interestingly, CDKN1A is a validated target of the miR-17 family (Fontana et al. 2008; Hong et al. 2010), suggesting a direct role for CDKN1A in the redifferentiation phenotype of 17KC animals. Although the work by Grabliauskaite and colleagues suggests that CDKN1A does not promote redifferentiation, higher levels of acinar CDKN1A in 17KC mice may contribute to locking redifferentiated cells into an acinar state and resisting reentry into ADM. A necessary experiment will be to return to the PanIN study and stain for CDKN1A to see if levels within acinar cells are higher in 17KC versus KC mice.

Extending the idea of balance between ADM and redifferentiation, another interesting question is whether cluster-dependent differences in PanIN formation would be even greater in an adult-induced model, such as the published iKras model (Collins et

al. 2012a). It is known that activating $KRAS^{G12D}$ in the adult pancreas, as opposed to with development, creates a weaker phenotype that additionally requires pancreatitis to generate PanINs (Guerra et al. 2007). This suggests that mature pancreatic tissues are more resistant to $KRAS^{G12D}$-driven ADM. It is possible that the resistance of 17KC mice to PanIN formation is overcome by the aggressive metaplasia that occurs with activation of $KRAS^{G12D}$ during development, and that adult tissues might be even more responsive to regenerative pressure in the absence of *mir-17~92*. Indeed, perhaps it is the resistance of adult tissues to ADM that preserves acinar cells in 17KC mice, whereby developmentally driven PanINs lacking *mir-17~92* redifferentiate in adulthood and find themselves now resistant to the effects of $KRAS^{G12D}$, subsequently persisting as acinar cells.

mir-17~92 and transforming growth factor-β

Activation of TGF-β is a watershed event in pancreatic tumorigenesis because of its prevalence in ADM, which drives the majority of PanIN formation. I have personally observed that TGF-β signaling is strongly increased in ADM lesions but largely absent from early stage PanIN lesions, and this signaling may be stronger in 17KC animals (Figure 2.4i-l). TGF-β is a known driver of EMT in a variety of contexts, and it is confirmed to have this activity in cultured pancreatic duct cells (Shin et al. 2011). Besides stimulating EMT, TGF-β signaling also triggers growth arrest by the induction of CDKN1A (Petrocca et al. 2008b) and has been suggested to antagonize ERK signaling by a SMAD-independent mechanism (Giehl et al. 2000). Thus TGF-β simultaneously

stimulates invasiveness and growth inhibition through several downstream effectors. It is well known that this tumor-suppressive role of TGF-β is bypassed in later stages of disease by the mutation of SMAD4 (Bardeesy et al. 2006b; Ijichi et al. 2006; Izeradjene et al. 2007).

Published literature on the oncogenic role of *mir-17~92* and its paralogs characterizes them as potent suppressors of TGF-β signaling, particularly by suppression of CDKN1A (Petrocca et al. 2008a; Petrocca et al. 2008b), SMAD4 (Fuziwara and Kimura 2014), and TGFBR2 (Volinia et al. 2006). Therefore, upregulation of the *mir-17~92* cluster is predicted to bypass TGF-β-driven growth arrest during ADM. This may suggest that growth arrest occurs at higher rates in the ADM lesions of 17KC animals. Perhaps this contributes to reduced PanIN burden in these animals. Interestingly, it has been shown in $BRAF^{V600E}$-driven thyroid cancer that *mir-17~92* is upregulated by the NOTCH effector HES1 and suppresses SMAD4 via miR-19 to drive proliferation (Fuziwara and Kimura 2014). Similarly, I observe prominent nuclear HES1 staining in ADM and PanIN lesions (Figure 2.4e-h), suggesting that NOTCH1-driven *mir-17~92* may act to suppress SMAD4 in KC animals, thus increasing the number of cells that successfully bypass cell cycle arrest. If this is the case, an interesting question will then be whether arrested cells persist in a mesenchymal state, undergo mesenchymal-to-epithelial transition (MET) to achieve an arrested PanIN state, or regress to an acinar differentiation state. The final identity of these cells could have major ramifications on disease progression, and could differ between KC and 17KC animals.

mir-17~92 potentiation of ERK signaling - speculation

As for how *mir-17~92* might normally act to enhance $KRAS^{G12D}$ signaling through ERK, that remains an important question. Ultimately, an unbiased approach to identifying miRNA targets would compare the results of prediction algorithms with RNA sequencing data to identify significantly downregulated transcripts that are also predicted targets of *mir-17~92* cluster. Without RNA sequencing data there is a significant lack of support for choosing any particular predicted target as a likely player in the PanIN phenotype. However, in the absence of that data, speculation may suggest interesting relationships.

Dual-specificity phosphatases (DUSPs) are well known to regulate the phosphorylation status of numerous MAPK family proteins, including ERK, JNK, and p38 (Rios et al. 2014). Several DUSPs have been implicated in pancreatic cancer progression and survival via modulation of MAPK signaling, including DUSP1 (Liu et al. 2014), DUSP6 (Furukawa et al. 1998; Furukawa et al. 2005; Xu et al. 2005), and DUSP10 (He et al. 2014a). Because each DUSP simultaneously regulates multiple MAPK proteins, predicting their activity in a given cellular context is impossible, but validated relationships from the literature and predicted miRNA-mRNA interactions suggest a mechanism for *mir-17~92*'s regulation of ERK signaling, which is explored in the following discussion and summarized graphically in Figure 2.17.

DUSP10 is a validated target of miR-92, and its suppression is required for JNK signaling and the proliferation of human pancreatic cancer cell lines (He et al. 2014a). The miR-17 family has been validated to suppress DUSP2 to drive ERK phosphorylation

in endometrial stromal cells (Lin et al. 2012). DUSP2 and DUSP7 preferentially suppress ERK activity over JNK or p38 MAPKs (Keyse 2008), and DUSP7 is a predicted target of both the miR-17 and miR-19 families by TargetScan analysis. Interestingly, MYC has been shown to regulate DUSP2 and DUSP7 in pluripotent stem cells (Chappell et al. 2013), and MYC itself drives transcription of *mir-17~92* (O'Donnell et al. 2005), suggesting that *mir-17~92* may be a necessary facilitator of ERK signaling downstream of MYC in cancer contexts. An intriguing study showed that DUSP10, which preferentially dephosphorylates JNK and p38, also inhibits ERK activity in a phosphatase-independent manner by binding and sequestering phosphorylated ERK in the cytoplasm and preventing its activation of downstream targets or transcription (Nomura et al. 2012). Lastly, a tantalizing study showed that TGF-β induces ERK dephosphorylation in pancreatic cancer cells through the SMAD4-independent activity of an unidentified phosphatase (Giehl et al. 2000). Thus, DUSP2, DUSP7, and DUSP10 represent a cancer-relevant anti-ERK regulatory network that may be potently and coordinately suppressed by multiple miRNAs of the *mir-17~92* cluster (Figure 2.17). In particular, this network integrates signaling from key players in KRAS-driven pancreatic cancer, including KRAS signaling, TGF-β signaling, and MYC. Confirmation of these relationships will be critical follow-up experiments to the body of work presented in this chapter.

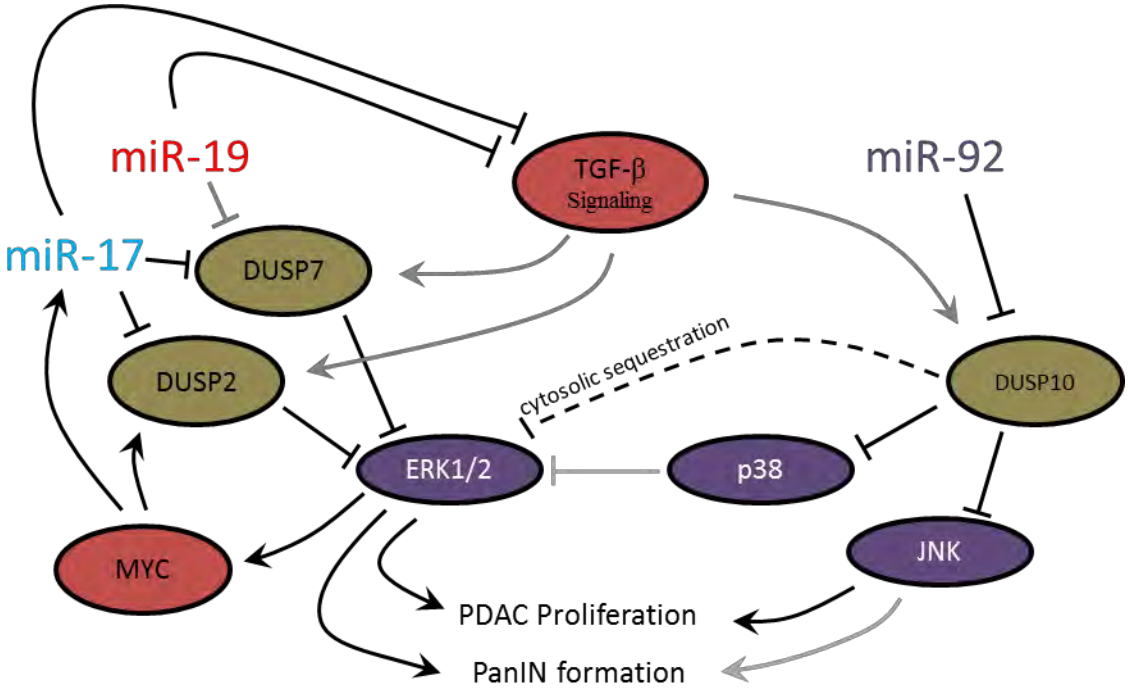


Figure 2.17. A potential mechanism for *mir-17~92* promotion of ERK activity.
Grey bars are predicted miRNA-mRNA target relationships or uncertain biological activities of proteins. Black bars are validated relationships from the literature. DUSP10 regulation of ERK is dashed to denote phosphorylation-independent suppression. MYC drives the expression of all members of *mir-17~92*, not just miR-17.

miR-19 promotes pancreatic cancer invasion

Promoting tumor progression by heterozygous deletion of *Tp53* creates tumors in both KPC and 17KPC mice. Tumors across these two genotypes are similar in size, pathological subtype, metastatic capability, proliferation, and apoptosis. The singular difference attributable to loss of the *mir-17~92* cluster appears to be a reduced invasiveness of primary tumors *in vivo*. I determined that tumors were less invasive *in vivo* by stratifying mice into metastatic or localized groups and observing that mice which were sacrificed for severe illness arising from localized disease survived longer and with larger tumors in the absence of *mir-17~92*. The rationale for this stratification is that metastasis and local invasion may represent biologically distinct processes, whereby entry into the circulation may present different and unique challenges compared to extended invasion through dense desmoplastic stroma to reach adjacent organs. This is supported by the observation that 17KPC tumors are not significantly different from KPC tumors in their ability to metastasize to the liver or peritoneum. Admittedly, histological evidence of invasion appears with equal frequency between the two groups, suggesting that invasion is eventually achieved and represents the common endpoint of mice dying from localized disease; however 17KPC tumors grow larger and require more time in order to reach equivalently invasive stages compared to KPC tumors.

These findings were nicely paralleled *in vitro* by my observations that KPC and 17KPC cell lines performed equivalently in growth and survival measurements, but 17KPC cell lines were particularly deficient in their invasive capabilities in a transwell assay. This correlates to a reduction in the presence of invadopodia, which are critical

structures for the execution of invasion through extracellular matrix. It is also consistent that gelatin degradation generally aligns with the prevalence of invadopodia formation between cell lines. For example, 9025#2 forms more invadopodia than 8745#3, and is also more effective at degrading gelatin.

Interestingly, however, 9025#2 is less invasive than 8745#3 in a transwell assay. This might suggest at first that although 9025#2 cells are more capable of degradation, they may be deficient in chemotaxis, but the migration assay confirms that they are migration-competent and, indeed, perform far better than 8745#3 cells. This highlights the complex and coordinated nature of cancer cell invasion. For instance, I have not characterized whether 17KPC or KPC cell lines exhibit differences in their adhesion to particular substrates. Specifically, 9025#2 cells may be more migratory than 8745#3 cells in an uncoated transwell assay, but they may be less capable of moving on Matrigel components, which would render their increased invadopodia and gelatin degradation advantage irrelevant. Moreover, these cell lines still exhibit predominantly epithelial morphology as well-differentiated ductal adenocarcinomas, and the dynamics of cell separation or collective migration have not been assessed.

Although I observe significant genotype differences in transwell invasion, invadopodia, and gelatin degradation, the specific contributions of these or other deficits within individual 17KPC cell lines dictate their net invasive capacity. While it seems clear that 17KPC tumors and cell lines are invasion defective, I must also acknowledge that there may be untested factors that are different by genotype and are also critical to 17KPC biology.

My finding that invasion can be suppressed with just miR-19 antagomirs greatly simplifies the range of potential targets that could mediate and inform our understanding of the 17KPC invasion defect. Critically, I have not yet determined whether invadopodia formation is suppressed by treatment with miR-19 antagomirs, although these experiments will be performed in the near future in collaboration with the Turner lab at SUNY Upstate. Moreover, I plan to send cell line RNA for sequencing to determine which transcripts are significantly downregulated in KPC cell lines compared to 17KPC cell lines, in order to facilitate the identification of relevant miR-19 targets. Awaiting these data, we can theorize as to the potential mechanisms of *mir-17~92*-driven invasiveness by considering known biology governing invadopodia.

SRC is a major regulator of invadopodia rosette formation (Oikawa et al. 2008). Several components of invadopodia are targets of SRC phosphorylation, including cortactin, CAS, ASAP, and paxillin (Thomas et al. 1995; Brabek et al. 2004; Bharti et al. 2007). Since its initial characterization as a SRC substrate, paxillin has been variously shown to be phosphorylated downstream of EGF (Hetey et al. 2005), TGF-β (Tumbarello and Turner 2007), and AKT (Zhao et al. 2010), all of which are pathways relevant to pancreatic carcinogenesis. Phosphorylation is responsible for paxillin's localization to peripheral actin and focal adhesions (Nakamura et al. 2000), where it acts to scaffold proteins required for invadopodia assembly (Deakin et al. 2012). Interestingly, it has recently been shown that MAP3K2 can trigger the ubiquitination of paxillin and force its cytoplasmic redistribution away from focal adhesions (Ameka et al. 2014). MAP3K2 is predicted to be powerfully and coordinately regulated by the *mir-17~92* cluster: its

3'UTR contains five predicted binding sites for the miR-17 family (one 8mer site, three 7mer-m8 sites, and one 7mer-A1site) and two sites for miR-19 (one 8mer and one 7mer-m8). This suggests that MAP3K2's expression and activity may be higher in 17KPC cell lines as a result of *mir-17~92* loss, potentially promoting paxillin delocalization and inhibiting invadopodia stability. Based on immunofluorescence in the cell lines thus far analyzed, it is not clear that paxillin localization is significantly different between KPC and 17KPC cell lines, but this will need to be quantified to rule out slight, but significant differences not appreciable by qualitative assessment.

Regulators of Rho GTPase activity, including Rho GAPs and GEFs, also present clear opportunities for miRNA regulation of cytoskeletal dynamics during invasion. DLC1 is a Rho GAP that inhibits tumor growth and invasion (Healy et al. 2008; Zhou et al. 2008). DLC1 has even been shown to regulate paxillin function in focal adhesions (Kaushik et al. 2014). DLC1 is a predicted target of miR-19 (one 8mer) but is not predicted to be targeted by any other members of the *mir-17~92* cluster, opening up the possibility that this Rho GAP could be a miR-19-specific target responsible for invasive differences in 17KPC cell lines. Given that paxillin is phosphorylated downstream of AKT (Zhao et al. 2010), another obvious candidate gene to interrogate will be PTEN, which antagonizes AKT activity, is a validated target of miR-19, and is a key component of the oncogenic activity of the *mir-17~92* cluster in lymphoma (Olive et al. 2009).

PanIN formation is a reversible process

Previous to the work presented in this chapter, the only documented way to achieve PanIN regression and pancreas regeneration after oncogenic insult was by direct inhibition of $KRAS^{G12D}$ or ERK signaling. Because $KRAS^{G12D}$ inhibition is clinically ineffective, and small molecule MEK inhibitors carry toxicity (Welsh and Corrie 2015), the implication that the *mir-17~92* cluster controls ERK signaling opens up opportunities to explore alternative approaches to the medical treatment of precursor lesions. If the critical intermediary of *mir-17~92*-mediated ERK dephosphorylation is a dual-specificity phosphatase (DUSP), it could present an opportunity to treat PanIN lesions medically with gene therapy. Specifically, a pancreas-targeted adeno-associated virus (AAV) could be used to deliver a miRNA-resistant version of the critical DUSP to PanIN lesions. Such pancreatic-duct targeted AAVs are already in development for the treatment of cystic fibrosis in pigs and are being tested in mice (Guo et al. 2013; Griffin et al. 2014). Alternatively, since ERK activity is critical to PanIN maintenance, another approach could be to use such pancreas-tropic AAVs to deliver shRNAs targeting upstream activators of ERK, or ERK itself.

