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Integration of Transcriptomic and CRISPR-Cas9 Technologies Reveal FOXA2 as a Tumor Suppressor Gene in Pancreatic Cancer

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Integration of Transcriptomic and CRISPR-Cas9 Technologies Reveal FOXA2 as a Tumor Suppressor Gene in Pancreatic Cancer

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cellular and Integrative Physiology

by

Christina Vorvis

2016

ABSTRACT OF THE DISSERTATION

Integration of Transcriptomic and CRISPR-Cas9 Technologies Reveal FOXA2 as a Tumor Suppressor Gene in Pancreatic Cancer

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Doctor of Philosophy in Molecular, Cellular and Integrative Physiology

University of California, Los Angeles, 2016

Professor Dimitrios Iliopoulos, Chair

Pancreatic ductal adenocarcinoma (PDAC) has a median survival of six months and a five-year survival of <5%, making it one of the most lethal human cancers. This poor prognosis is due to the uniformly advanced disease stage at the time of diagnosis and to its profound resistance to existing therapies. Ductal adenocarcinomas makes up between 75 to 92% of pancreatic neoplasms. Premalignant lesions, known as pancreatic intraepithelial neoplasms (PanINs) are of ductal origin and found in close physical proximity with advanced malignant tumors. PanINs are thought to be precursors of ductal adenocarcinoma, as they progress toward increasingly atypical histological stages. Previous studies have implicated the role of different signaling pathways in the pathogenesis of pancreatic cancer, however the role of transcriptomic alterations have not been well characterized. The aim of this dissertation was to characterize the role and function of a new transcription factor gene, called FOXA2, in

the pathogenesis of pancreatic cancer. Our preliminary analysis revealed that FOXA2 gene was highly down-regulated in pancreatic cancer tissues relative to control tissue samples. Interestingly, FOXA2 gene has not been implicated previously in pancreatic oncogenesis. FOXA2 is a transcription factor that was initially identified in hepatocytes, where it binds in the promoter areas of important liver-enriched genes transthyretin, alpha 1-antitrypsin and albumin. Our data revealed that FOXA2 is significantly down-regulated in pancreatic cancer, according to qPCR and immunohistochemical analysis in human pancreatic tissues. In order to study the role of FOXA2 deletion *in vivo,* we utilized the CRISPR/Cas9 system to delete FOXA2 from the PANC-1 cell line (FOXA2Δ). Subcutaneous injections in immunodeficient mice demonstrated FOXA2Δ had a significantly higher weight and volume than the control tumors. Moreover, we identified a negative regulator of FOXA2 expression, microRNA-199a, which targets directly and reduces FOXA2 mRNA and protein expression levels in pancreatic cancer. Overexpression of microRNA-199a was observed in human pancreatic cancer tissue by qPCR and *in-situ* hybridization methods. In an effort to identify downstream targets of FOXA2, we transiently knocked-down FOXA2 with an siRNA and performed a gene expression array in a human pancreatic cell line. The expression data revealed plasminogen urokinase activator receptor (PLAUR) was significantly up-regulated upon FOXA2 inhibition. PLAUR is a marker of invasiveness and is responsible for the invasive phenotype we observed upon FOXA2 knockdown.

This dissertation of Christina Vorvis is approved.

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CHAPTER ONE

Molecular Mechanisms Involved in Pancreatic Oncogenesis

Cellular Components of the Pancreas

Cellular components of the pancreas can be regarded under six categories (151): (1) *acinar* and (2) *ductal*, which together constitute the exocrine component of the organ responsible for the production of digestive enzymes (trypsin, chymotrypsin, lipases, and others) and their transportation to the duodenum, respectively; (3) *islets of Langerhans*, the endocrine component, which is responsible for the production of hormones involved in regulation of glucose and other metabolic activities; (4) *ambiguous cells*, such as centroacinar cells, which are less well characterized; (5) *supportive elements*, which encompass fibrous tissue, vessels, nerves, and other connective tissue elements; and (6) *"potential"cells,* such as stem cells, the existence of which is evidenced based on in vitro studies, but their representatives in normal human pancreata have not yet been clearly identified in histologic sections.

Pancreatic Cancer Incidence

Pancreatic ductal adenocarcinoma (PDAC) is the predominant form of pancreatic neoplasms and accounts for >85% of the clinical cases (106). The disease develops via acinar-ductal metaplasia and neoplastic precursor lesions (127). In the United States alone, 48,960 new cases of pancreatic cancer were expected to occur in 2015, with an estimated 40,560 deaths, about the same number in women (19,850) as in men (20,710). Over 96% are cancers arising from exocrine pancreas. Endocrine carcinomas are often diagnosed at a younger age and exhibit a better prognosis. From 2007 to 2011, mortality rates increased slightly by 0.3% per year. For all stages combined, the 1- and 5-year relative survival rates are 28% and 7%, respectively. For the small

percentage of people diagnosed with local disease (9%) the 5-year survival is 26%, while more than half of patients (53%) are diagnosed at a late stage for which 1- and 5-year survival rates reach 15% and 2%, respectively (1). These numbers are a staggering example of the poor prognosis associated with PDAC.

Invasive Ductal Adenocarcinoma

Invasive ductal carcinoma is the most common neoplasm of the pancreas, constituting >85% of pancreatic tumors that come to clinical attention. It is one of the deadliest of all cancers, with a 5-year survival <5% (79). It is also responsible for a substantial number of cases with carcinomas of unknown primacy (137), because it is often widely disseminated at the time of diagnosis, and the primary tumor may not be obvious unless carefully investigated. In fact, because of its infiltrative pattern, early dissemination, and rapid fatality, the primary tumor seldom forms a compact lesion that measures more than 5 centimeters. Along the same lines, only 20% of the cases are deemed resectable at the time of diagnosis (41).

Invasive ductal adenocarcinoma has some morphologic characteristics that are fairly unique to this type of cancer. It has a well-differentiated glandular pattern (**Figure 1**) but also has a strong capacity for infiltration. It is not uncommon for individual malignant glands to be identified far away from the main tumor, as these glands have a remarkable affinity for perineurial and vascular invasion. Perineurial invasion is identified in close to 80% of the cases and is thought to contribute to back pain, one of the more common symptoms of pancreas cancer. Vascular invasion is also common, and may be responsible for the rapid spread of the carcinoma cells.

Another aspect of pancreatic ductal adenocarcinoma is the degree of intratumoral heterogeneity, which is reflected in the frequent mixture of glandular and solid

(nonglandular) patterns as well as in the high degree of genetic instability (66). The cells in pancreatic adenocarcinoma are typically cuboidal with variable amount of cytoplasm that contains mucin and mucin-related glycoproteins, and may occasionally demonstrate predominance of a specific organelle creating distinctive patterns such as “foamy-gland” (with swollen, altered mucin), “cell-cell” (abundant glycogen), and “oncocytoid” or “hepatoid” variants with prominent mitochondria or lysosomes, respectively (133).

One of the most characteristic features of ductal adenocarcinoma is desmoplastic stroma (**Figure 2**). Perhaps more so than in any other cancers, pancreatic adenocarcinoma is associated with abundant fibroinflammatory changes in the stroma, to a degree that there is often a lot more stromal tissue than invasive carcinoma cells in a given tumor. This phenomenon creates major problems for both diagnosticians and researchers. This is an important pitfall, in particular for studies that utilize “global” arrays, which do not discriminate between the cellular compartments of the specimen; thus the importance of immunohistochemical or in situ hybridization methods in verification.

Key Signaling Pathways in Pancreatic Cancer

K-Ras Signaling Pathway

Over two decades ago, the K-Ras gene was identified. Activating mutations of K-Ras constitute one of the most frequently activated oncogenes, with 25% of all human tumors harboring these mutations (46). K-Ras encodes a ~21 kDa small GTPase, which cycles between GTP-bound active and GDP-bound inactive states. GTPase-activating proteins (GAPs) induce hydrolysis of GTP resulting in K-Ras inactivation. Activating mutations of K-Ras found in human PDAC impair intrinsic GTPase activity of the K-Ras

protein and prevent its interaction with GAPs. This leads to constitutive activation of K-Ras and persistent stimulation of downstream signaling pathways including sustained proliferation, metabolic reprogramming, anti-apoptosis, remodeling of the tumor microenvironment, evasion of the immune response, cell migration and metastasis (139). K-Ras is conceived as a principal initiator of pancreatic cancer, based on the early occurrence of K-Ras genetic alterations (mostly point mutations at codon G12) in PDAC formation (85), as well as their prevalence at the time of diagnosis (>90% of the clinical cases) (98). In the same direction, recently developed genetically engineered mouse models expressing mutated K-Ras in the pancreas, sufficiently initiate development of neoplastic precursor lesions that are histologically identical to those in humans (68). A recent clinical trial, describes a clinically applicable siRNA delivery method that seems to overcome the major obstacles of toxicity and organ accessibility. The miniature biodegradable implant, siG12D-LODER™ in combination with chemotherapy displayed potential efficacy in patients with locally advanced pancreatic cancer. Though, the prolonged clinical benefit warrants further evaluation of this agent in combination with chemotherapy (57). However, K-Ras is still widely considered undruggable (16) and attention is now focused on the therapeutic exploitation of its effector pathways that contribute to PDAC initiation, progression and maintenance.

Over the last several years, a handful of miRNAs have been identified as regulators of the K-Ras signaling pathway in pancreatic oncogenesis. Data obtained by locked nucleic acid in situ hybridization (LNA-ISH) and real-time quantitative polymerase chain reaction showed that miR-217 is frequently down-regulated in PDAC tissues. Manipulation of miR-217 expression provides evidence for its role as a tumor suppressor that exerts its function by specifically targeting the K-Ras oncogene (196). Another study identified miR-96 as a potent regulator of K-Ras signaling. As evidenced

by a subset of *in vitro* and *in vivo* assays, miR-96 directly targets K-Ras, negatively regulates the phosphorylated AKT signaling pathway downstream of K-Ras and exerts anti-proliferative, pro-apoptotic, and anti-metastatic effects (187). Mechanistic studies also demonstrated that down-regulation of miR-126 and let-7d contributes to PDAC transformation by posttranscriptional up-regulation of K-Ras (81). LNA-ISH analysis was used to confirm increased production of miR-21 in human PDAC. MiR-21 has recently been linked to the activation of the transcription factor AP-1 in response to RAS (154) and thus not surprisingly, precursor lesions originating from the K-Ras(G12D) model display high concentrations of this miRNA. Accordingly, it has been shown that activated K-Ras(G12D) stimulates the miR-21 promoter in human pancreatic cells.