Such methods will have to await the final development of the requisite AAV tools, but they could be our first attempt at non-surgical prophylactic therapy in healthy at-risk individuals. In particular, at-risk individuals with a family history of pancreatic cancer could be put on a gene therapy regimen designed to regress a significant bulk of their PanIN lesions back to healthy exocrine tissue. Given the long timeline of pancreatic

carcinogenesis, a histological reset such as this could result in tremendous gains of life, even if it was not curative. Moreover, gene therapy has the potential to be radically less toxic and more efficacious compared to prophylactic inhibitor therapy, given that it could be delivered in a tissue-specific manner and in a few doses, as opposed to chemical inhibitors, which must be dosed continuously and systemically. I am particularly drawn to the idea that PanINs can now be medically targeted using such a prophylactic approach, because these lesions, particularly PanIN-1, possess far more predictable biology compared to later stages of disease where severe disruption of gene expression and tumor evolution render generalized therapy ineffective.

The diversity of late-stage tumor biology is the reason why targeted therapy of invasive or metastatic disease is an entirely separate and far more difficult problem. Pancreatic cancer invasiveness is a critical barrier to curative therapy for patients, because tumors frequently invade nearby tissues before causing symptoms that trigger detection. In particular, the mechanism of very early invasiveness, seen around ADM lesions and early stage PanINs, is an even greater mystery for its seeming disregard for the classical paradigm of carcinoma progression (Rhim et al. 2012). According to the work of Rhim and colleagues, ADM allows epithelial-derived cells to disseminate into the stroma and even into the circulation where they can seed and survive in the liver long before the advent of carcinoma. The presence of circulating pancreatic cells in humans with benign precursor lesions has since been confirmed (Rhim et al. 2014b). This is starkly different from other carcinomas like colon cancer that progress through benign hyperplastic stages to ductal carcinoma *in situ* prior to invading. Indeed, this suggests a

unique biology for pancreas cancer, where a significant number of cancer cells may exist outside the pancreas even in those patients with localized disease. This makes a great deal of sense with the clinical data that clean surgical resection offers only a 25% chance of surviving five years: clearly pancreatic tumors are not bound by their surgical margins. However, this is merely conjecture, as the malignant potential of these early-invasive cells or their effects on tumor progression have not been demonstrated. Suffice to say that our ability to use chemotherapy and radiation to combat metastatic disease is severely limited by the biological diversity of metastatic cells, and our best chance at clinical benefit in the near future may be interventions against PanINs.

The interplay of *mir-17~92* and TGF-β signaling in the context of oncogenic KRAS is complex, but suggests a link between early metaplastic events and the aggressiveness of late stage tumor progression. Importantly, we now appreciate that epithelially-derived cells expressing $KRAS^{G12D}$ migrate into the stroma and can be found near ADM and PanIN lesions (Rhim et al. 2012). These cells possess myofibroblast-like morphology and likely play active roles in the biology of the investing stroma around PanINs and tumors. It follows that the stroma around KPC and 17KPC tumors therefore likely differs due to the genetic differences in these EMT-derived cells. Specifically, *mir-17~92*-deficient cells that maintain a mesenchymal state would be predicted to respond more strongly to TGF-β signaling. Indeed, it has been shown that miR-19 blunts the TGF-β response of hepatic stellate cells and reduces their expression of αSMA and type I collagen (Lakner et al. 2012). Therefore cells derived from early EMT events in 17KPC mice would contribute to the formation of a denser stroma because of enhanced collagen

production compared to similar cells in the KPC group. Subsequently, tumors formed in the 17KPC context would find themselves within a denser stroma compared to KPC tumors, which would impair their ability to invade adjacent tissues. This conceptual model agrees with published work demonstrating that the reactive stroma around pancreatic cancer probably acts to limit tumor aggressiveness (Rhim et al. 2014a). Therefore early metaplastic events and the genetics governing them may exert a stronger influence over the clinical progression of late stage disease than previously appreciated, and this emphasizes the need to understand and if possible intervene in these early stages.

PanIN regression and ADM dynamics – future experiments

The finding that PanINs can regress under certain physiological conditions, including *mir-17~92* loss, and subsequently be replaced by healthy exocrine tissue, suggests that PanINs may revert to acinar tissue directly through an event similar but opposite to ADM. However, histological stains, which capture a single moment in time, may be unable to distinguish such a redifferentiation event from ADM. Confirmation of acinar redifferentiation from PanINs would be genetically possible by the addition of a secondary recombination system, such as flippase recombinase (Flp) (Lee et al. 2012a), to complement the Cre-lox system of the KC model.

For instance, a fusion transgene composed of the promoter region of *Muc1* and the coding sequence of Flp (*Muc1-Flp*) would be predicted to activate Flp expression in cells that express *Muc1*: namely, early PanIN-1 lesions but not normal ducts or acini (Gold et al. 2007). The Flp recombinase recognizes flippase recognition target (FRT)

sites in DNA and deletes the intervening genetic sequence in a mechanism similar to that of Cre-lox (McLeod et al. 1986). Therefore a *Muc1-Flp* allele could be combined with a gene encoding red fluorescent protein (RFP) with an upstream FRT-stop-FRT cassette, similar to the *LSL-Kras*^{G12D} allele. This 'FSF-RFP' allele could itself be driven from the ubiquitously expressed *Rosa26* locus, which has been used extensively to drive gene expression in mouse models (Casola 2010). Importantly, a similar allele has been described already and generates no background fluorescence (Rhim et al. 2012). The full genetic model for PanIN lineage tracing would therefore consist of *LSL-Kras*^{G12D}*, Ptf1a-Cre, Rosa26-FSF-RFP, Muc1-Flp*, with or without *mir-17~92*$^{flox/flox}$ (suggesting the shorthand KCRF and 17KCRF). These KCRF mice would be expected to generate PanINs similarly to the well-described KC model, but would additionally activate RFP upon metaplasia downstream of *Muc1* activation. Subsequently, *Rosa26-RFP* would remain constitutively active in all derived cells, including any differentiated acinar cells that derived from mature PanINs.

miRNAs are thought to provide generally subtle regulation of gene expression, with the average miRNA-mRNA interaction generating less than a two-fold reduction in protein levels (Baek et al. 2008). This suggests that the regeneration phenotype of 17KC mice reflects the enhancement of an underlying process inherent to KC mice, rather than the acquisition of a completely new ability. It would therefore not be surprising to discover a low basal rate of PanIN regression in young KCRF mice in the absence of ERK inhibition or *mir-17~92* loss. My prediction is that this reversion is normally

outbalanced by full $KRAS^{G12D}$ signaling through ERK such that acinar regeneration is not observed by measuring total acinar content over time in KC mice.

An additional benefit of the KCRF model is that it could be used to track invasive or metastatic cells derived from PanINs and tumors, allowing a higher resolution view of the impact of *mir-17~92* deletion on tumor progression. KCRF mice could be bred to incorporate the $Tp53^{flox}$ allele ('KPCRF'), which would not only provide a beneficial tool for tracking metastatic cells, but also provide an opportunity to use *in vivo* imaging to monitor tumor progression and spread. Importantly, both the *Rosa26-FSF-RFP* allele and the *Muc1-Flp* transgene can theoretically be bred to homozygosity, which is an important advantage in mouse genetics where Mendelian inheritance can make the generation of littermate pairs inefficient. Altogether, the KCRF model presents an exciting opportunity to confirm the reversibility of 17KC PanINs and more deeply explore the nature of the 17KPC invasion deficiency.

Future *in vivo* experiments exploring the role of *mir-17~92* in pancreatic tumorigenesis should additionally delete the *mir-106b~25* cluster now that we understand it to be so highly upregulated in murine pancreatic tumors. The work I present in this chapter – particularly the demonstration the miR-19 antagomirs suppress invasion – supports a miR-19-centric model of pancreatic cancer invasion; yet I have not determined whether other miRNAs of the cluster play a role in the invasive phenotype. Expression from the *mir-106b~25* cluster may have prevented me from observing defects related to miR-17 or miR-92 family activity and is therefore a confounding variable in the above experiments. Although I believe I have arrived at meaningful interpretations of the

available data, I would be eager to see the results of dual cluster deletion on PanIN formation and tumor invasion.

At the commencement of these studies, no conditional allele for *mir-106b~25* existed. Such an allele would be ideal, but germline deletion of *mir-106b~25* is, in fact, developmentally tolerated (Ventura et al. 2008). Ventura and colleagues demonstrate that combined deletion of *mir-106b~25* and *mir-106a~363* displays no developmental abnormalities, so follow-up studies could even be performed using *mir-17~92*$^{flox/flox}$, *mir-106b~25*^{null}, *mir-106a~363*^{null} mice in the KC context to thoroughly determine the effect of the loss of these miRNAs in pancreatic carcinogenesis. Moreover, with the rapidly evolving technology of the CRISPR-Cas9 system, it is becoming much faster and more cost-effective to generate novel and complex mouse models in-house. For example, miRNA cluster alleles could be cloned with lox-p sites flanking specific subsets of the clusters in order to facilitate tissue-specific deletion of particular miRNA families. These alleles could be tested for efficacy *in vitro* and rapidly transitioned *in vivo* using CRISPR-mediated recombination strategies. The development of CRISPR-Cas9 technology is beginning to finally give mouse genetics the power and speed that has previously been restricted to more facile model organisms, like *Drosophila*.

Final thoughts

The work presented in this chapter is consistent with our understanding of miRNAs as subtle modulators of gene expression. Specifically, the PanIN regeneration phenotype of 17KC animals, although dramatic, is a slow process that occurs over the

course of months. Additionally, the invasion defect of 17KPC tumors manifests as a slight survival benefit and a slight overall decrease in 17KPC cell line invasiveness. Indeed, my ability to demonstrate these differences rests largely in the scale of the experiments: sufficient mice were enrolled that stratification of the study data for localized disease still resolved a significant survival difference, and profiling eleven cell lines generated significant invasion differences, whereas statistical testing of eight-line subsets of the data fails to do so (data not shown).

An advantage of my experimental design is that it examined the role of an entire cluster of miRNAs that contains multiple members of the same miRNA families, many of which target the same transcripts. This fact improved my chances of seeing phenotypic effects, but the subtlety of the resultant phenotypes should serve as a cautionary tale to future investigators. miRNAs are certainly critical regulators of cellular processes, but assessing their role by individual deletion *in vivo* is likely to rarely yield meaningful results unless the miRNA being investigated is already known to be a dominant regulator of a particular process *and* its activity is unlikely to be substituted by that of another miRNA. Barring that, the investigator should be prepared to dissect potential redundancies, as in my analysis of the expression of the *mir-17~92* paralogs.

Tumor progression is an incredibly complex process, involving myriad signaling pathways that are interwoven at every level. Moreover, the specific cellular context significantly impacts the effect of a given pathway. Accordingly, the expression and impact of miRNAs on the various stages of tumor progression is similarly variable, and should not be considered as simply ‘tumor suppressive’ or ‘oncogenic.’ Understanding

the mechanisms behind miRNA effects in a transcriptome-specific context is an absolute necessity, but also an enormous challenge: consider, for instance, the fact that *mir-17~92* can potentially suppress CDKN1A, TGFBR2, SMAD4, PTEN, BIM, MYCN, and a host of GAPs and GEFs regulating RAS family proteins, but these signaling pathways themselves are under constant flux and selective pressure that varies according to cell type, extent of dysplasia, microenvironment, and host physiology. Ultimately, targets must be validated using robust RNA sequencing or proteomic technologies, but even these are limited by their resolution in a field that now considers each cell as an independent and potentially lethal entity. All of this is compounded by random mutations in cancers that generate unpredictable shifts in molecular interactions, at times rendering our best understanding of normal biology insufficient. To a certain extent, we can expect cancers to follow Murphy's law, where anything that could go wrong, will go wrong. These complexities and uncertainty are strong arguments for a focus on cancer prevention, although we should never abandon our attempts to understand and treat advanced disease.

I have demonstrated that loss of *mir-17~92* drives the regression of PanINs and their replacement with healthy acinar tissue. This work comes on the heels of similar studies demonstrating that PanIN regression can occur even in the continuing presence of oncogenic $Kras^{G12D}$ through downregulation of ERK signaling. Now, after decades of research, we are beginning to gain insight into the ways PanINs may be medically treated to prevent pancreatic adenocarcinoma. Perhaps soon we will begin to see a decrease in the number of pancreatic cancer cases in this country thanks to innovations in the medical

regression of PanINs. Previously, all talk of progress focused on detection, surgery, and improved chemotherapeutics, but these treatments carry significant morbidity and risk of mortality. It is hard to envision a way that a procedure as radical as the Whipple can be made much safer, but perhaps with a greater understanding of the very early stages of pancreatic neoplasia, we can prevent patients from ever needing it. My findings regarding the role of *mir-17~92* in PanIN maintenance reassure me that this is possible, and they give me hope that soon this will be a flourishing field of research that will have a positive impact on the care of the pancreatic cancer patient.

Materials and Methods

Cell Culture

All cell lines were grown in high glucose DMEM (Life Technologies #11965) supplemented with 10% fetal bovine serum (Atlanta Biologicals #s11150) and 100U/ml penicillin/streptomycin (herein 'complete media') (Pen/Strep: Life Technologies #15140). Cell lines were maintained at subconfluent densities on tissue culture-treated dishes and periodically thawed fresh from frozen stocks to maintain low passage number.

Proliferation assays were carried out by plating $2.5x10^4$ cells per well of a 24-well plate and counting cells at regular intervals by dissociation in 0.25% trypsin (Invitrogen #25200) and trypan blue exclusion (Invitrogen #15250061). Doubling time was calculated by first plotting the $\log_2$ of the cell number against time and fitting a linear regression to the data. The slope of this line has units of doubling events/time, thus the doubling time is derived by taking the inverse of the slope (i.e. time/doubling event).

Migration assays were carried out by plating $2.5x10^4$ cells in serum-free media into 8um-porous migration inserts (Fisher #08-774-162) in a 24-well plate. Cells were allowed to settle and adhere onto insert membranes for 20 minutes prior to the addition of 750ul of complete media to the outside of the insert. Cells were then allowed to migrate towards the serum gradient for 24 hours prior to methanol fixation and staining with Giemsa (Sigma-Aldrich #48900). Nonmigrating cells were wiped from the interior surfaces of the insert prior to membrane separation and mounting on glass slides. Cells were counted across five 100x fields under the microscope and averaged across two

membranes for each experiment. Invasion assays involved the parallel seeding of cells onto invasion inserts (Fisher #08-774-122), which require rehydration in serum-free media for one hour prior to seeding, but are otherwise processed identically. Invasion index was calculated as the number of invading cells divided by the number of migrating cells multiplied by 100%.

Serum-starvation survival assays were performed by plating $5x10^4$ cells per well of a 24-well plate. One day after seeding, cells were treated with complete media or serum-free media and incubated for a further 24 hours. At this time floating cells from the media and one PBS wash were collected and combined with trypsinized cells from the plate surface. This pool of live and dead cells was then pelleted by centrifugation and resuspended in 100ul of complete media supplemented with 0.04% trypan blue. Percent survival in fed and starved conditions was calculated as live cells divided by total cells multiplied by 100%. 'Baseline survival' is survival in the fed state, and 'serum-starvation survival' is survival in the starved state, divided by survival in the fed state, multiplied by 100%, and therefore measures the additional death that is attributable to the absence of serum.

Soft agar colony formation was measured by mixing 2x complete medium, made from DMEM powder (Invitrogen #12100) in a 1:1 ratio with 1.4% agarose (Denville #CA3510-8) in distilled water. This mixture is created and maintained at 42˚C to prevent agarose polymerization, and is used to coat 10cm plates and create a polymerized non-adherent surface ('hard agar plates'). A second mixture of 2x complete medium and 0.8% agarose is then made and used to resuspend a pellet of $3x10^5$ cells, which are

subsequently distributed across three hard agar plates and allowed to polymerize before feeding with 8mls of complete media. Colonies are allowed to form for 14 days before counting. Quantification consists of counting the total number of colonies greater than three cell diameters wide, across eight 50x fields.

Antagomir transfections began with seeding of $2.5x10^5$ cells per well of a 6-well plate 24 hours before transfection. miRCURY LNA Power Inhibitors (Exiqon #4101004-100, 4103258-100, and 199006-100; herein 'antagomirs') were transfected by combining 35 picomoles of antagomir with serum-free media to a final volume of 100ul. This was then combined with 20ul of Superfect (Qiagen #301305) and vortexed for ten seconds prior to incubation at room temperature for ten minutes. Cells were washed in PBS immediately prior to the addition of transfection complexes. The transfection mixture was combined with 600ul of complete media, the PBS wash was aspirated from the cells and the transfection mixture then added drop-wise. Transfection proceeded for three hours at 37˚C before an additional 700ul of complete media was added to protect against dehydration. Cells were fed with fresh media 24 hours after transfection and at 48 hours were plated into assays.