Moreover, EGFR production, which is intensified in PDAC, promotes miR-21 expression in PDAC-derived cells (42). It has been recently reported that miR-206 is abrogated in PDAC specimens and cell lines and exerts tumor suppressive effects through combinatorial targeting of the K-Ras and Annexin A2 (ANXA2) oncogenes. This study revealed the role of miR-206 as a negative regulator of oncogenic K-Ras-induced nuclear factor-κB (NF-κB) transcriptional activity, resulting in a concomitant reduction of the expression and secretion of pro-angiogenic and pro-inflammatory factors. Suppression of the potent prolymphangiogenic factor vascular endothelial growth factor C takes place through an NF-κB-independent mechanism. Notably, re-expression of miR-206 in PDAC cells seems to be sufficient to inhibit tumor blood and lymphatic vessel formation, thus leading to a significant delay of tumor growth and progression (88).

Cell Cycle Signaling Pathway

Synthesis of cyclin D and activation of cyclin-dependent kinases 4/6 (CDK4/CDK6) constitute an early response to growth factor stimulation (97, 134). Subsequently, cyclin D-CDK4/6 phosphorylates the retinoblastoma (Rb) protein, a well-known tumor suppressor that binds and inhibits the transcription factor E2 factor (E2F) (94). As a result, E2F is released and mediates transcription of genes whose products are required for cell cycle progression and DNA replication (26). Cyclin D1 overexpression and p16 inactivation are very common events in pancreatic cancer, highlighting the importance of disrupting G1 progression to disease development (27, 50). The inactivation of multiple regulatory pathways of the cell cycle may account for the aggressiveness of pancreatic cancer.

Extensive studies indicate that miRNAs play critical roles in pancreatic cancer development and progression. Zhao and colleagues revealed that miR-192 promotes cell proliferation and facilitates cell cycle progression through the G1 to S-phase in pancreatic cancer cell lines. The growth-promoting effects of miR-192 enforced expression were indicated in the *in vitro* colony formation assay and in a xenograft tumor model *in vivo*. Interestingly, reduced expression of CDK inhibitors and RB family members occurred upon miR-192 overexpression, while increased expression of positive cell cycle regulators such as cyclin D1, cyclin D2, CDK4, CDC2 and S-Phase Kinase-Associated Protein 2 was observed (193). Another study demonstrated that miR-301a promotes pancreatic cancer cell proliferation, at least in part, by directly targeting the 3'UTR of Bim (28). Bim serves as an apoptotic stimuli sensor and initiates apoptosis through activation of multi-domain pro-apoptotic proteins such as Bak and Bax (171).

Activation of the extracellular signal-regulated kinases (ERK) pathway is known to protect pancreatic tumor cells from apoptosis, as well as to regulate their progression in the cell cycle (18). MiR-424-5p was found to be frequently up-regulated in pancreatic cancer specimens and modulate ERK1/2 signaling pathway by negatively regulating suppressor of cytokine-induced signaling 6 (SOCS6). Data further supporting this view, show that down-regulation of miR-424-5p inhibits the expression of SOCS6 downstream targets, such as Bcl-2 and Myeloid Cell Leukemia 1 (MCL1), thus attenuating the ERK pathway activity (175). A recent study unveiled the involvement of miR-193b-mediated de-regulation of the K-Ras axis in pancreatic carcinogenesis. Specifically, miR-193b was found to function as a cell-cycle brake in PDAC cells by inducing G1-phase arrest and reducing the fraction of cells in S phase, thereby leading to decreased cell proliferation. Malignant transformation phenotype of PDAC cells was also modulated by miR-193b via anchorage-independent growth suppression. Mechanistically, K-Ras was verified as a direct effector of miR-193b, through which the AKT and ERK pathways were modulated and cell growth of PDAC cells was suppressed (82).

Notch Signaling Pathway

Molecular knowledge of the Notch signaling pathway with respect to pancreatic cancer is considered important for discovering new drugs and the design of novel therapeutic strategies against pancreatic cancer. Notch signals are known to affect stem cell self-renewal and differentiation, and have been suggested to play a role during pancreatic carcinogenesis. It is conceived that activation of Notch and K-Ras pathways displays synergistic effects in the initiation of pancreatic carcinogenesis (40). Interestingly, the concurrent inhibition of the EGF and Notch pathways results in decreased cell proliferation, with concomitant increase in cell apoptosis. Blockage of Notch signaling

cascade has also been shown to result in attenuation of NF-κB activity and up-regulation of the p21 and p27 (166).

A growing number of miRNAs has been shown to crosstalk with the Notch pathway in human PDAC. Preclinical studies have shown that TP53 directly regulates miR-34, which further downstream targets Notch, indicating a role in the maintenance and survival of PDAC initiating cells. Markedly, treatment of pancreatic cancer stem cells (CSCs) with chromatin-modulating agents resulted in suppression of the miR-34a putative targets Bcl-2, CDK6 and Sirtuin 1 (130). Moreover, restoration of miR-34 expression in pancreatic CSCs down-regulates Notch-1 and -2, while tumor initiating cells display high levels of Notch-1 and -2, consistent with the loss of miR-34 expression. These results imply that self-renewal of pancreatic tumor initiating cells relates to direct modulation of Notch by miR-34 (80). As illustrated by Brabletz and colleagues, miR-200 members target Notch pathway components, such as Jagged1 and the mastermind-like coactivators Maml2 and Maml3, thereby mediating enhanced Notch activation by the activator of EMT, ZEB1. These results provide a link for the latter and its cancer promoting properties to Notch activation (20). Furthermore, examination of mouse xenografts lacking expression of the pancreatic stem cell marker, DCLK1, revealed increased miR-145, let-7a and miR-200 levels resulting in significant reduction of pluripotency and EMT-related transcription factors, along with inhibition of Notch1 via miR-144 (153).