Histological Stains

Mouse tissues were fixed in 10% neutral-buffered formalin for at least 24 hours prior to processing through ethanol to paraffin blocks. Tissues were cut in 5um sections onto charged glass slides and allowed to dry overnight before staining. Prior to staining, paraffin slides were melted at 60˚C for 15 minutes and cleared through Xylenes (Fisher

#X3P) to 100% ethanol. Slides were then rehydrated through a graded alcohol series to distilled water. For immunohistochemical stains, antigen retrieval was performed using a citrate buffer (Vector Labs #H3300) by microwave heating at low power for 15 minutes. When peroxidase-labeled antibodies were to be used, endogenous peroxidases were inactivated by incubation in a 3% hydrogen peroxide solution for seven minutes. Tissues were then washed twice in TBS-T prior to blocking. When using mouse primary antibodies, an optimized Mouse-on-Mouse kit (Vector Labs #BMK-2202) was used according to the manufacturer's instructions. For all other antibodies, tissues were blocked in PBS supplemented with 10% normal goat serum. A list of all antibodies and conditions used for immunohistochemical stains appears in Table 2.1. Stains were developed using ABC (Vector Labs #PK-6101) and Nova Red (Vector Labs #SK-4800) kits. Counterstaining was performed with hematoxylin and slides were dehydrated through a graded alcohol series and xylenes prior to being mounted in Permount (VWR #100496).

Hematoxylin and eosin stains were performed according to field standards. The quadchrome stain was developed by David Driscoll and consists of a hybrid protocol derived from Sirius Red staining for collagen and Alcian Blue staining for mucin. Briefly, slides are rehydrated through a graded alcohol series to distilled water. They are then incubated in Weigert's Hematoxylin for one hour before being rapidly washed in acidified water (15 dips in 0.5% acetic acid). This is followed by Sirius Red staining for one hour, washes in acidified water, Alcian Blue staining for 30 minutes, washes in

Table 2.1 Conditions of immunohistochemical stains.
Blocking for all stains was performed with PBS supplemented with 10% normal goat serum overnight at 4˚C (pSMAD3 was blocked 4 hours at room temperature). RT: room temperature; O/N: overnight.

		Antibody			
	Stain	Cat. Number	Concentration	Time	Temp
Co-immunofluorescence	anti-Insulin	Dako A0564	1:500	4hr	RT
	anti-Glucagon	Dako A0565	1:50	4hr	RT
	Alexa-fluor-594 anti-rabbit	ab150080	1:250	1hr	RT
	FITC anti-guinea	Jackson Immuno: 706-095-148	1:500	1hr	RT
Immunohistochemistry	anti-ki67	ab66155	1:600	4hr	RT
	anti-CC3	cs-9664	1:800	4hr	RT
	anti-αSMA	ab5694	1:400	1.5hr	RT
	anti-Hes1	CST-11988	1:5000	4hr	RT
	anti-pSMAD3	ab52903	1:200	O/N	4°C
	anti-pERK	CST-9101	1:400	1hr	RT

acidified water, and dehydration to xylenes prior to mounting. In this stain, collagen appears bright red, mucin is blue, nuclei are black, and cytoplasm is a weak yellow.

qRT-PCR

Quantitative RT-PCR for miRNAs was performed as previously described (Fiedler et al. 2010). Briefly, total RNA was isolated by homogenization of PBS-washed adherent cells in TRIzol reagent (Invitrogen #15596). RNA was extracted to the aqueous phase using chloroform and precipitated in 70% isopropanol overnight at -20˚C to ensure capture of small RNAs. Pellets were washed in 70% ethanol prior to solubilization in DEPC-treated water and long-term storage at -80˚C.

cDNA libraries were created by first treating RNA with DNAse (Life Technologies #AM1907) to eliminate genomic contaminants. DNA-free RNA was then polyadenylated with *E. coli* poly-A polymerase (New England Biolabs #M0276) at 37˚C for 30 minutes prior to the generation of cDNA using a reverse transcription kit (Invitrogen #18080). During the RT reaction, a pool of special primers was used to generate cDNA copies of polyadenylated miRNAs. These specially designed primers encode a 20-nt universal tag, followed by a 15-nt oligo-dT segment, and finally a 2-nt tag that tethers the primer to the 3' end of polyadenylated miRNAs. A pool of all 12 possible combinations for the 2-nt tag was used at a final concentration of 50uM to capture all miRNAs during cDNA synthesis. A complete list of RT primer sequences appears in Table 2.2. All subsequent steps of the cDNA synthesis were conducted according to kit

Table 2.2 Primers used in PCR reactions.
Primer sequences are provided for all qPCR primers used in the study. In all cases primers were derived according to the sequence of the mature mouse miRNA based on miRBase records. The primer for U6 snRNA is complementary to human and mouse U6 RNA.

Primer Name	Primer Sequence (5' - 3')
miR-17	CAAAGTGCTTACAGTGCAGGTAG
miR-106a	CAAAGTGCTAACAGTGCAGGTAG
miR-106b-5p	TAAAGTGCTGACAGTGCAGAT
miR-20a	TAAAGTGCTTATAGTGCAGGTAG
miR-20b	CAAAGTGCTCATAGTGCAGGTAG
miR-93	CAAAGTGCTGTTCGTGCAGGTAG
miR-19a/b	TGTGCAAATCTATGCAAAACTGA
miR-18a	TAAGGTGCATCTAGTGCAGATAG
miR-18b	TAAGGTGCATCTAGTGCTGTTAG
miR-92	TATTGCACTTGTCCCGGCCTG
miR-25	CATTGCACTTGTCTCGGTCTGA
miR-363	AATTGCACGGTATCCATCTGTA
snoRNA234	GATTTAACAAAAATTCGTCACTACCACTGAGA
U6 snRNA (mmu/hsa)	CATCTCGAGCTAATCTGGTGGG
RT Primer 1	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT AA
RT Primer 2	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT AC
RT Primer 3	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT AG
RT Primer 4	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT AT
RT Primer 5	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT CA
RT Primer 6	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT CC
RT Primer 7	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT CG
RT Primer 8	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT CT
RT Primer 9	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT GA
RT Primer 10	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT GC
RT Primer 11	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT GG
RT Primer 12	ACGCATCTATGCGCATATCG TTTTTTTTTTTTTTT GT
Universal Reverse Primer	ACGCATCTATGCGCATATCG

directions. Lastly, samples were incubated with RNase for 20 minutes at 37˚C prior to long-term storage at -20˚C.

PCR for miRNAs used a slightly modified rapid program to amplify the very short miRNA cDNAs: denaturation at 94˚ for 15 seconds, annealing at 55˚C for 30 seconds, and extension at 70˚C for 34 seconds, repeated 40 times . The reverse primer for all miRNA reactions was the sequence of the universal tag present in all 12 RT primers. The forward primer for each miRNA was the mature miRNA sequence. A full list of primers used to detect miRNAs in the study is presented in Table 2.2. PCR reactions were carried out on an ABI Step One Plus machine in 10ul volumes using SYBR Green (VWR #95072).

C_T values were calculated for all miRNA PCR reactions at a uniform threshold of absorbance across all experiments and controlled to C_T values for the endogenous reference (snoRNA234 in mouse-only experiments, U6 in human and cross-species comparisons). The expression of individual miRNAs is presented as relative snoRNA234 units in order to convert the ΔC_T value for each miRNA into a relative molar measure (calculated as $2^{\Delta CT}$). Once all miRNAs are expressed in this manner, the expression levels of individual miRNAs can be straightforwardly summed to generate a measure of the relative abundance of all miRNAs within a shared miRNA family, allowing intra-family comparisons between treatment groups or genetic cohorts of cell lines. Because of seed family redundancy, this analysis is critical when considering the potential overall effect of a miRNA family composed of many differently expressed individual miRNAs.

Mouse Breeding, Euthanasia, and Necropsy

All mice generated in this study were housed in a dedicated facility in accordance with guidelines set forth by the Institutional Animal Care and Use Committee. The alleles for *mir-17~92*^{flox} (Ventura et al. 2008), *Ptf1a-Cre* (Kawaguchi et al. 2002), *LSL-Kras*^{G12D} (Jackson et al. 2001), and *Tp53*^{flox} (Jonkers et al. 2001) have been described previously.

For the precursor lesion study, *mir-17~92*$^{flox/wt}$*, Ptf1a-Cre* mice were maintained on a C57/Bl6 background and crossed to *mir-17~92*$^{flox/wt}$*, LSL-Kras*^{G12D} mice on a mixed background to generate littermate KC and 17KC animals. For the tumor survival study, *mir-17~92*$^{flox/wt}$*, Tp53*$^{flox/flox}$*, Ptf1a-Cre* mice were maintained on a C57/Bl6 background (F_0 generation) and crossed together to generate littermate *mir-17~92*$^{flox/flox}$*, Tp53*$^{flox/flox}$*, Ptf1a-Cre* and *mir-17~92*$^{wt/wt}$*, Tp53*$^{flox/flox}$*, Ptf1a-Cre* mice (F_1 generation). Littermate *mir-17~92*$^{flox/flox}$*, LSL-Kras*^{G12D} and *mir-17~92*$^{wt/wt}$*, LSL-Kras*^{G12D} mice were generated in a similar fashion. These mice were then used to generate mating cages for the survival study. Thus, 17KPC and KPC mice (F_2 generation) could be bred with high efficiency while preserving genetic relatedness through littermate control of the F_1 generations. Over the course of the study, experimental animals were generated from mating cages formed from at least three separate F_1 generations, further protecting study results from effects of random genetic drift between KPC and 17KPC breeders.

Health status was monitored at least three times per week for all animals. Major signs of illness warranting euthanasia included severely reduced mobility with lack of arousal upon handling, hunching, abdominal distension, wasting, palpable abdominal mass, and hyperventilation. Comorbidities contributing to euthanasia decisions included

pallor, squinting, grooming deficits, and abnormal gait. Prior to euthanasia extensive notes were gathered regarding general appearance and reasons for sacrifice. Upon necropsy, the disposition of the internal organs was noted prior to dissection, including distension of any tubular organs, fibrosis, adhesions, metastases, strictures, necrosis, jaundice or developmental abnormalities. Tumors were measured in three orthogonal directions (length, width and depth) and tumor volume was calculated according to the equation $V = \frac{4}{3}\pi(lwd)$. The presence of adhesions or frankly invasive disease was noted, as well as the color, firmness, and location of the tumor within the pancreas relative to other structures. Tumors were then subdivided into pieces according to the amount of available tissue and the following priority list: 1) fixation (esp. along areas of adhesion or likely invasion), 2) cell line, 3) flash frozen samples for protein, RNA, and DNA.

CHAPTER III

Partial Loss of *Dicer* Sensitizes Tumors to Anoikis and Inhibits Metastasis

Introduction

The lethal nature of pancreatic cancer is largely due to its early invasive and metastatic nature, which prevents the majority of patients from undergoing potentially curative surgery (Hariharan et al. 2008). Understanding the mechanisms of tumor progression, particularly as they relate to the processes of invasion and metastasis, is critical to the future of care for the metastatic patient. A major step in a carcinoma's metastatic program is the ability to invade through basement membranes, escape the epithelial compartment, and cross into the circulation (Hanahan and Weinberg 2011). Subsequent to this, cells must survive in the liquid environment of the circulatory system, continue to evade the immune system, reattach at distance sites, invade into foreign tissues, and then survive and grow to form a competent metastasis (Chambers et al. 2002; Chaffer and Weinberg 2011). These multiple steps exert strong selective pressure on tumor cells, and metastatic seeding can be a very inefficient process (Luzzi et al. 1998). Nevertheless, for highly metastatic cancers like pancreatic cancer, tumor cells are evidently proficient at overcoming such hurdles, and we must better understand the means by which they achieve this.

miRNAs govern nearly every cellular pathway and provide an additional means of understanding tumor origins and behavior (Lu et al. 2005). Broad characterization of human tumors reveals a general downregulation of miRNA biogenesis in many solid

tumors (Lu et al. 2005), indicating a tumor-suppressive role for miRNAs. This is supported by observations that widespread shortening of 3'UTRs also occurs in cancer (Mayr and Bartel 2009), and suppression of miRNA processing machinery can promote tumor progression in several cancers (Kumar et al. 2007; Hill et al. 2009; Kumar et al. 2009; Lambertz et al. 2010).

Until very recently, the broad role of miRNAs in pancreatic cancer has not been explored (Morris et al. 2014; Wang et al. 2014). Generally, mutations in *DICER* or other components of the miRNA biogenesis pathway are not seen in PDAC, and miRNA *up*regulation is more common than downregulation in this disease (Bloomston et al. 2007; Zhang et al. 2009; Frampton et al. 2014). It is also known that miRNAs are broadly required for the development and maintenance of pancreatic cell lineages (Lynn et al. 2007; Morita et al. 2009; Morris et al. 2014). These data collectively suggest that miRNAs play an oncogenic role in the promotion of pancreatic tumorigenesis and that mutations in the miRNA processing machinery are selected against during tumor evolution. A single study has provided preliminary data for the role of *Dicer* in precursor lesion development that largely agrees with the published data from other tumor contexts (Morris et al. 2014; Wang et al. 2014), but to date no functional study of the role of *Dicer* in pancreatic tumorigenesis or progression has been described, nor has any explanation been proposed for the preservation of miRNA biogenesis in human pancreatic tumors.

This chapter describes my investigation into the requirement for *Dicer* in pancreatic tumorigenesis. In agreement with the published literature, I demonstrate that complete loss of *Dicer* creates instability in mature pancreatic lineages, resulting in

degeneration of the exocrine pancreas. *Dicer* heterozygosity is developmentally tolerated, and I show that partial loss of *Dicer* is also permissible for tumor development, but that *Dicer*-heterozygous tumors are deficient in their ability to metastasize to distant sites. *In vitro* experiments link this failure of metastasis to an increased susceptibility to anoikis, which is cell death triggered by loss of attachment and is analogous to the stress of surviving in the circulation. Efforts to describe miRNA signatures predictive of cell line phenotypes and metastasis are ongoing.

Results

Dicer is broadly required for normal vertebrate development (Harfe et al. 2005; Yang et al. 2005; Lynn et al. 2007; O'Rourke et al. 2007). Therefore I made use of the *Ptf1a-Cre* allele to selectively delete conditional alleles of *Dicer* in the pancreas (Mudhasani et al. 2008). In agreement with the published literature, I found that complete ablation of *Dicer* in the pancreas is not tolerated, as evidenced by the progressive deterioration and loss of the exocrine compartment in $Dicer^{flox/flox}$, *Ptf1a-Cre* animals (Figure 3.1b,d,f) (Morita et al. 2009). I observed this at postnatal day 3 as hypomorphic acinar cells with reduced zymogen contents compared to $Dicer^{flox/wt}$, *Ptf1a-Cre* animals (Figure 3.1a,b). This progressed to a pronounced loss of exocrine architecture by six months with residual islands of acinar tissue, ducts, islets, and lymph nodes existing within a vast expanse adipose tissue (Figure 3.1f). Staining of three-day-old pancreata revealed extensive apoptosis in $Dicer^{flox/flox}$ pancreata and a compensatory increase in the proliferative rate of acinar cells (Figure 3.2).