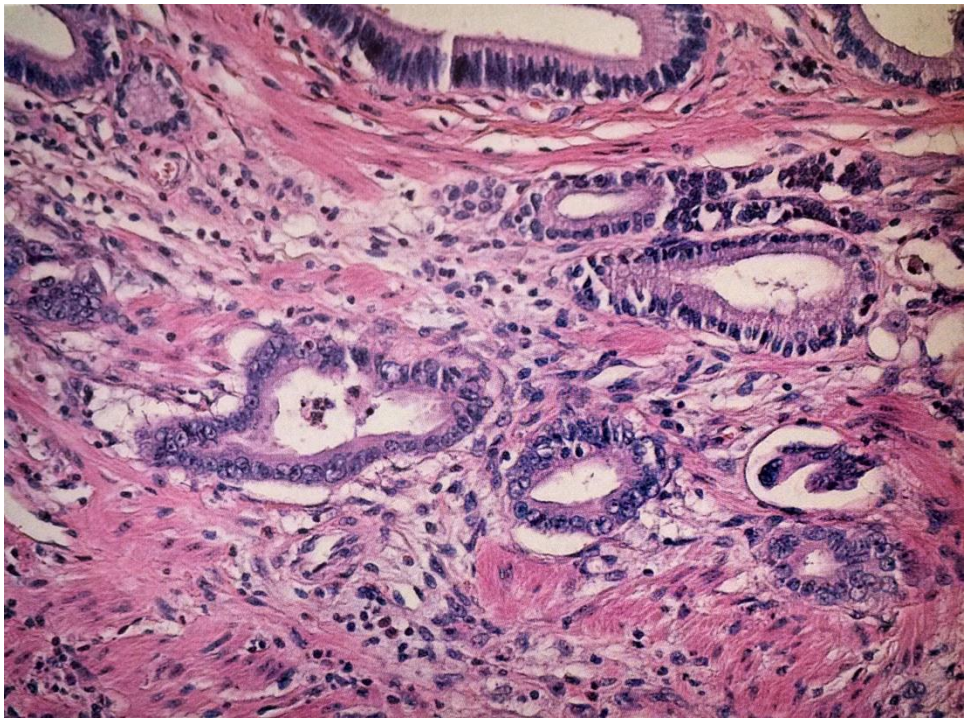

Figure 1.1. Invasive Ductal Adenocarcinoma.

Invasive ductal adenocarcinoma, well differentiated. Widely separated, well-formed glandular units lined by one to two layers of cuboidal cells showing nuclear atypia. (*Adapted from N.V. Adsay et al.)*

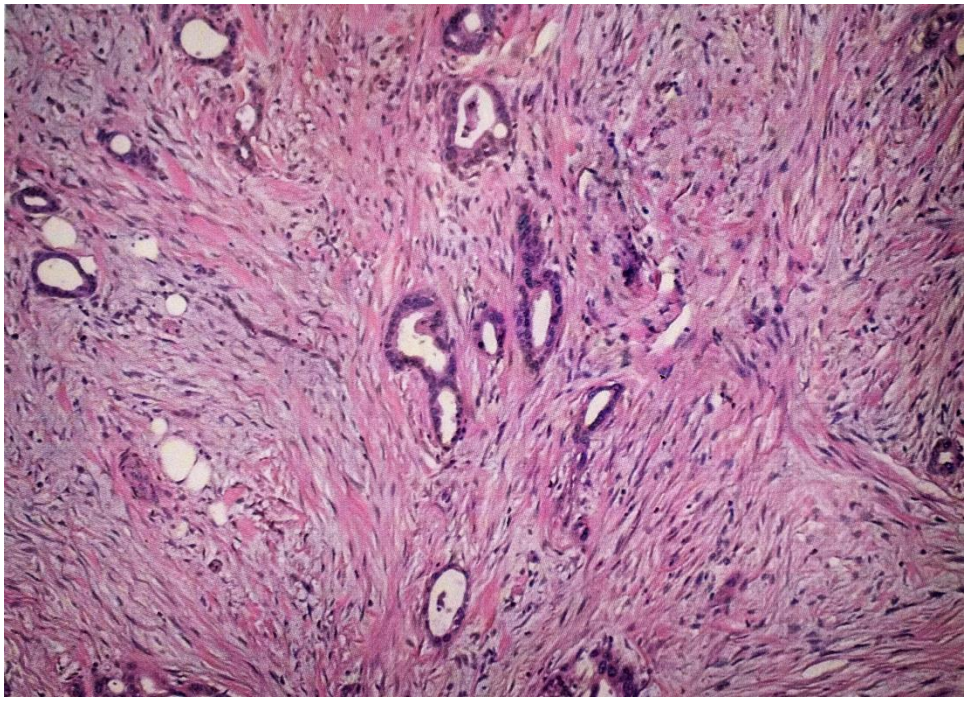

Figure 1.2. Desmoplasia.

Desmoplasia is the fibroinflammatory reaction of stroma which is often abundant in ductal adenocarciona. The tumor consists of well-formed glands surrounded by abundant desmoplastic stroma. (*Adapted from N.V. Adsay et al.)*

CHAPTER TWO

MicroRNA Signaling Pathways in Pancreatic Cancer

Overview

While new therapeutic options are emerging for non-hematologic malignancies molecular-targeted therapies for pancreatic cancer have failed to make any positive impact on patient survival. In 1993, the Nobel-Prize winning discovery of small interference RNAs (siRNAs) led to an outburst of knowledge on RNA interference and gene regulation (103). Since then, thousands of research studies have described the functional role of small non-coding RNAs, named miRNAs, in a vast panel of human pathologies. In 2002, a small genomic region in chromosome 13q14 consisting of miR-15a and miR-16-1 genes was found to be commonly deleted in chronic lymphocytic leukemia, suggesting a link between miRNAs and human tumors (23). Typically acting as negative regulators of gene expression, miRNAs have provided new directions for research on mechanisms underlying disease, serving as potential targets for therapeutic intervention.

MicroRNA Biogenesis

MiRNAs pair to the messages of protein-coding genes, ultimately regulating gene expression through mRNA cleavage or translational inhibition (11)-(105). The miRNA biogenesis pathway (**Figure 1**) has an important role in gene regulatory networks, considering that a) more than 1,000 individual miRNA genes have been identified; b) an individual miRNA can target hundreds or thousands of different mRNAs and c) an individual mRNA can be coordinately suppressed by multiple different miRNAs. Numerous studies have revealed that miRNAs mediate essential processes such as cell proliferation, apoptosis and inflammation (30) Importantly, miRNAs have emerged as

critical players in cancer initiation and progression by modulating pathological aspects related to tumor development, growth, metastasis, and drug resistance (37)-(178). Pointing to this direction, overexpression of tumor suppressor miRNAs or inhibition of oncogenic miRNAs has shown therapeutic potential in model systems (86). Mounting evidence highlights the aberrant expression of miRNAs in various types of cancer (116, 160) supporting the notion that miRNAs potentially serve as better indicators of tumor prognosis than conventional protein-coding gene arrays.

MicroRNA Mechanism of Action

MicroRNAs can bind with complementarity or imperfect complementarity to each strand of the double stranded RNA and depending on that can regulate the gene expression (**Figure 2**)(113). There are three major currently-known mechanisms for miRNA-mediated gene regulation: translation repression, direct mRNA degradation and miRNA-mediated mRNA decay (116, 160). Which mechanism controls gene expression is entirely dependent on the degree of miRNA complementarity to their targeted mRNAs. MicroRNAs bind, in most cases, with imperfect complementarity to their targeted mRNAs and guide mRNA translation repression. However, there are also several miRNAs which directly degrade their targeted mRNAs. The exact mechanism for miRNA-mediated translation repression is still unknown but most likely miRNA-RISC complex inhibit the initiation and/or elongation of protein translation by interacting with various translation factors. Furthermore, it is shown that miRNAs mediate gene expression by guiding mRNA decay through de-adenylation and de-capping process of targeted mRNAs, which is completely different from normal translation repression

and/or direct mRNA degradation (32). It is well known that the 3' poly(A) tail and 5' cap are very important for mRNA stability and avoiding mRNA decay. When miRNAs guide the removal of the 3' poly(A) tail and 5' cap of the targeted mRNAs, these targeted mRNAs will be degraded by cellular enzymes. In a majority of cases, miRNAs bind to their targeted mRNAs at the 3' UTR with multiple sites. However, miRNAs targeted to the 5' UTR and/or the open reading frame (ORF) can also repress gene expression (148). MicroRNAs interact with their targeted mRNAs primary through the six to eight nucleotides at the 5'end of miRNAs, which is perfectly bound to the targeted mRNAs. This region is called 'seed' sequence in miRNAs and is high conserved in a same miRNA family from species to species (146). The majority (61%) of miRNA genes are located in the intronic region of protein-coding genes; however can also be in regions of exons or intergenes. Interestingly, more than 50% of miRNA genes can be found in cancer- associated genomic regions or in fragile sites, suggesting that miRNAs play an important role in the pathogenesis of neoplasias (24).

MiRNA-Mediated Pathways Implicated in Pancreatic Oncogenesis

Due to the devastating natural course of pancreatic adenocarcinoma, research has focused on the identification, classification and biology of this cancer (9)-(76). Recent advances in pancreatic cancer biology have emerged important roles for miRNAs in regulating tumor responses. However, regulation of the molecular pathways underlying pancreatic tumor initiation and progression, as well as miRNAs function in mediating signals within the tumor microenvironment still remain poorly explored.