Based on these findings, $Dicer^{flox/flox}$, *Ptf1a-Cre* animals did not provide a developmentally normal baseline from which to measure differences in tumor formation, leading me to compare $Dicer^{flox/wt}$ animals to $Dicer^{wt/wt}$ littermates in the tumorigenesis study. It must be noted, however, that $Dicer^{flox/wt}$ animals also exhibit some abnormal changes in the form of sporadically 'vacuolated' acinar cytoplasm (Figures 3.1g, 3.3c). Vacuolization of the acinar compartment does not occur in $Dicer^{flox/wt}$, *Ptf1a-Cre* animals (Figure 3.3a), but it does occur in the context of *Tp53* loss (Figure 3.3b). In these contexts I do not observe any deterioration of the pancreas architecture or pancreatic insufficiency

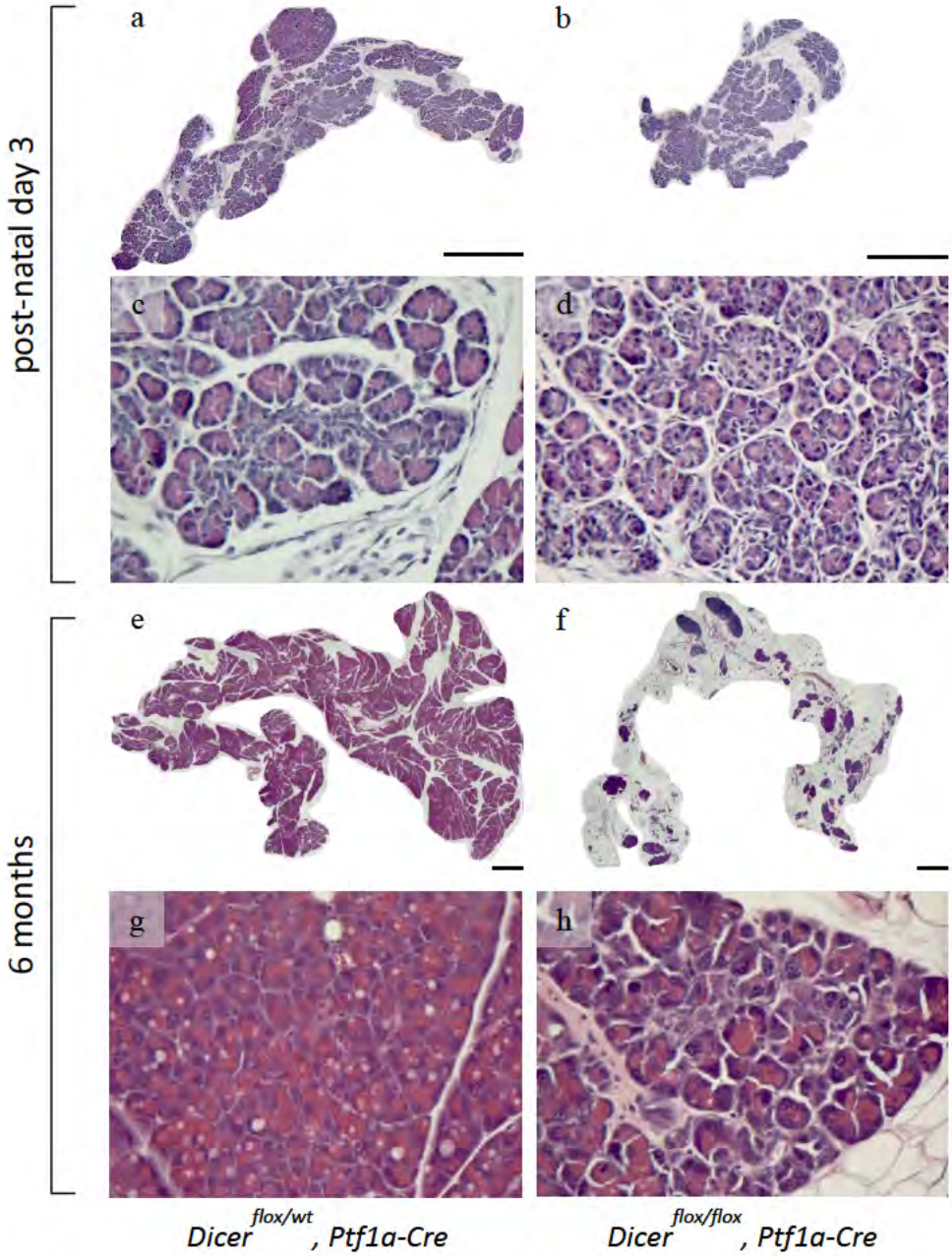


Figure 3.1. Complete loss of *Dicer* in the exocrine pancreas causes early developmental defects and progressive degeneration.
Attempts to ablate *Dicer* result in smaller pancreata in three-day-old pups (**B,D**), which display reduced acinar cytoplasm and disorganized ductal networks compared to heterozygous animals (**A,C**). By 6 months these animals' pancreata have degenerated into islands of unhealthy acinar tissue separated by adipose from isolated ductal and endocrine components (**F,H**, compare to **E,G**). Residual acinar tissue displays inconsistent zymogen content and architectural defects (**H**). *Dicer*-heterozygous pancreata exhibit normal architecture but aberrant vacuolization of acinar cytoplasm (**G**).
Scale bars on stitched whole organ images represent 1mm.

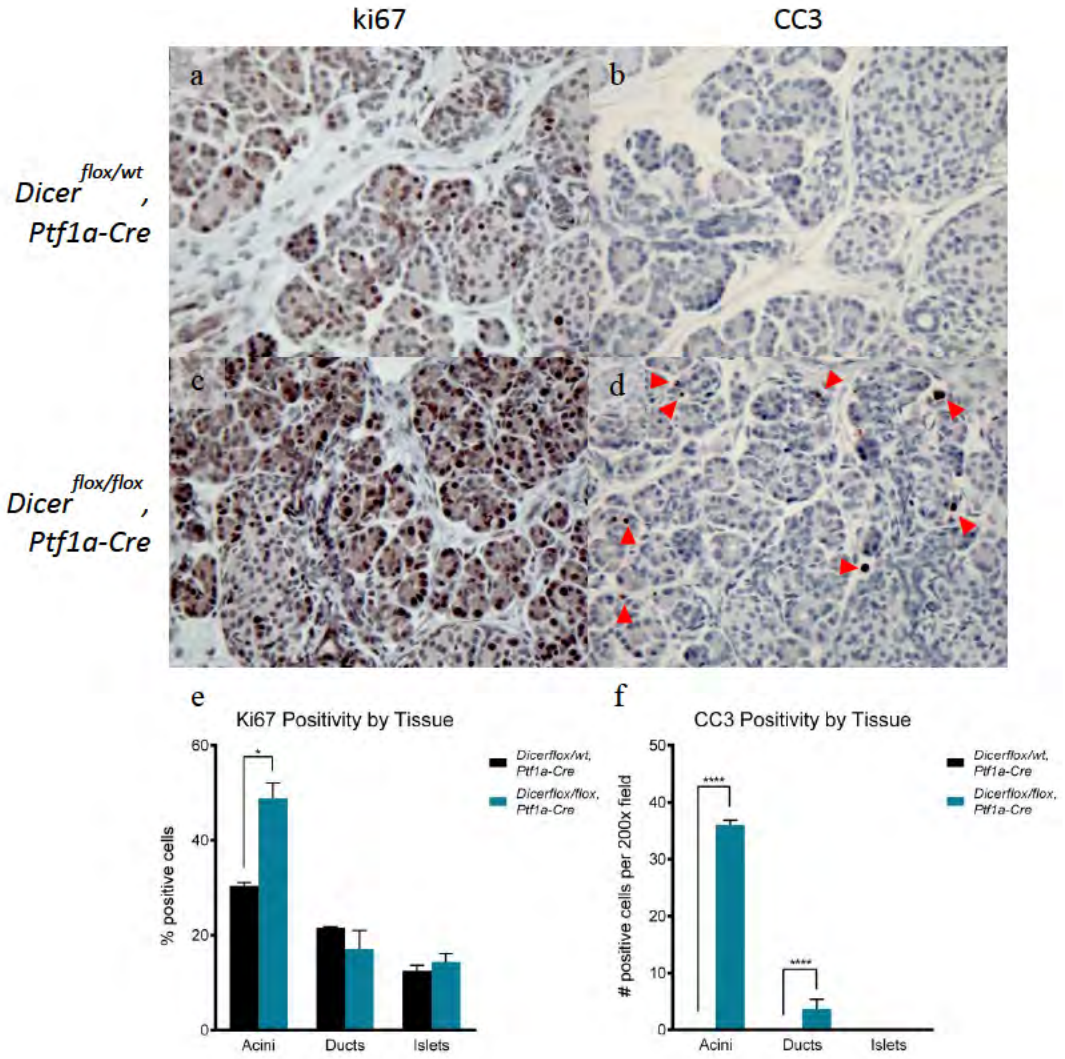


Figure 3.2. Complete loss of *Dicer* in the exocrine pancreas causes apoptotic cell death and compensatory proliferation.
Immunohistochemical staining of pancreata from three-day-old pups demonstrates higher rates of ki67 positivity within the acinar compartment, but equivalent rates in ducts and islets (**A**,**C**,**E**). Cleaved caspase 3 staining is completely absent in heterozygous animals, but is highly prevalent in *Dicer*^{null} pancreata (**B**,**D**,**F**).
p values: * < 0.05, **** < 0.0001

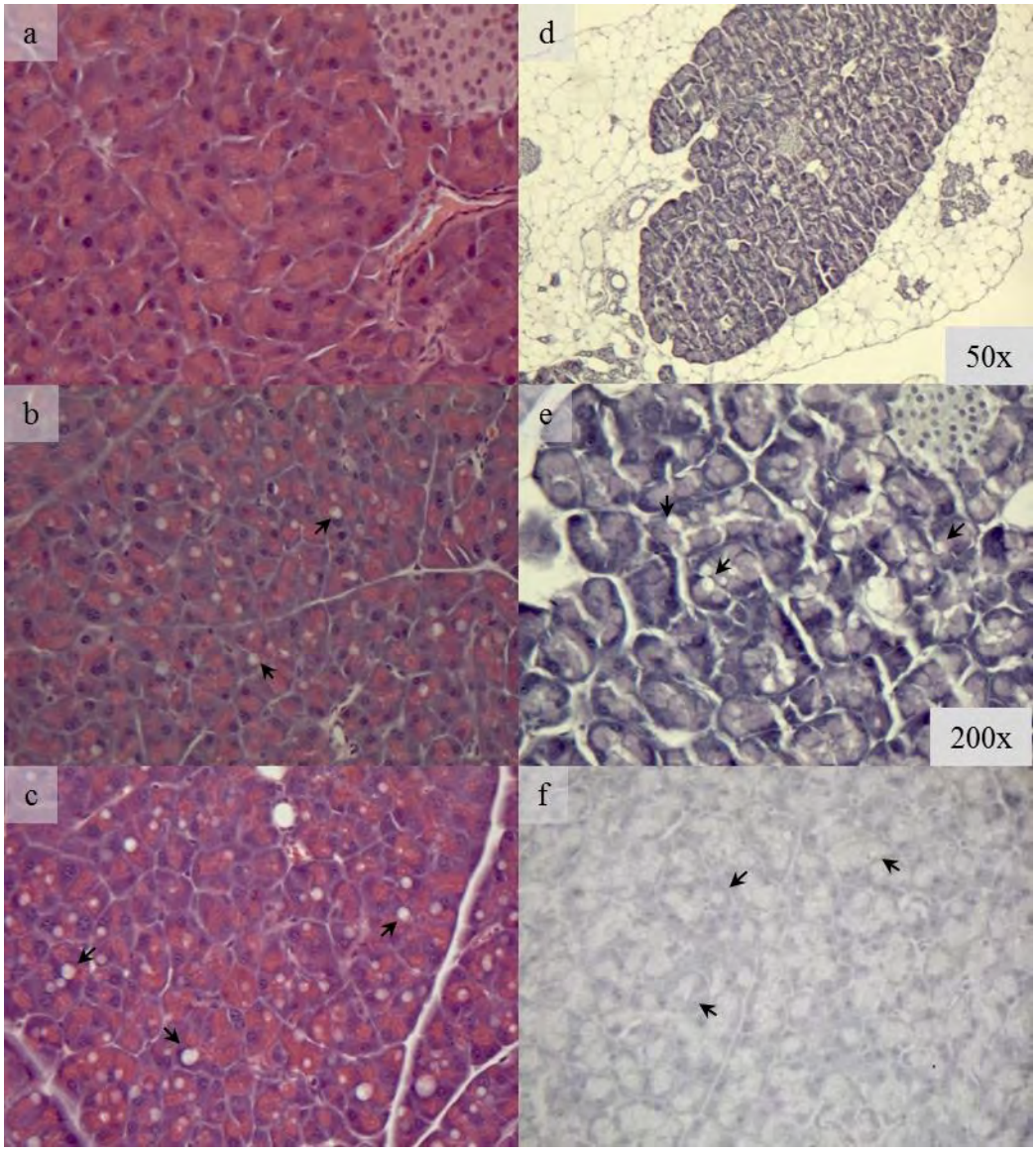


Figure 3.3. 'Vacuolization' and degeneration are dependent on *Tp53* and *Dicer* status.
H+E staining was performed on the pancreata of mice from different backgrounds to examine the role of *Dicer* and *Tp53* in vacuolization and fatty replacement. (**A**) *Dicer*$^{flox/wt}$, *Ptf1a-Cre*: note the absence of acinar vacuoles. (**B**) *Tp53*$^{flox/flox}$, *Ptf1a-Cre.* (**C**) *Dicer*$^{flox/wt}$, *Tp53*$^{flox/flox}$, *Ptf1a-Cre.* (**D,E**) *Dicer*$^{flox/flox}$, *Ptf1a-Cre*. (**F**) Oil Red O stain of *Dicer*$^{flox/wt}$, *Tp53*$^{flox/flox}$, *Ptf1a-Cre*. Arrows highlight vacuoles.

in aged animals (data not shown). Exocrine degeneration and fatty replacement occurs only upon complete genetic ablation of *Dicer* (Figure 3.3d,e). These 'vacuoles' are currently of an unknown composition or origin, but I have confirmed that they are neither lipid positive by Oil Red O stain (Figure 3.3f), nor glycogen positive by Periodic Acid Schiff stain (data not shown).

To induce tumor formation, I used the RCAS-*tva* system of retroviral gene delivery. The RCAS virus (replication competent avian sarcoma-leukosis virus long terminal repeat with a splice acceptor) is an engineered vector capable of infecting cells expressing the avian cell surface receptor *tva* (Bates et al. 1993; Young et al. 1993). Transgenic mice expressing *tva* under the control of the elastase promoter are therefore susceptible to pancreas-specific infection by RCAS viruses (Lewis et al. 2003a). The RCAS virus itself is designed to drive expression of the inserted gene of interest by the viral long terminal repeat (Gorman et al. 1982; Norton and Coffin 1987). Using this system, our lab has used RCAS virus expressing polyoma virus middle-T antigen (RCAS-PyMT) to induce tumors in the pancreas (Lewis et al. 2003a; Ahronian and Lewis 2014; Sano et al. 2014). PyMT is an oncogene (Chowdhury et al. 1980) that acts to recruit signal transduction molecules to endomembranes (Schaffhausen and Roberts 2009). It is capable of activating the SRC family proteins c-SRC and YES (Bolen et al. 1985; Thomas et al. 1993), PI3K (Whitman et al. 1985; Summers et al. 1998), and RAS via its recruitment of SHC and GRB2 (Campbell et al. 1994). Induction of tumors using the RCAS-PyMT system allows the generation of diverse carcinoma subtypes, including

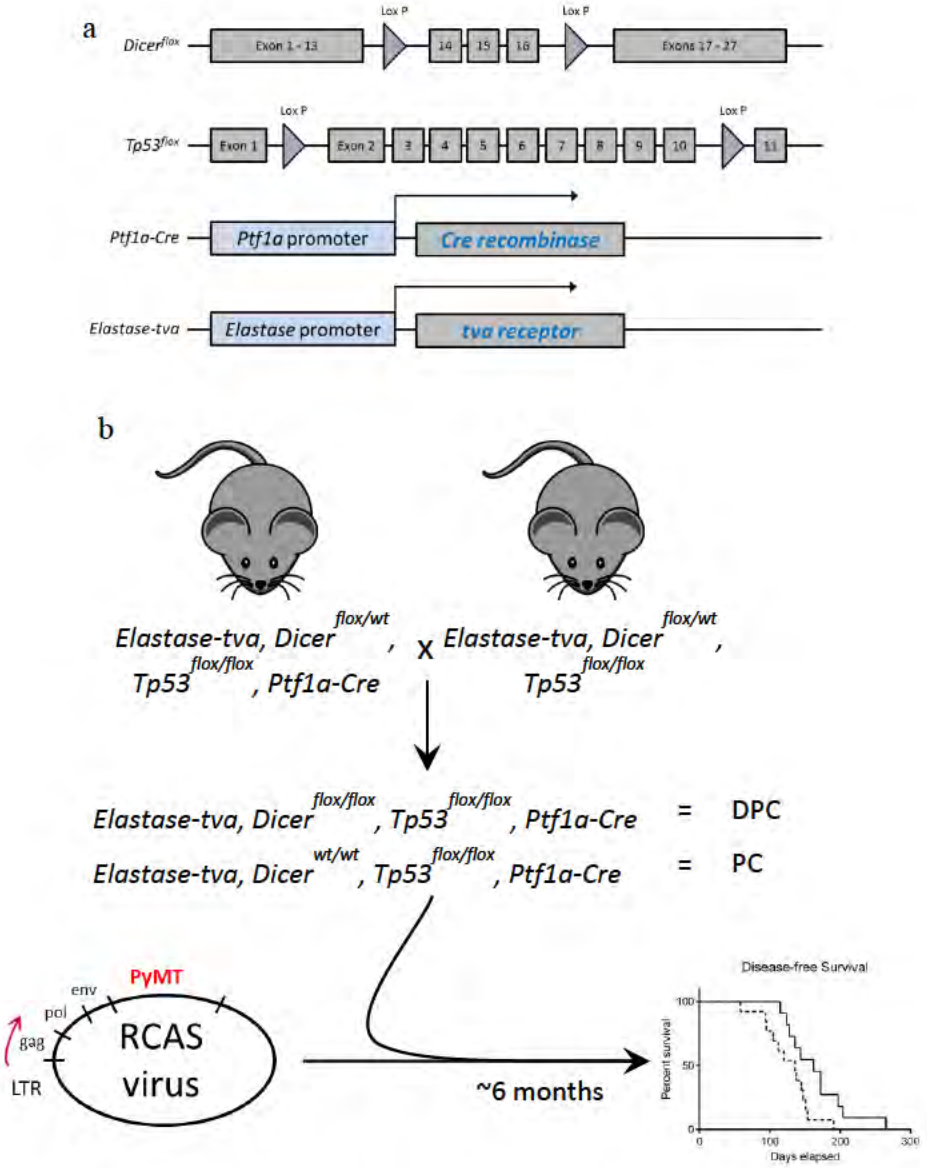


Figure 3.4. Pancreatic tumors are sporadically induced by the RCAS-*tva* system of viral gene delivery.