MiRNAs as Potential Biomarkers in Pancreatic Cancer

While the actual diagnosis of pancreatic cancer is most often done by traditional methods, such as biopsies of tissues and ultrasound guided fine needle aspiration, the detection of miRNAs in the plasma/serum could represent a non-invasive diagnostic method. In 2008, remarkable insights into circulating miRNAs as biomarkers for cancer classification and prognostication were reported (123). One of the most important advantages of using circulating miRNAs as biomarkers is their stability in plasma and serum, where they are most likely protected from RNase degradation by binding to Argonaute proteins (6). Eighty seven differentially expressed miRNAs have been identified as potential valuable markers for the assessment of cancer recurrence in patients and for people with a familial risk of PDAC (13). In addition, miR-221 has been proposed as a useful biomarker for predicting malignant outcomes (87), while miR-486-5p and miR-938 seem to discriminate PDAC patients from healthy controls and those with chronic pancreatitis. Remarkably, the diagnostic ability of miR-486-5p was comparable to that of Carbohydrate antigen 19-9, which stands for the most reliable diagnostic serum marker (100). However, further investigation is required to prove serum miRNAs as clinically useful diagnostic tools.

Crosstalk between miRNAs and Transcription Factors in Pancreatic Cancer

Transcription factors affect downstream gene transcription of signal transduction pathways triggered by genetic and epigenetic changes linked to the aggressive nature of cancer (131). Accumulating data suggest that miRNAs exert a widespread impact on regulating either directly or indirectly the expression of transcription factors in pancreatic

cancer and vice versa. Inhibiting miR-22 with antisense oligonucleotides (ASOs) enhances SP1 transcription factor (SP1) and estrogen receptor 1 (ESR1) expression levels in pancreatic cancer cell lines (152). The constitutive activation of NF-κB, which regulates important genes and thereby affects many cellular processes, is also known to contribute to the aggressive behavior of pancreatic cancer (51). MiR-146a overexpression has been shown to result in inhibition of the invasive capacity of pancreatic cancer cells with concomitant downregulation of NF-κB (110). A positive feedback loop as a mechanism for persistent NF-κB activation, in which miR-301a represses NF-kB repressing factor (Nkrf) gene to elevate NF-κB activity, which in turn promotes miR-301a transcription, has been recently proposed. Accordingly, miR-301a inhibition or Nkrf up-regulation in pancreatic cancer cells led to reduced NF-κB target gene expression and attenuated xenograft tumor growth (117). Signal transducer and activator of transcription 3 (STAT3) is activated in primary pancreatic cancer and is involved in various physiologic functions (34), including apoptosis, cell cycle regulation, angiogenesis, and metastasis. Mechanistic studies showed that miR-20a regulates STAT3 at the posttranscriptional level, leading to attenuation of cell proliferation and invasion of pancreatic carcinoma (181). Dual luciferase assays revealed that STAT3 is directly targeted by miR-130b, which was further confirmed by the inverse expression of miR-130b and STAT3 in pancreatic cancer specimens (195). Recently, another transcription factor named Forkhead Box M1 (FOXM1) is perceived to play an important role in pancreatic cancer progression (176). Notably, FOXM1 overexpression is responsible for acquisition of the EMT and cancer stem cell phenotypes, which is mediated in part by the regulation of miR-200b expression (8). In addition, FOXC1

transcription factor has been recently proposed as a target of miR-138-5p, the latter exerting anti-proliferative effects both *in vitro* and *in vivo* (186). Reporter expression and chromatin immunoprecipitation assays shed insights into a novel mechanism where increased zinc, mediated by the zinc importer ZIP4, transcriptionally induces miR-373 in pancreatic cancer to promote tumor growth. Further analysis of miR-373 *in vivo* oncogenic function revealed that it is mediated through its negative regulation of tumor protein 53 inducible nuclear protein 1 (TP53INP1), Large Tumor Suppressor Kinase 2 and CD44 (192). Moreover, TP53 has been described to be downregulated by miR-155, accelerating pancreatic tumor development (56). MiR-222 and miR-203 are also able to target p53 and affect its function as a crucial regulator of the cell cycle (58).

FOXA2 Transcription Factor

Forkhead box protein A2 is a 455-amino acid member of the forkhead class of DNA-binding proteins. It contains a highly conserved winged-helix DNA-binding domain with a transcriptional activation domain on either side (135). FOXA2 is produced at the onset of pancreatic development in the foregut endoderm (126). During gastrulation, FOXA2 is initially detected in the anterior primitive streak of the E6.5 (embryonic day 6.5) mouse embryo followed by expression in the node, notochord, floorplate of the neural tube and endoderm (83, 84, 99, 126, 144, 145). In mice, targeted deletion of FOXA2 results in early embryonic lethality shortly after gastrulation (5, 169). More recently, chromatin immunoprecipitation coupled to massively parallel sequencing (ChIP-Seq) was used to identify *in vivo* approximately 11,000 and 7,000 FOXA2 binding sites in the mouse adult liver and beta islets, respectively (70, 167). FOXA2 contains an AKT2/PKB

phosphorylation site at residue 156 in the N-terminus of the forkhead domain (140). Work from Stoffel and colleagues have introduced a concept regarding FOXA2 in nutrient metabolism. Specifically, they found that the transcriptional activity of FOXA2 was blocked by treatment with insulin, and that this regulation requires an intact phosphorylation site for the insulin-activated kinase AKT2/ PKB at residue T156 of mouse FOXA2 (172, 173). In a subsequent study, they found that FOXA2 is acetylated at the conserved residue Lys259, following inhibition of histone deacetylases class I-III and the cofactors p300 and SirTi which are involved in FOXA2 acetylation and deacetylation, respectively. This demonstrated that fasting state and glucagon stimulation are sufficient to induce FOXA2 acetylation (161). Thus, the importance of FOXA2 in lipid metabolism is well established. To our knowledge, we are the first group to investigate the role of FOXA2 in PDAC oncogenesis.