(**A**) Schematics for genetic alleles used in this study are provided for reference. (**B**) Mice were bred to generate *Dicer*$^{flox/flox}$ and *Dicer*$^{wt/wt}$ littermates, which were subsequently injected at post-natal day three with RCAS-PyMT virus and sacrificed at the onset of illness.

acinar carcinoma, ductal adenocarcinoma, and poorly differentiated forms (Lewis et al. 2003a). Using this system, I sought to determine whether miRNA biogenesis would differentially impact the formation of these subtypes.

I intraperitoneally injected litters of *Elastase-tva, Dicer*^{variable}*, Tp53*$^{flox/flox}$*, Ptf1a-Cre* mice at postnatal day 3 with live chicken fibroblasts producing high titer RCAS-PyMT virus ('*Dicer*^{variable}' signifies that pups were derived from the crossing of *Dicer*$^{flox/wt}$ parents). I then compared the survival of *Elastase-tva, Dicer*$^{wt/wt}$*, Tp53*$^{flox/flox}$*, Ptf1a-Cre* to *Elastase-tva, Dicer*$^{flox/wt}$*, Tp53*$^{flox/flox}$*, Ptf1a-Cre* littermates (herein 'PC' and 'DPC' respectively) (Figure 3.4).

I observed that PC and DPC mice exhibit similar rates of overall survival and tumor size (Figure 3.5a,b). Moreover , the distribution of tumor pathologies was also similar between groups with similar rates of development of acinar and ductal carcinomas, in agreement with published literature regarding PyMT expression in the exocrine pancreas (Figure 3.5c-f) (Lewis et al. 2003a; Du et al. 2009). However, DPC mice experience significantly lower rates of metastatic disease than PC mice (Figure 3.5j). Metastases were only observed in the liver and infiltrated into the liver parenchyma (Figure 3.5g) or grew intravascularly (Figure 3.5h).

An extensive panel of cell lines derived from tumors in the survival study was characterized *in vitro* for performance in a variety of cancer-related phenotypes. Proliferative capacity, measured by direct cell number over time, shows no difference between PC and DPC cell lines (Figure 3.6a). The ability of cell lines to grow suspended in an agarose matrix was universally low across PC and DPC cell lines except for

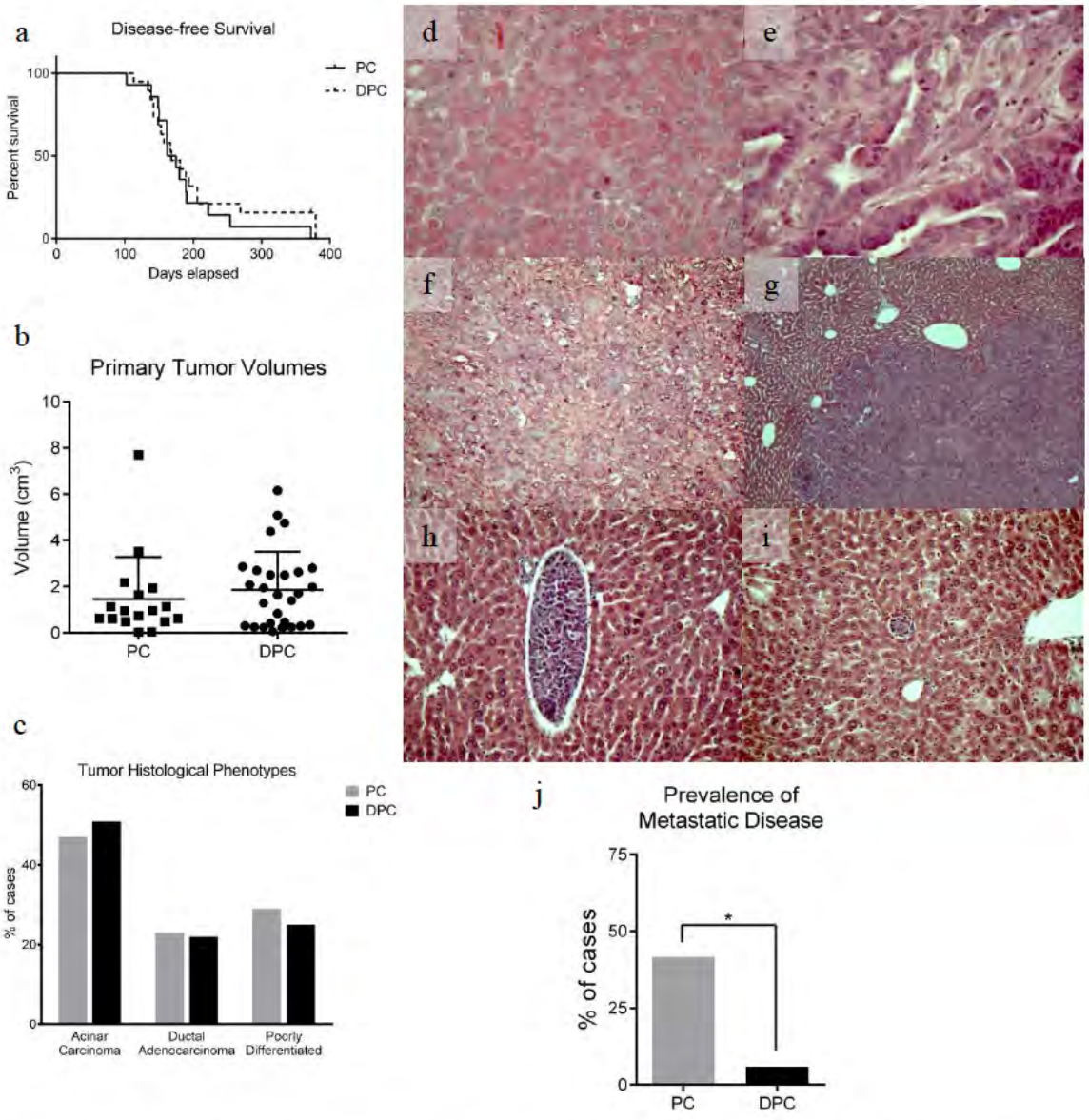


Figure 3.5. Partial loss of *Dicer* inhibits pancreatic cancer metastasis to the liver. Overall survival (**A**), tumor burden (**B**), and distribution of carcinoma subtypes (**C**) do not differ significantly between PC and DPC mice. Examples of acinar carcinoma (**D**), ductal adenocarcinoma (**E**), and poorly differentiated carcinoma (**F**) are provided. Liver metastases in both groups can manifest as infiltrative disease (**G**), or intravascular growth (**H**). Small intravascular cellular aggregates were not counted as successful metastases if they contained fewer than 20 cells (**I**). Overall, DPC mice were less likely to present with metastatic disease at sacrifice (**J**).

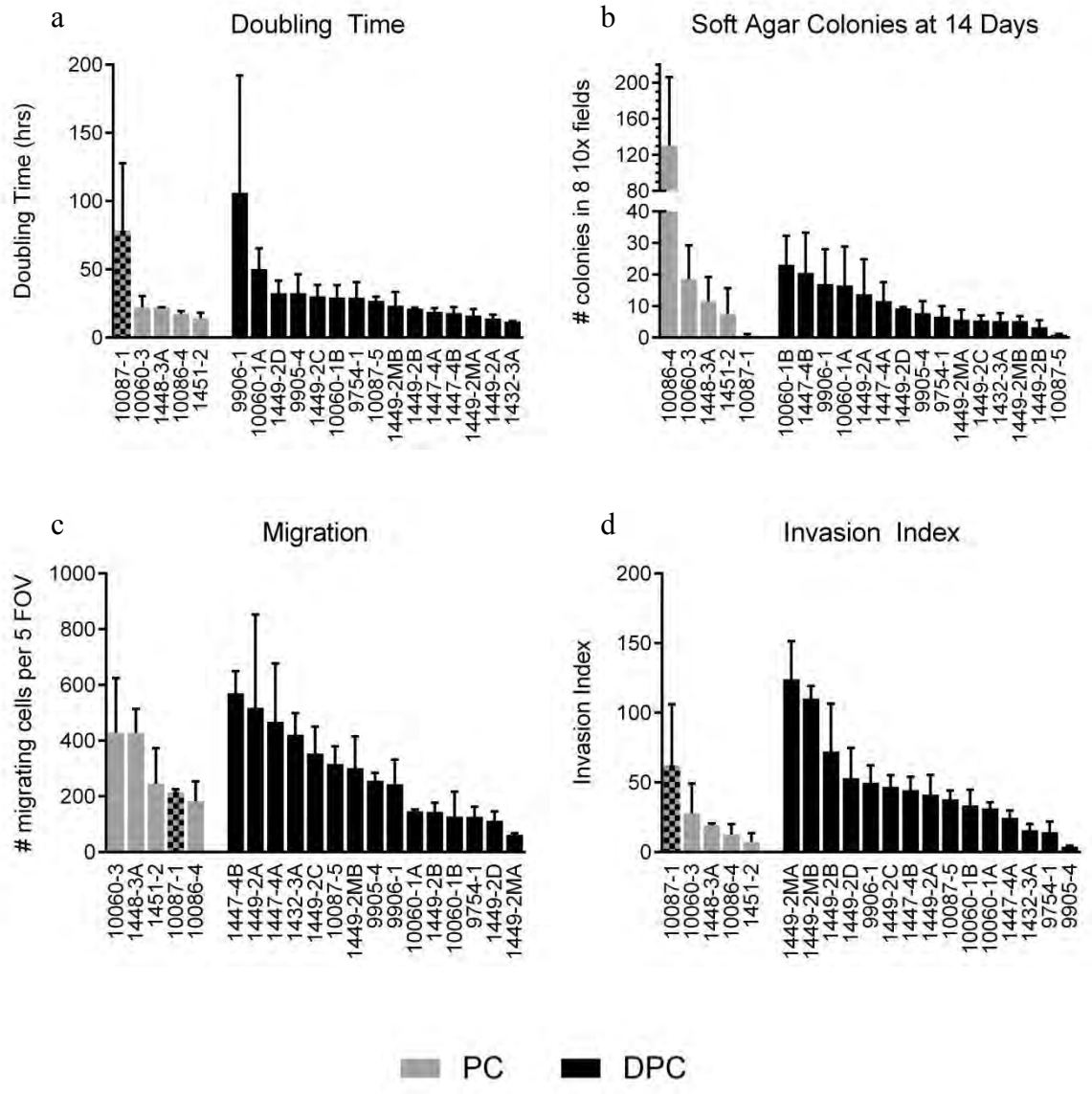


Figure 3.6. PC and DPC cell lines exhibit a spectrum of behaviors in proliferative and chemotactic assays *in vitro*.
No significant differences exist between PC and DPC cell lines in measures of proliferative rate (**A**), anchorage-independent growth (**B**), migration (**C**), or invasion through Matrigel (**D**). The cell line 10087#1 was isolated from a PC mouse outside the survival study and is not included in statistical analyses (see discussion).

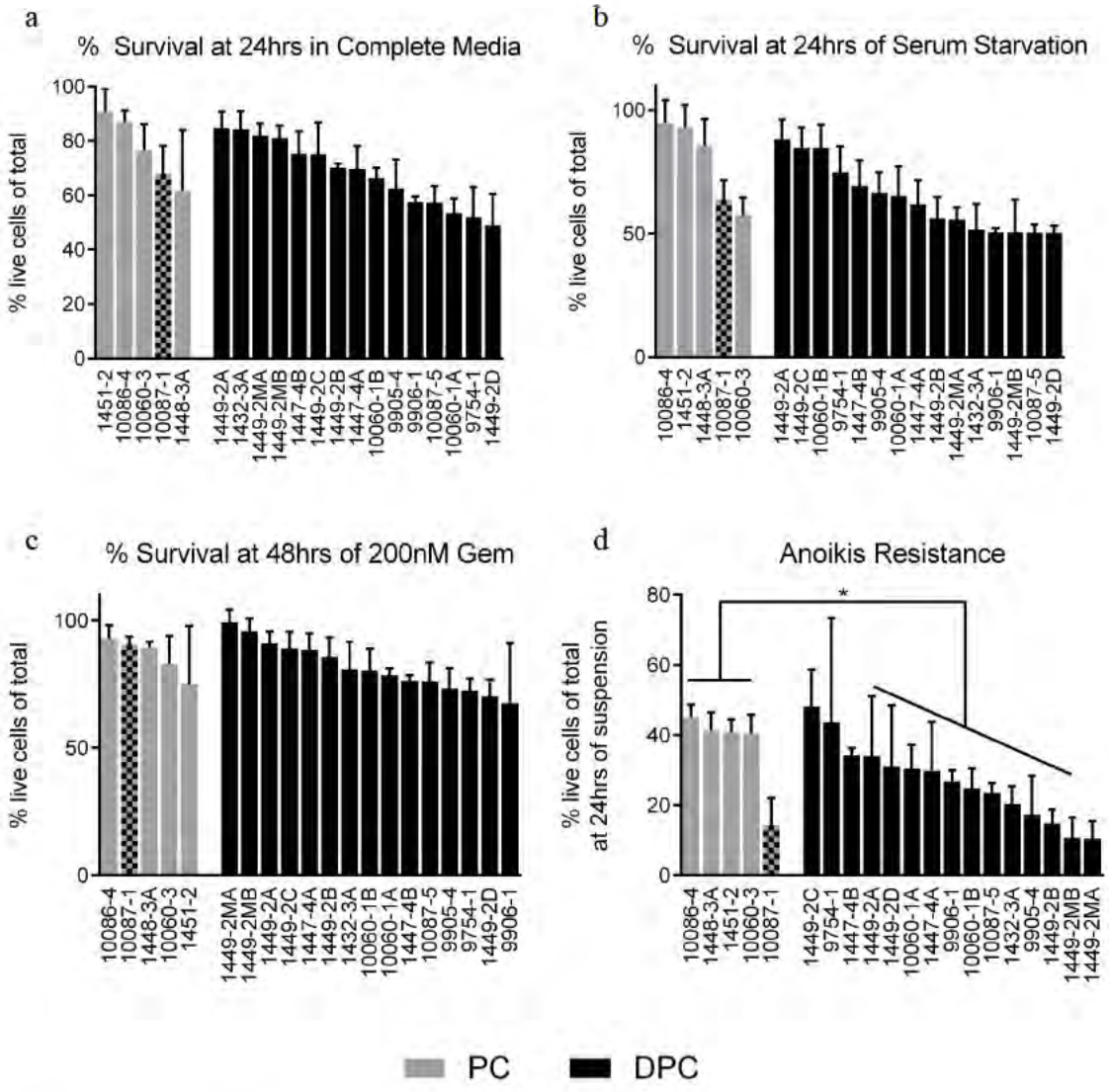


Figure 3.7. DPC cell lines are sensitive to anoikis.
No significant differences exist between PC and DPC cell lines in baseline cell death rate (**A**), susceptibility to serum-starvation (**B**), or susceptibility to gemcitabine (**C**). DPC cell lines as a group are significantly more sensitive than PC cell lines to cell death upon loss of attachment (**D**). The cell line 10087#1 was isolated from a PC mouse outside the survival study and is not included in statistical analyses (see discussion). Student's t test p value: * < 0.05

10086#4, and no significant differences existed by genotype (Figure 3.6b). Migration and invasion capacity, measured in a transwell assay, were also not significantly different between PC and DPC cell lines (Figure 3.6c,d). When assayed for their resistance to cell death in a variety of conditions, DPC cell lines were similar to PC cell lines in their baseline survival and resistance to serum starvation and gemcitabine (Figure 3.7a-c), but they were significantly more sensitive than PC lines to cell death upon loss of attachment ('anoikis'; Figure 3.7d). Therefore, greater sensitivity to anoikis may underlie the reduced capability of DPC tumors to metastasize *in vivo*.

Discussion

Dicer$^{flox/flox}$ pancreas degeneration

It is not surprising that complete loss of *Dicer* is not tolerated in the pancreas. At this point, miRNAs are known to be broadly required for tissue development, homeostasis, and cellular survival (Bernstein et al. 2003; Lynn et al. 2007; Lambertz et al. 2010; Zhang et al. 2014a). What is interesting, perhaps, is that *Dicer$^{flox/flox}$, Ptf1a-Cre* animals possess a growing pancreas at birth, but this tissue then slowly regresses and is replaced by adipose with time, leaving behind intact islets and isolated portions of the ductal system with residual, abnormal acinar tissue. This is a weaker phenotype than that observed by Lynn and colleagues, who used *Pdx1-Cre* to delete *Dicer* in the pancreas and observed severe exocrine degeneration at embryonic stages (Lynn et al. 2007). *Pdx1* is a transcription factor responsible for the differentiation of both endocrine and exocrine cell lineages of the pancreas (Gu et al. 2002), and is expressed earlier in development than *Ptf1a*, which contributes predominantly to exocrine differentiation (Kawaguchi et al. 2002). My work and the work of Lynn and colleagues together suggest that the timing of *Dicer* deletion is important for its developmental tolerability.

At this point I do not know the precise mechanism of the acinar regression observed in these animals. It could be due to cell death and subsequent replacement by infiltrating adipose from adjacent visceral depots. This is supported by the higher rates of CC3 staining observed in the pancreata of *Dicer$^{flox/flox}$, Ptf1a-Cre* pups. Another intriguing possibility is the transdifferentiation of pancreatic parenchyma *into* adipose.

This has been reported once before in animals which were engineered to lose *Myc* and which also expressed pancreatic lineage tracing genes (Bonal et al. 2009). This group was able to demonstrate that adipocytes present in the setting of a degenerating pancreas had previously expressed the pancreas-specific transcription factor *Ptf1a*, suggesting that these cells had originated from pancreatic exocrine progenitors. The observation of vacuolization in DPC pancreata initially offered an enticing clue that this could be relevant to my model, but whatever the nature of the observed acinar vacuoles, they are not lipid positive. Indeed, the persistence of these vacuoles into late age and the absence of exocrine degeneration or intercalating adipocytes in *Dicer*$^{flox/wt}$, *Tp53*$^{flox/flox}$, *Ptf1a-Cre* animals do not support the idea that this could represent a transition state between acinar and adipocyte differentiation. Nevertheless, aged human pancreata exhibit areas of adiposity that suggest the potential for acinar cells to transdifferentiate to adipocytes in response to stress or some other stimulus. From the perspective of cellular stress, it is possible that these vacuolar artifacts represent an aberrant response that is normally regulated or prevented in the setting of fully competent miRNA biogenesis. One possibility that leaps to mind is that these could be evidence of deregulated autophagy, but I have yet to stain these vacuolated tissues for autophagy markers.

Carcinoma subtypes

I chose to use the RCAS-*tva* system of tumorigenesis partly because of its ability to induce a variety of different tumor types: acinar, ductal, and poorly differentiated carcinoma. One of the questions I wanted to ask was whether miRNA biogenesis was

more generally required in one carcinoma subtype or another, hypothesizing that this would be reflected in a shift of subtype prevalence in DPC animals compared to PC animals. The fact that this was not observed suggests that miRNA biogenesis is not preferentially required by one carcinoma more than another.

Circulating tumor cells, anoikis, invasion, and metastasis

The major question of this project was whether single-copy loss of *Dicer* could negatively impact tumorigenesis. To this end, I was pleased to find that DPC tumors progressed to metastatic disease less frequently than PC tumors. Based on the developmental abnormalities of $Dicer^{null}$ pancreata, I initially suspected that the failure to metastasize would correlate with an aggressive cell death response. In my initial analysis of standard cancer cell phenotypes *in vitro*, including proliferation, soft agar colony formation, migration, invasion, and survival response to serum starvation, I did not observe any significant differences by genotype, although there was substantial variability across the entire panel of cell lines in most phenotypes. I noticed with some surprise that nearly all of the cell lines tested were quite deficient in soft agar colony formation compared to pancreatic cell lines I had worked with in the past (see Figures 3.6 and 2.10). Moreover, at the completion of the soft agar assays, there was also no evidence of intact single cells to account for the cells which were seeded and observable at the beginning of the assay, suggesting that they had died and degraded. Because I observed robust resistance to cell death in serum starvation and standard growth conditions for PC

and DPC cell lines, I wondered if the failure to form soft agar colonies represented sensitivity to a specific form of cell death caused by the loss of attachment signaling.

'Anoikis' (a neologism from Greek morphemes that roughly translates as 'homelessness') describes the process of cell death in response to loss of extracellular attachment signals (Frisch and Francis 1994). When I suspend PC and DPC cell lines in media over a non-adherent surface, I observe that both cell lines display aggressive cell death over 24 hours, but DPC cell lines are more sensitive than PC cell lines. This provided a potential explanation of the *in vivo* data, suggesting that the reason for the reduced metastasis of DPC tumors is the accelerated death of circulating tumor cells (CTCs). Notably, in the course of assessing liver metastases in this study, I had observed frequent and numerous intravascular inflammatory foci in the livers of PC and DPC mice (Figure 3.5i), which I did not observe in the $Kras^{G12D}$-driven mouse model of pancreatic cancer that I used in the studies described in Chapter 2. I now suspect that these foci represent an inflammatory reaction to dying CTCs that is characteristic of mice possessing tumors driven by RCAS-PyMT. I also observed that a portion of all metastases observed in either arm of the study grew intravascularly, further suggesting that PyMT-driven CTCs spend a significant amount of time in the vasculature prior to extravasation.

One mouse in particular, 1449#2, provided an exciting opportunity to explore the interrelationship of anoikis, extravasation and metastasis. At sacrifice, this DPC mouse possessed four large and distinct primary tumors and two distinct liver metastases. I was fortunate to be able to successfully derive cell lines from all four primary tumors

(1449#2A-D) and both liver metastases (1449#2MA and 1449#2MB), all of which are characterized in Figures 3.6 and 3.7. Fascinatingly, 1449#2MA and 2MB are simultaneously the most invasive and the most anoikis-sensitive lines across the entire study of twenty cell lines.

Based on the enhanced sensitivity of DPC cell lines to anoikis and their overall metastatic deficiency, I had originally suspected that the 2MA and 2MB lines would display increased resistance to anoikis compared to other DPC lines, hypothesizing that this would promote prolonged intravascular survival and facilitate metastasis. Strikingly, the extreme sensitivity of these cell lines to anoikis suggests that their survival in the circulation would have been severely challenged; instead it was probably their extreme invasive capacity – the highest observed of all cell lines in the study – that facilitated their rapid escape from the vasculature and successful metastasis. It is interesting to note that three of the top five anoikis-resistant DPC cell lines come from the 1449#2 mouse. This suggests that the presence of anoikis-resistant CTCs is still predictive of metastasis, but it may suggest a polyclonal model where anoikis-resistant CTCs do not themselves possess invasive capacity. Instead, anoikis-resistant CTCs could, through cell-cell contacts, provide survival signals to invasion-competent, anoikis-sensitive CTCs, thereby giving them more time to extravasate and seed metastases. Such a model would agree with my observations of intravascular tumor growth and could suggest that these are a necessary staging area for metastatic spread in this model.

10087#1 and Tp53 context

The cell line derived from mouse 10087#1 constitutes an outlier in the study because it was isolated from a Cre-negative animal which was in good health at the time of sacrifice. This animal had been injected as part of an experimental litter with RCAS-PyMT and was sacrificed to obtain control pancreas tissue alongside an experimental littermate that was showing signs of illness. It was at this time that 10087#1 was noted to have a tumor in the head of the pancreas in addition to grossly visible metastases in the liver. This tumor therefore developed in the context of intact *Tp53* and *Dicer,* making its biology distinct from that of the other PC mice. Moreover, this mouse was sacrificed in good health, which is why this animal is not included in the survival analysis and this cell line is excluded from statistical testing for differences between PC and DPC cell lines, although it is charted in Figures 3.6 and 3.7 for comparison. The behavior of this cell line in culture has implications for the influence of *Tp53* status on pancreatic cancer biology.

The cell line 10087#1 exhibits severely impaired growth compared to nearly all of the other cell lines in the study, clearly shown by its long doubling time and its exaggerated failure to form colonies in soft agar. It is sensitive to anoikis and serum starvation, but resistant to gemcitabine, and its invasive capacity is elevated, suggesting a behavioral profile similar to the metastatic cell lines 1449#2MA and 1449#2MB. These characteristics also describe the typical clinical picture of pancreatic cancer in humans (slow growing, metastatic, invasive, and resistant to gemcitabine). *TP53* is not completely lost in human pancreatic cancers, and single-copy loss is typically complemented by mutational inactivation of the second allele, suggesting an oncogenic

role for mutant TP53 in pancreatic cancer progression or maintenance (Redston et al. 1994). Indeed, the mutated allele $Tp53^{R172H}$ has been shown to cooperate with oncogenic $Kras^{G12D}$ to drive metastasis (Hingorani et al. 2005), and mutant *TP53* has been shown to promote gemcitabine resistance in human pancreatic cancer cells (Fiorini et al. 2015). Together these studies agree with the behavior of the 10087#1 cell line, and suggest that it likely possesses a mutated allele of *Tp53*. They also suggest that the metastatic cell lines from 1449#2 may have accessed similar biological pathways via some *Tp53*-independent mechanism. A recent study has demonstrated that the pro-metastatic nature of mutant *Tp53* in murine PDAC results from its ability to antagonize TP73 and alleviate TP73's transcriptional repression of the pro-metastatic growth factor receptor PDGFR (Weissmueller et al. 2014). This suggests that in the complete absence of TP53, PC and DPC cell lines would be expected to be less metastatic due to the suppression of PDGFR by TP73. Perhaps the metastatic cell lines of 1449#2 have re-expressed PDGFR to enhance their invasive capacity through silencing of *Tp73*. An in-depth comparison of the transcriptional profiles of these cell lines could reveal convergent biology between these metastatic cell lines and human pancreatic cancers.

Mechanism of anoikis sensitivity - speculation

Many classically cancer-associated growth and survival pathways have been implicated in anoikis resistance, including activation of cell surface receptors such as EGFR, IGF1R, and integrins, with subsequent downstream activation of MAPK and PI3K signaling (Galante et al. 2009; Buchheit et al. 2014). SRC activity is known to drive

anoikis resistance in pancreatic cancer cell lines (Connelly et al. 2010), and its activation can occur downstream of focal adhesion kinase (FAK) activity (Duxbury et al. 2004a; Duxbury et al. 2004b). In pancreatic cancer specifically, it has been shown that MAP3K7 is important to anoikis resistance through its induction of noncanonical WNT signaling (Yu et al. 2012b), and HMGA1 promotes anoikis resistance of MIA PaCa-2 and BxPC3 pancreatic cancer cell lines via PI3K-AKT pathway upregulation (Liau et al. 2007). These studies reveal the early stages of our understanding of anoikis, as they deal broadly with identifying the major operant signaling pathways in a few cellular contexts, but fall short of identifying unique aspects of anoikis that do not overlap with other general cell death pathways. Subsequently, there is little to go on when attempting to interpret a specific deficit in anoikis resistance that does not affect cell death responses to other stimuli, such as I observe in my characterization of DPC cell lines.

It has also been suggested that anoikis resistance depends on the ability of the upstream driver oncogene to regulate the activity of RHOB, which is a tumor suppressor that impairs soft agar colony growth and promotes anoikis. Specifically, oncogenic RAS, but not active SRC, is capable of suppressing RHOB activity (Jiang et al. 2004). This may suggest why PyMT-driven cancer cell lines, which are predominantly driven by SRC signaling, are generally deficient in soft agar colony formation and sensitive to anoikis, but this does not offer much interpretation for the additional anoikis sensitivity of DPC lines compared to PC lines. A very interesting idea has been proposed that suggests miRNA biogenesis may broadly govern cancer stress responses through an ability to

buffer aberrant gene expression triggered by mutated gene promoters and enhancers (Ebert and Sharp 2012).

The idea that Ebert and Sharp propose flows partly from the consideration of an analogy: mutations in protein coding sequences can be 'buffered' by chaperone proteins like HSP90 to generate normal cellular functions. Similarly, mutations in upstream genetic elements could contribute to aberrant transcriptional activation of genes, which could be buffered by miRNA suppression. Integral to this concept is the idea of miRNA thresholds (Mukherji et al. 2011), wherein stochastic bursts of transcription in the absence of upstream signaling (i.e. 'leaky' gene expression) is suppressed by an abundance of miRNAs targeting the comparatively low number of leaky transcripts. However, transcription driven by 'intentional' cellular signaling generates high levels of transcripts that overwhelm miRNA suppression to achieve translation. Therefore miRNAs act in part to suppress random transcriptional events by requiring a threshold level of transcription before gene expression can occur. However, in the context of impaired miRNA biogenesis, such as occurs in come cancers, stochastic transcription resulting from mutations in upstream repressive elements may achieve translation because of a reduced miRNA threshold. Ebert and Sharp propose that this random gene expression contributes to selective pressure against cancer cells that have partially lost DICER expression and accelerates the development of more malignant phenotypes.

However, the interrelationship of transcriptional noise, physiological robustness and selective pressure could be very different in the specific context of pancreatic cancer, compared to other cancers where DICER is a validated tumor suppressor. Pancreatic

cancer is characterized by pronounced genomic instability that is observable in both early and late stages of disease progression (Hingorani et al. 2005; Hruban et al. 2008; Campbell et al. 2010). This aggressive instability is driven at least partly by a high prevalence of telomere shortening and tumor suppressor loss in early PanINs. Combined with the hypoxic, ischemic and inflammatory microenvironment around PanINs, this suggests that pancreatic cancer cells innately possess a robust mutator phenotype and experience significant selective pressure at baseline. In such a setting, preserving miRNA function could be advantageous by providing robustness to an already highly dysfunctional cellular physiology. Indeed, given the long timeline of pancreatic cancer development and the high rate of passenger mutations known to occur in disease progression, loss of translational control by impaired miRNA biogenesis could be lethal by suddenly exposing cumulative mutations in gene regulatory elements to selective pressure. Similarly, a pancreatic cell line engineered to have baseline deficiencies in miRNA biogenesis may be less capable of coping with acute stress due to aggressively mutated gene regulatory elements that promote aberrant gene expression when translational precision is needed. Considering this, it could make a great deal of sense that DPC cell lines would be less able to cope with the intensely selective pressure of anoikis compared to PC lines, which would be predicted to have higher levels of miRNA processing and therefore more robust control of their transcriptional response to stress.

Profiling of human pancreatic cancers has to date focused on gene amplifications, chromosomal rearrangements, miRNA profiles, and the analysis of transcriptomes and proteomes (Buchholz et al. 2005; Prasad et al. 2005; Lee et al. 2007; Jones et al. 2008;

Zhang et al. 2009; Campbell et al. 2010; Yachida et al. 2010; Que et al. 2013; Kim et al. 2014). However, no comprehensive study of mutations in upstream genetic elements including promoters and regulatory elements has yet been performed. Perhaps such an analysis would reveal a higher than average rate of mutations in pancreatic cancers in these genomic regions compared to other carcinomas, which might suggest a need for downstream regulation of gene expression by miRNAs. Hypomethylation of gene promoters is known to increase gene expression in cancer, and could exert selective pressure to maintain miRNA biogenesis as a means to balance deregulated transcription. However, evidence exists to support both hypermethylation as well as hypomethylation of gene promoters in pancreatic cancer, suggesting that the apparent requirement for miRNA biogenesis in this context is not related to the widespread hypomethylation of gene promoters (Omura et al. 2008; Tan et al. 2009; Yi et al. 2013). Further work is needed to determine the impact of passenger mutations in pancreatic cancer and what relationship they may play in governing transcriptional control. Such an analysis could lead to an improved understanding of the broad need for miRNA biogenesis in pancreatic cancer.

Final thoughts

The results of this study indicate that *Dicer* plays an important role in conferring physiological robustness to pancreatic cells, both in normal tissue homeostasis as well as in a cancer context. I have thoroughly characterized the behavior of a large panel of cancer cell lines from the tumor study, and have demonstrated a deficit in anoikis

resistance for DPC cell lines that aligns with the metastasis deficiency of DPC tumors. However, the mechanism of this deficiency remains to be determined. I have discussed the potential for general miRNA expression levels to buffer cellular responses to stress, but I have not yet determined whether specific miRNA-mRNA relationships could be differentially regulated in DPC cells that would explain their sensitivity to anoikis.

In pursuit of this, I am collaborating with Mike Lee to perform multivariate analysis of small RNA sequencing from five PC cell lines and five DPC cell lines. The goal of this analysis is to compare each cell line's miRNA expression profile to its phenotypic profile and determine across the entire set if predictive relationships exist between particular miRNAs and particular phenotypes. Our hope is that we will discover miRNA networks that could implicate particular cellular pathways or proteins in the anoikis resistance of PC cell lines. We could then validate a model based on these relationships by analyzing the miRNA expression of cell lines which have been phenotypically characterized but were not included in the sequencing set. However, if no relationships exist between the sequencing data and the degree of anoikis resistance, it would suggest that this phenotype is governed by something that we have yet to measure. This scenario could support the miRNAs-as-buffers model previously discussed, but that would also be very difficult to validate, requiring fairly nuanced biochemical analyses of a large number of cell lines to determine differences in transcriptional and translational rates in response to stress. It will certainly be interesting to see where the data leads over the next few months.