MiRNA-199a-3p

MicroRNAs (miRNAs), a class of non-coding RNAs, have emerged as critical players in cancer initiation and progression by modulating many pathological aspects related to tumor development, growth, metastasis, and drug resistance. Studies have found that miRNAs control many cellular processes through involvement in development, proliferation, the stress response, apoptosis, cell cycle progression, and differentiation (4, 11, 12, 43, 112). The major function of miRNAs is to post-transcriptionally regulate gene expression depending on recognition of complementary sequence residing in target mRNAs. Commonly, a particular miRNA recognition sequence could be found in a number of genes, which allows a single miRNA to regulate multiple functionally

connected genes simultaneously and/or chronologically. Furthermore, a single gene can be targeted and regulated by multiple miRNAs. Other studies have demonstrated that miRNAs are aberrantly expressed or mutated in many cancers, suggesting that the primary functions of miRNAs are as oncogenes or tumor suppressors (15, 189). In the last decade, investigations have revealed that the expression of miRNA-199a is altered in several human cancers (54, 102, 183). Specifically, the expression of miRNA-199a is increased in ovarian cancer cells and cervical carcinomas (54, 183), in accordance to our preliminary data in PDAC. Furthermore, miRNA-199a can reduce cellular resistance to cytotoxic drugs (54). More recently, Nonaka et al. found that miR-199a expression in serum was significantly higher in the colorectal cancer patients than the non-cancer patients, suggesting that circulating miR-199a could be a novel serum biomarker for colorectal cancer (132). Until now, the role of miR-199a in PDAC remains largely unknown.

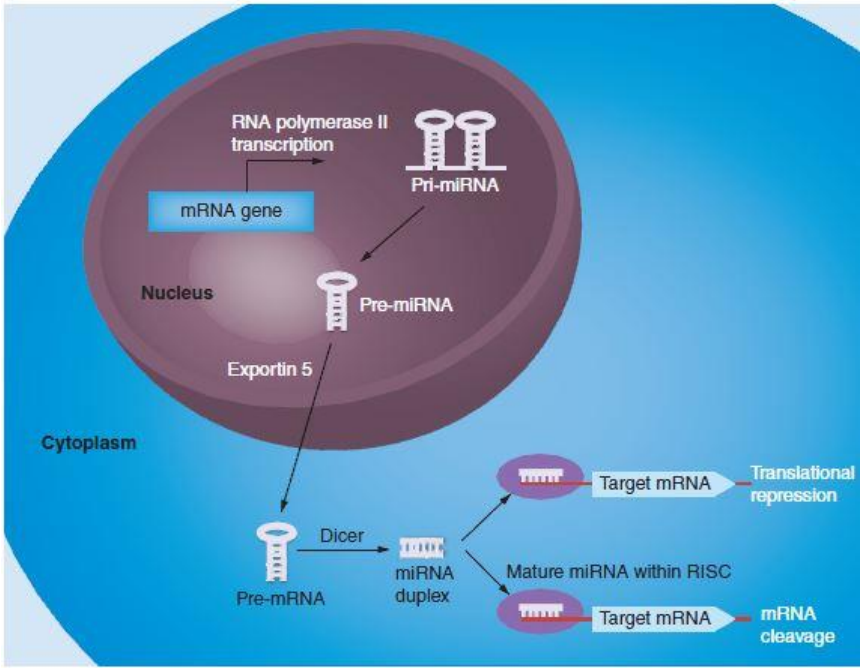

Figure 2.1. Schematic Representation of miRNA Biogenesis and Mechanism of Action.

RNA precursors called pri-miRNAs are processed by the microprocessor complex into pre-miRNA hairpins, transported into the cytoplasm and further processed by Dicer into miRNA duplexes. Following strand separation, the mature miRNAs are loaded onto the RISCs to guide the repression of protein synthesis or mRNA degradation. Direct binding in the 3' untranslated region of genes and specifically through sequence complementarity between nucleotides 2 and 8 of miRNAs (called seed sequence) represents their major post-transcriptional mechanism of action. A particular miRNA recognition sequence could be found in a number of genes, which allows a single miRNA to regulate multiple functionally connected genes simultaneously and/or chronologically.