Materials and Methods

Cell Culture

All cell lines were grown in high glucose DMEM (Life Technologies #11965) supplemented with 10% fetal bovine serum (Atlanta Biologicals #s11150) and 100U/ml penicillin/streptomycin (Life Technologies #15140). Cell lines were maintained at subconfluent densities on tissue culture-treated dishes and periodically thawed fresh from frozen stocks to maintain low passage number.

Proliferation, migration, invasion, serum starvation, and soft agar colony formation assays were carried out as described previously (Chapter II; Materials and Methods). Gemcitabine survival experiments were plated at 10^5 cells per well of a 24-well plate and allowed to adhere for 24 hours prior to treatment with 200nM gemcitabine. Forty-eight hours after treatment, live and dead cells were counted by trypan blue exclusion. Anoikis assays were performed by plating a suspension of $2.5x10^5$ cells per well of a 24-well plate pre-coated with poly(2-hydroxyethyl methacrylate) ('poly-HEMA'; Sigma-Aldrich #P3932). Suspended cells were plated in a 1:1 mixture of 2x complete media and 2% methylcellulose to prevent cell-cell interactions (Sigma-Aldrich #M7140). Twenty-four hours after plating, cells were collected, separated from methylcellulose medium by centrifugation and counted by trypan blue exclusion.

DF1 chicken fibroblasts were maintained at 39˚C in a humidified tissue culture incubator. Production of RCAS virus was initiated by transfection of 5ug of RCAS-PyMT into a subconfluent 10cm plate of cells using Superfect (QIAGEN #301305). Viral titers were confirmed by serial dilution of viral supernatant onto naïve DF1 cells and PCR

of genomic DNA to confirm the presence of the RCAS envelope protein. Titers were required to be high enough to generate detectable infection of naïve DF1 cells at a dilution of $1:2x10^7$. Prior to injection into mice, cells were trypsinized, suspended in complete media, and counted. Cells were then pelleted by centrifugation and suspended in serum-free media at a final concentration of $1x10^8$ cells/ml. Primer sequences for the RCAS PCR reactions are: Forward: CAGTCTCTCCCTAACATTAC; Reverse: CTACCTTGTGTGCTGTCGACC.

Histological Stains

Mouse tissues were fixed, processed, cut and stained as described previously (Chapter II; Materials and Methods).

Mouse Breeding, Euthanasia, and Necropsy

All mice generated in this study were housed in a dedicated facility in accordance with guidelines set forth by the Institutional Animal Care and Use Committee. The alleles for *Ptf1a-Cre* (Kawaguchi et al. 2002), $Tp53^{flox}$ (Jonkers et al. 2001), and $Dicer^{flox}$ (Mudhasani et al. 2008) have been described previously. $Dicer^{flox}$ mice were a generous gift from Steve Jones at the University of Massachusetts.

For the tumor study, *Elastase-tva,* $Dicer^{flox}$, $Tp53^{flox}$ mice were bred to homozygosity and subsequently crossed *Elastase-tva,* $Dicer^{flox/wt}$, $Tp53^{flox/flox}$ mice to generate littermate PC and DPC animals. Three day-old pups were injected with DF1 chicken fibroblasts producing high-titer RCAS-PyMT, as previously described (Ahronian

and Lewis 2014). Animals were then monitored as described above for signs of illness and euthanized appropriately.

APPENDIX A

Preclinical Assessment of a Triple-Drug Regimen for the Treatment of PDAC

Introduction

Preclinical trials are absolutely necessary for the discovery of new therapeutic regimens to treat advanced pancreatic cancer. The work of a previous graduate student in the lab, Victoria Appleman, demonstrated that $KRAS^{G12D}$ or $BRAF^{V600E}$-driven pro-survival signals are partly mediated by autocrine signaling through the IGF1 receptor (IGF1R) by IGF2 (Appleman et al. 2012). She demonstrated that pancreatic cancer cells are resistant to inhibitors of MEK or IGF1R signaling when given singly, but a combination of the two inhibitors can stimulate growth arrest and cell death. Based on these promising data, we decided to attempt the therapeutic treatment of mice using MEK and IGF1R inhibitors in combination with gemcitabine.

We designed and executed a two-step preclinical trial beginning with a toxicity study to explore the tolerability of a variety of treatment regimens. Based on the results of the toxicity study, a treatment study was performed that suggests a slight benefit of combined MEK/IGF1R treatment with gemcitabine over gemcitabine alone, but these data do not achieve significance. This study requires follow-up to confirm treatment efficacy in target tissues and to explore whether adaptations in tumors reflect selective pressure to overcome the intervention. The completion of this study will inform our understanding of the therapeutic potential for IGF1R inhibition in the treatment of pancreatic cancer.

Results

We first performed a toxicity trial in healthy C57/Bl6 animals to investigate the tolerability of various experimental cocktails of MEK and IGF1R inhibitors with gemcitabine. The dosages and abbreviations for these cocktails are given in Table A.1. Four mice were enrolled into each of the twelve arms of the study. We chose to use the inhibitors in a daily gavage regimen to limit toxicity associated with less frequent larger doses and also to enhance pharmacological efficacy. The IGF1R inhibitor BMS-754807 and the MEK inhibitor PD-0325901 were used at 50 and 5mg/kg/day, respectively. We additionally tested two gemcitabine dosages: 75 and 150mg/kg. Gemcitabine was delivered by intraperitoneal injection twice weekly, at least three days apart, at which time the animals were also weighed. Treatment proceeded for four weeks in age-matched C57/Bl6 mice specifically purchased from Jackson Labs for the study. At the end of four weeks, animals were euthanized and weighed, and samples were obtained for biochemical (serum, frozen pancreas and frozen liver) and histological profiling (pancreas, liver, kidney, bowel, heart, lungs).

Overall, treatment regimens were well-tolerated as determined by weight maintenance and overall health (Figure A.1). PBG150 was deemed toxic because of ubiquitous weight loss and 50% mortality during treatment. Histological analysis of the tissues of these mice revealed unremarkable histology in the lungs, heart, kidneys and liver, but prominent dysplasia in bowel crypts (Figure A.2). Crypt dysplasia was observed in all high-dose gemcitabine groups and in low-dose gemcitabine combined with MEK inhibition (Table A.2), and consisted largely of enhanced nuclear

Table A.1 Drug regimen dosages.
Drug dosing was based on an average mouse weight of 25g, such that a gavage of 100ul delivered 50 and 5mg/kg of the BMS and PD drug, respectively. The two different gemcitabine dilutions in phosphate buffered-saline (PBS) were similarly calculated to deliver 75 or 150mg/kg in 100ul volume (i.e. 19 or 38mg/mL based on a 25g average body weight).

	Drug Regimen Code:	V	P	B	PB	G75	PG75	BG75	PBG75	G150	PG150	BG150	PBG150
Oral Gavage (100ul)	BMS-754807 in PEG:H2O			+	+			+	+			+	+
	PEG:H2O	+	+			+	+			+	+		
	PD-0325901 in HPM-T		+		+		+		+		+		+
	HPM-T	+		+		+		+		+		+	
IP Inj. (100ul)	38mg/mL Gemcitabine in PBS									+	+	+	+
	19mg/mL Gemcitabine in PBS					+	+	+	+				
	PBS	+	+	+	+								

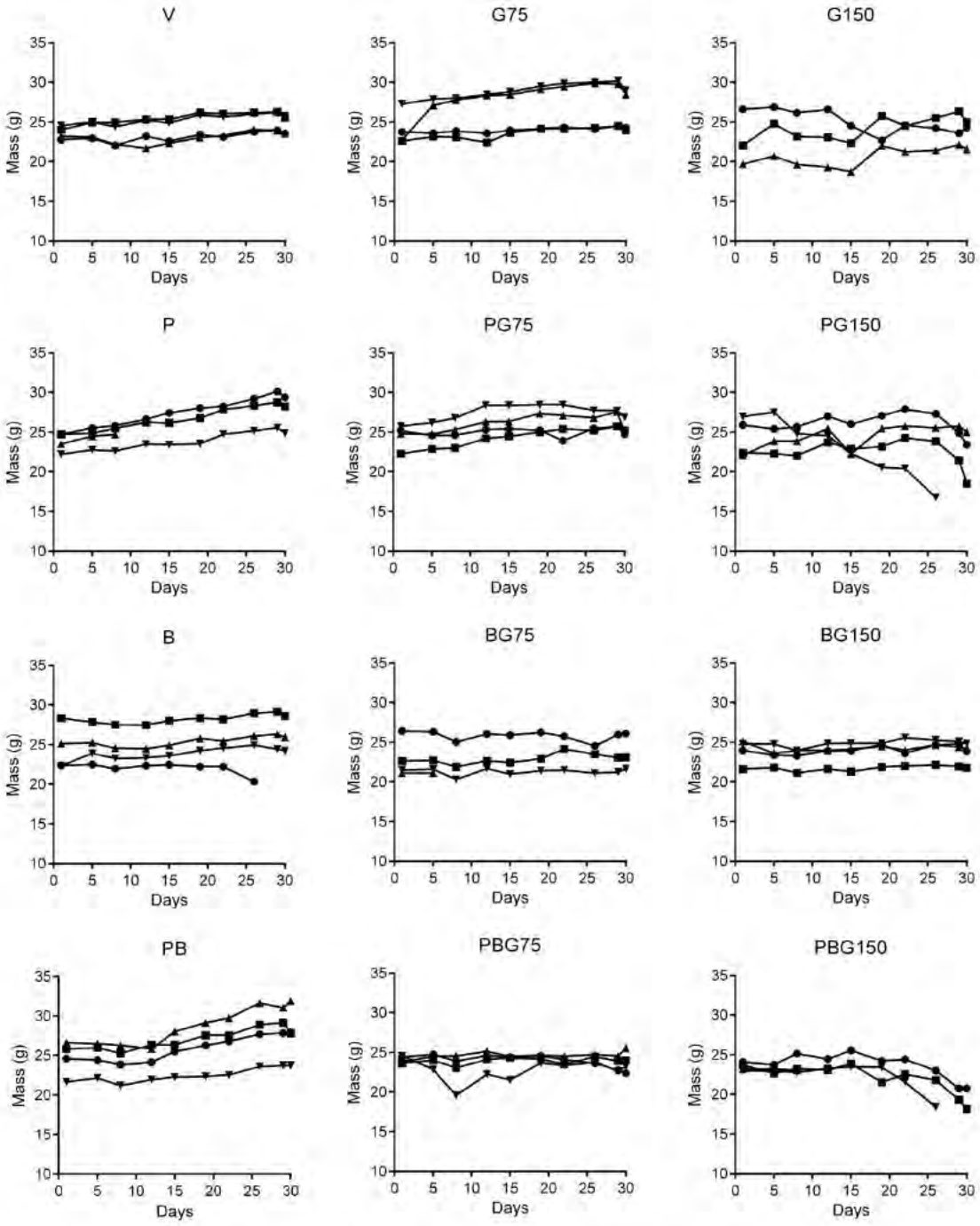


Figure A.1 Weight trends are stable for low-dose gemcitabine regimens.
Twice-weekly weights are shown for the duration of treatment (day 1 is weight at enrollment, day 30 is weight at sacrifice). Triple-drug therapy with high-dose gemcitabine (PBG150) caused weight loss in all animals and early death in two. Triple-therapy with low-dose gemcitabine was well-tolerated.

Table A.2 Histological analysis of tissues from the toxicity study.
'-': unremarkable histology; 'B': Ballooning degeneration; 'D': Dysplasia; 'I': Adipose Inflammation; 'H': Hydronephrosis

Animal	Pancreas	Liver	Kidneys	Bowel	Heart	Lungs
V#1	-	-	-	-	-	-
V#2	-	-	-	-	-	-
V#3	-	-	-	-	-	-
V#4	-	-	-	-	-	-
P#1	-	-	-	-	-	-
P#2	-	-	-	-	-	-
P#4	-	-	-	-	-	-
B#2	-	B	H	-	-	-
B#3	-	B	-	-	-	-
B#4	-	B	-	-	-	-
PB#1	-	B	-	-	-	-
PB#2	-	B	-	-	-	-
PB#3	-	B	-	-	-	-
PB#4	-	B	-	-	-	-
G75#1	-	-	-	-	-	-
G75#2	-	-	-	-	-	-
G75#3	-	-	-	-	-	-
G75#4	-	-	-	-	-	-
PG75#1	-	-	-	-	-	-
PG75#2	-	-	-	-	-	-
PG75#3	-	-	H	D	-	-
PG75#4	-	-	-	D	-	-
BG75#1	-	B	-	-	-	-
BG75#2	-	B	-	-	-	-
BG75#4	-	B	-	-	-	-
PBG75#1	-	B	-	D	-	-
PBG75#2	-	B	-	D	-	-
PBG75#3	I	B	-	D	-	-
PBG75#4	I	B	-	D	-	-
G150#2	-	-	-	-	-	-
G150#3	-	-	-	-	-	-
G150#4	-	-	-	-	-	-
PG150#1	-	-	-	-	-	-
PG150#2	-	-	-	D	-	-
PG150#3	-	B	-	D	-	-
BG150#1	I	B	-	-	-	-
BG150#2	I	B	-	D	-	-
BG150#3	I	B	-	D	-	-
BG150#4	I	B	-	D	-	-
PBG150#1	-	-	-	D	-	-
PBG150#2	I	-	-	D	-	-

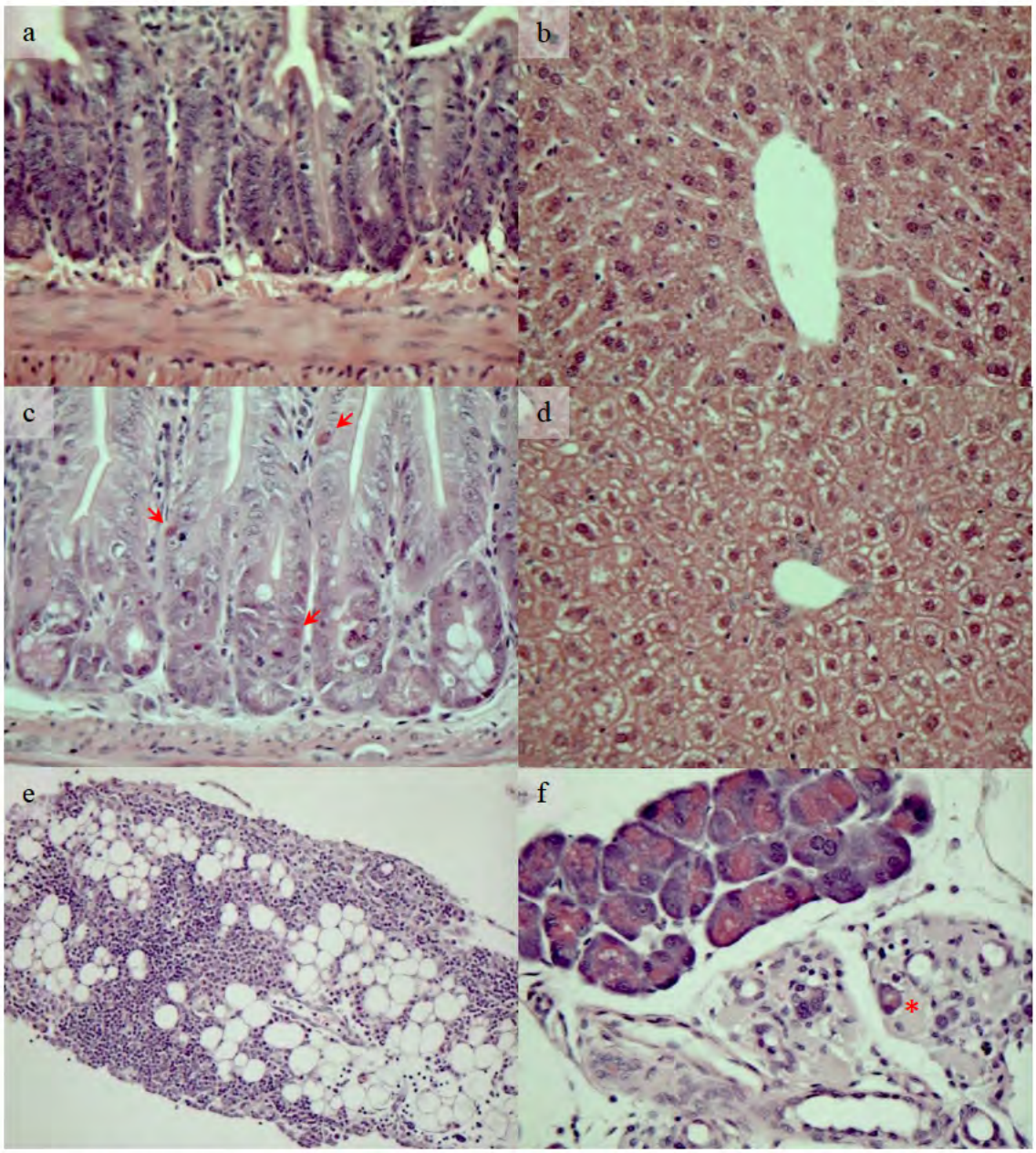


Figure A.2 Representative histological findings from the toxicity study.
Normal intestine (**A**) and liver (**B**) are provided for comparison to crypt dysplasia (**C**) and ballooning degeneration (**D**). Inflammatory processes were observed in adjacent loose connective tissue (**E**) and associated with vacuolization of acinar cells and hyalinization (**F**). Arrows denote eosinophils within intestinal epithelium, asterisk marks a degenerating acinus surrounded by fibrous and inflammatory stroma.

euchromasia, disruption of nuclear polarity, inflammatory infiltrates, and increased cell death (Figure A.2b). Dysplasia was most severe in PBG150 animals, which also produced lightly colored, greasy stools, indicating malabsorption as a primary cause of weight loss. Additional histological findings relevant to treatment regimens included inflammation in the visceral adipose of PBG75, PBG150, and BG150 animals, which may be associated with tissue damage (Figure A.2e,f). Ballooning degeneration of hepatocytes was also observed in all mice treated with IGF1R inhibitor (Table A.2, Figure A.2d). Based on these data, PBG75 was chosen as the best-tolerated triple-drug regimen to be bested in the treatment study.

During the treatment study, most mice maintained weight throughout treatment, with the notable exception of animals in the B cohort (Figure A.3). A disproportionate number of these animals succumbed to disease-related signs and symptoms (e.g. cachexia, jaundice, hunching, pain), but this association is not statistically significant. No statistically significant survival differences exist across the eight arms of the therapeutic trial, although there is the suggestion of a survival benefit of 22 days for the triple-treatment regimen compared to gemcitabine alone (Figure A.4).

Enrollment for the PBG groups was especially difficult due to occasional sepsis and death immediately following intraperitoneal injection (there were four iatrogenic mortalities for this group during enrollment). Necropsy of these animals revealed ascites and pruritic exudates in the IP cavity typically in the presence of an enlarged cecum that was visibly full of gas bubbles. Therefore death was due to the murine equivalent of ruptured appendicitis. This was most likely secondary to the small bowel pathology

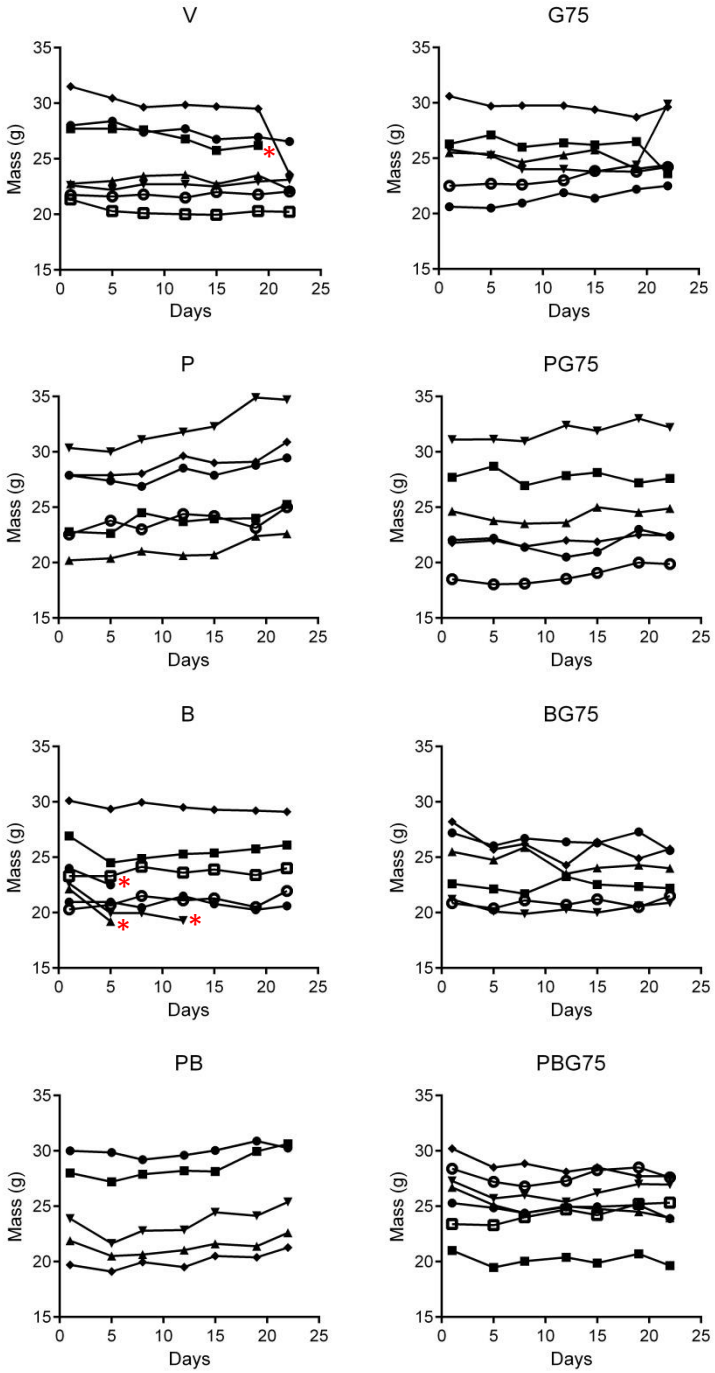


Figure A.3 Weight trends are stable for low-dose triple-therapy; unopposed IGF1R inhibition carries higher tumor-related mortality.
Weight measurements are plotted for the 21 days of active treatment. Asterisks highlight deaths related to disease during active therapy.

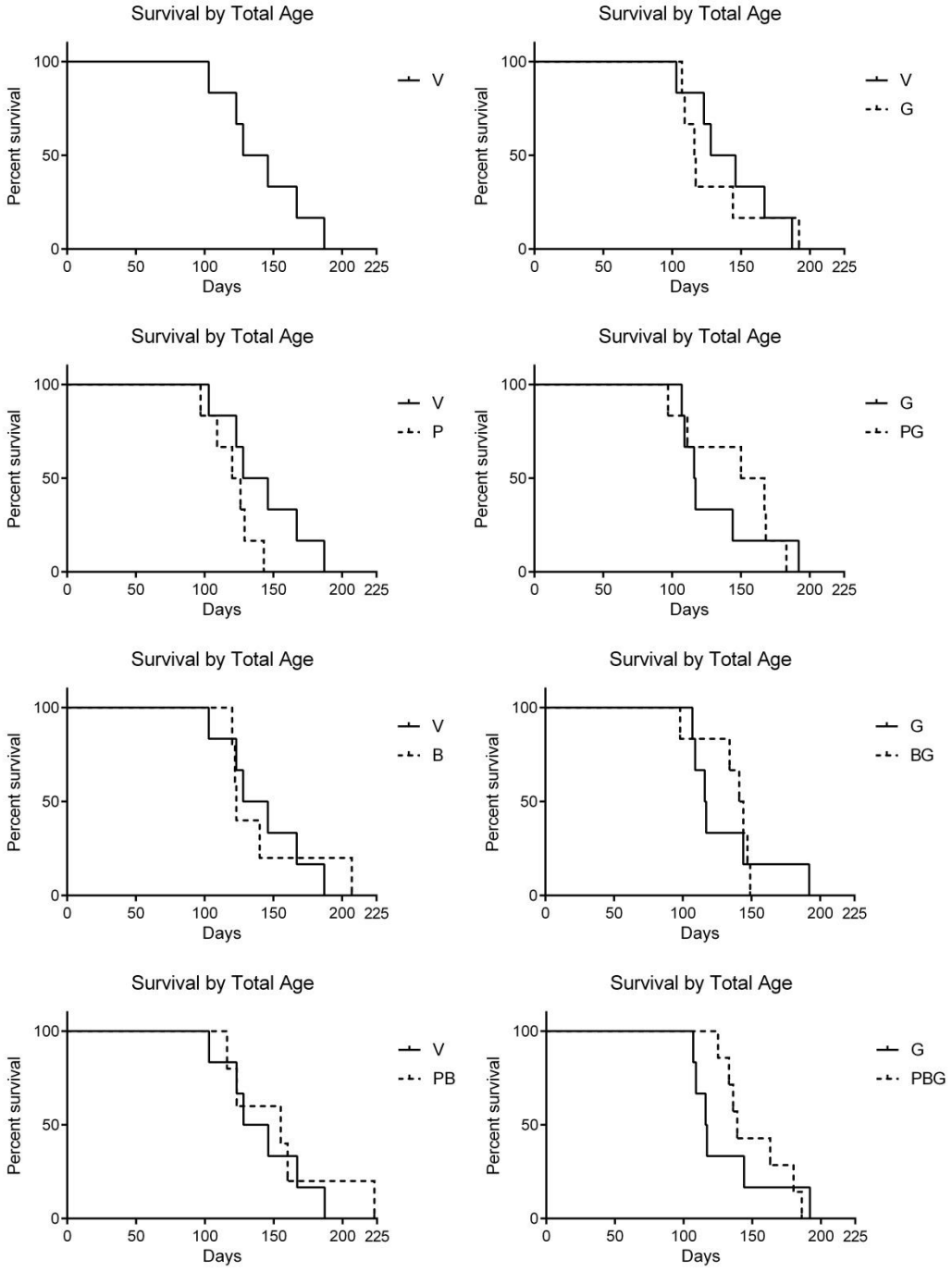


Figure A.4 No significant survival difference is seen with triple-drug therapy.
There is an observable right-shift of 23 days in the survival curve of PBG-treated mice compared to gemcitabine alone, but the difference is not significant.

observed for this regimen in the toxicity study (i.e. malabsorption in the small intestine allowed nutrients to enter the large bowel, stimulating floral expansion and gas production and creating a toxic, enlarged cecum vulnerable to needle puncture).

Discussion

Targeting downstream pathways of KRAS signaling remains one of the most promising avenues of therapeutic intervention for the treatment of advanced pancreatic cancer. Initial work performed *in vitro* suggested efficacy against PDAC cell lines when treating with a combination of MEK and IGF1R inhibitors alongside gemcitabine, but our preclinical trial failed to generate statistically significant results. The data do suggest a benefit of an additional 22 days of survival for the PBG regimen, but the lack of statistical significance may simply indicate that our study was underpowered to detect this magnitude of a difference. A repeat trial focusing on this regimen with a higher enrollment could reveal if this difference is repeatable and significant. Indeed, in the treatment of pancreatic cancer, such a modest improvement in survival would be consistent with therapeutic advancements of the past, and should not be lightly discarded for being small in magnitude. However, prior to any repeat trials, a significant body of work remains to be completed with regards to immunohistochemical characterization of the tumors from this trial, specifically addressing ki67 and CC3 positivity and AKT, ERK, and IGF1R activity. It is reasonable to hypothesize that if the treatment did generate a partial clinical response, that the tumors which recovered after treatment may exhibit signs of a specific resistance to the therapy, including overexpression of IGF1R, EGFR, or MEK, hyper-activation of $KRAS^{G12D}$, or overexpression of members of the ABC family of multi-drug-resistance transporters.

Future trials should also include a separate cohort of mice to be sacrificed on the last day of treatment, so that drug penetration and efficacy in tumor tissues can be

evaluated. One major factor in the chemotherapeutic resistance of pancreatic cancer is the physical barrier presented by the desmoplastic reaction around carcinoma cells. Dense networks of cross-linked collagen and a high oncotic pressure of the interstitial fluid inhibit drugs from diffusing out of the vasculature, through the stroma and into carcinoma cells (Neesse et al. 2011). This barrier is a significant challenge and several groups have sought ways of degrading it in order to improve drug access (Buckway et al. 2013; Meng et al. 2013). In lieu of such techniques, stromal investment must be accounted for when considering drug efficacy against a given tumor. Therefore stromal investment would need to be approximated by *in vivo* imaging at the time of enrollment in any future preclinical trials of the PBG regimen in order to accurately control for this variable across treatment groups. Together with a separate cohort for end-of-treatment histological analysis, this approach would yield definitive data on biochemical efficacy.

Another lingering curiosity from this study is the apparently more aggressive course of disease in animals treated with IGF1R inhibition alone ('B' groups). There is a 20% chance that this observation is simply due to chance, according to a Fisher's exact test, so a concrete interpretation is not strictly warranted, but if the finding is true, it does raise some concerns. For instance, in the three animals that died during treatment, all three exhibited severe signs of jaundice, although in one animal a tumor mass was not evident and in another the tumor was quite small, although all pancreata were notably fibrotic (data not shown). This may suggest a toxic effect of IGF1R inhibition against normal bile duct epithelium that only occurs in the context of a desmoplastic reaction. It is known that VEGF receptor signaling drives nitric oxide synthesis in endothelial cells to

stimulate smooth muscle cell relaxation (Isenberg et al. 2009), and perhaps (by an admittedly generous leap of the imagination) there exists a similar paracrine signaling between the normal bile duct epithelium and the investing desmoplastic stroma of the neoplastic pancreas, such that IGF1R inhibition results in pronounced bile duct stricture. Perhaps more simply, given that neoplastic cells escape the epithelial compartment during ADM and take on myofibroblast morphology (Rhim et al. 2012), this may suggest that IGF1R inhibition triggers contraction of these cells, perhaps through activation of PKC, although the mechanism of that interaction would contradict a classical view of RAS signaling. To date there are no studies suggesting any such relationship, but given that jaundice is a mitigating factor in recovery from pancreatic surgery (Roque et al. 2015), this may be a worthwhile field of exploration.

Materials and Methods

Mouse Breeding, Dosing, Euthanasia, and Necropsy

All mice generated in this study were housed in a dedicated facility in accordance with guidelines set forth by the Institutional Animal Care and Use Committee. The alleles for *Ptf1a-Cre* (Kawaguchi et al. 2002), *LSL-Kras*^{G12D} (Jackson et al. 2001), and *Tp53*^{flox} (Jonkers et al. 2001) have been described previously.

For the toxicity study, pure C57/Bl6 strain mice were obtained from Jackson Labs at ages between six and eight weeks. For the intervention study, *Tp53*$^{flox/flox}$, *LSL-Kras*^{G12D} mice on a mixed background were crossed to *Ptf1a-Cre* mice on a C57/Bl6 background to generate *LSL-Kras*^{G12D}, *Tp53*$^{flox/wt}$, *Ptf1a-Cre* mice for enrollment.

Health status was monitored at least three times per week for all animals and weights were measured on Mondays and Fridays for the duration of treatment in both studies. Major signs of illness warranting euthanasia included severely reduced mobility with lack of arousal upon handling, hunching, abdominal distension, wasting, palpable abdominal mass, hyperventilation, and weight loss greater than 10% of total body weight in 24 hours. Notes were recorded regarding external and internal signs of illness and samples were processed as previously described (see Chapter II, Materials and Methods).

Drug administration via daily oral gavage was performed by trained animal medicine staff. Drugs were prepared and delivered twice weekly to the mouse room for use by animal medicine to ensure optimum inhibitor stability and efficacy. Intraperitoneal injections were performed on Mondays and Fridays using a 28 gauge insulin syringe.

Drug Preparation

The IGF1R inhibitor BMS-754807 is dissolved in a 4:1 mixture of polyethylene glycol (PEG) and water. This occurs by first vortexing the desired amount of drug in a volume of PEG equal to 80% of the desired final volume until drug clumps have dissociated. Water is then added to bring the solution to its final volume and vortexing is continued for one minute to ensure complete solvation of the drug. The solution can then be 0.45um filter sterilized and is shelf-stable for several weeks at 4˚C.

The MEK inhibitor PD-0325901 is dissolved in a 0.5% solution of hydroxypropylmethylcellulose (HPM) supplemented with 0.2% Tween-80 ('HPM-T'). HPM powder disperses into water at high temperatures and dissolves at lower temperatures, and the final solution cannot be filter sterilized. Therefore assembling the liquid vehicle for the PD drug takes place in the hood and requires sterile technique. Briefly, an excess of water is heated to near-boiling on a stirrer-hot plate in the hood. A second flask is assembled with a stirring rod and the desired amount of HPM powder. With constant stirring, the desired volume of hot water is added to the HPM powder and allowed to mix until the powder has completely dispersed and there are no clumps. This solution is then allowed to cool very slowly to room temperature with stirring, upon which the HPM goes into solution. Tween-80 is then added and the HPM-T is left to stir overnight. PD drug is suspended in HPM-T by sonication, and stocks require periodic sonication to resuspend precipitated drug. Drug aliquots were sonicated and delivered to the animal facility every other day to prevent precipitation prior to gavage.

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