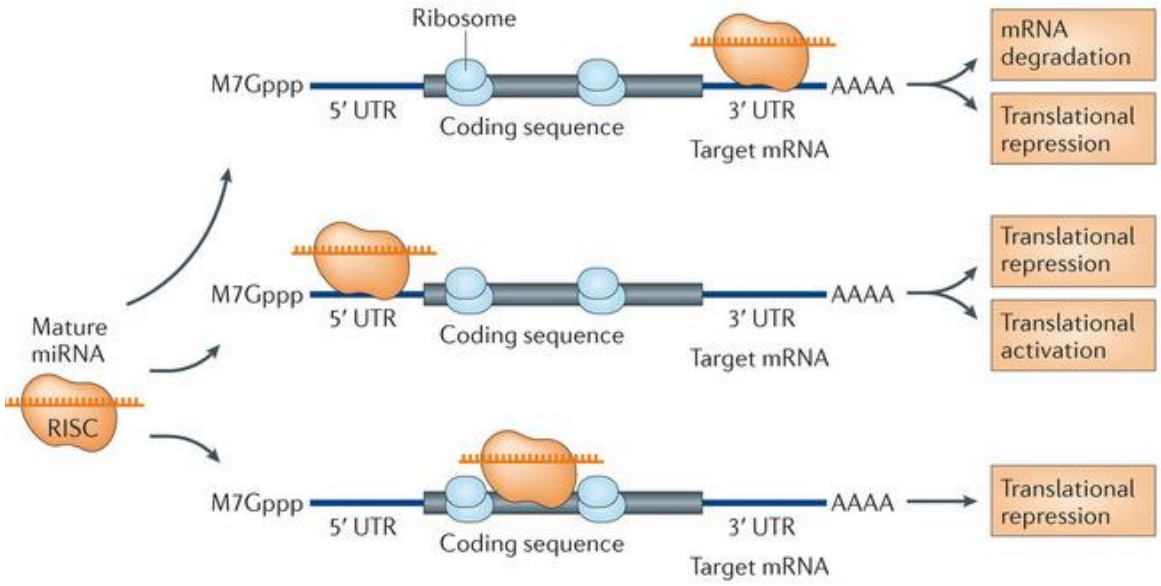

Figure 2.2. Mechanisms of Action of MicroRNAs.

A miRNA is recruited to the RNA induced silencing complex (RISC) and regulates the output of protein coding genes through diverse mechanisms. The interaction of miRNAs with the 3' untranslated region (3'UTR) of protein coding genes is considered as the main mechanism, which usually leads to a decrease in protein output either by mRNA degradation or by translational repression. (*Adapted from Ling H et al. Nature Rev Drug Discovery, 12, 847-865, 2013*).

CHAPTER 3

Novel Technologies for Studying Complex Molecular Mechanisms in Pancreatic Cancer

Nanostring Technology

The nCounter Analysis System is an automated, multi-application, digital color-barcode detection technology that is based on direct multiplexed measurement of gene expression and offers high levels of precision and sensitivity (<1 copy per cell). The technology uses molecular “barcodes” and single molecule imaging to detect and count hundreds of unique transcripts in a single reaction. Each color-coded barcode is attached to a single target-specific probe corresponding to a gene of interest. Mixed together with controls, they form a multiplexed CodeSet. The counting system includes the nCounter Prep Station, an automated liquid handling component for processing and preparing the samples for data collection. The fluorescent reporters are immobilized in the sample cartridge and the nCounter Digital Analyzer collects the data from samples in the form of images and the data is processed into output files (**Figure 1**) (55).

The nCounter Analysis System is a cutting-edge platform that enables research from basic discovery to the development and commercialization of molecular diagnostic tests on a single platform. The nSolver Analysis software is a tool available for users to perform quality check, normalize and analyze their data.

The company also provides nSolver Analysis software, a data analysis program that provides nCounter Analysis System users the ability to quality check, normalize and

analyze their data. Importantly, the company has plans to develop and sell diagnostic kits for use in molecular diagnostic testing in clinical laboratories.

The NanoString Technologies' nCounter Analysis System offers:

- A simple, cost-effective way to profile hundreds of mRNAs, miRNAs or DNA targets simultaneously
- High sensitivity and precision
- Digital detection of target molecules
- High levels of multiplexing eliminate the compromise between data quality and data quantity
- Gold-standard sensitivity and reproducibility for studies of hundreds of targets
- The system uses molecular "barcodes" and single-molecule imaging to detect and count hundreds of unique transcripts in a single reaction.
- Unlike other methods, the protocol does not include any amplification steps that might introduce bias to the results

In this study, the nCounter Human v3 miRNA Expression Assay was performed using RNA isolated from human pancreatic cancer and cancer adjacent normal tissues. The assay enabled us to look at the expression of 800 human miRNAs from miRBase v21 in each sample. The assay is a highly specific and sensitive method for miRNA profiling, which enabled us to identify differentially expressed miRNAs (>2-fold change sensitivity) between PDAC and normal samples with a low false-positive rate and excellent specificity. Furthermore, there is very low cross-hybridization between miRNAs which

demonstrates the superior specificity of the nCounter Human v3 miRNA Assay. Additionally, there are 6 positive controls and 8 negative controls used in this assay. The positive controls are probes that recognize synthetic mRNA targets included in the CodeSet at specified concentrations. The negative controls are probes that recognize synthetic mRNA targets not included in the CodeSet. There are also 5 miRNA references controls that are probes that recognize endogeneous mRNA targets commonly expressed in human tissues. There are also 3 positive and 3 negative ligation controls that are probes that recognize synthetic miRNA targets included (or not included) in the Sample Preparation Kit. Lastly, there are 5 spike-in controls that are probes that recognize exogenous miRNA targets to monitor upstream RNA isolation/purification.

More recently, the company has developed an nCounter PanCancer Pathways Panel. The PanCancer pathways panel is a novel set of 700 essential genes representing all major cancer pathways including key driver genes. Using a biology-guided, data-driven approach and applying basic co-expression principles, the company scored and ranked each gene based on its biological relevance to cancer and its role as an essential representation in one or more of the 13 canonical pathways. In 2016, Yoon and colleagues used an nCounter PanCancer Pathways Panel of 230 cancer-related genes to identify gene products affected by the bromodomain inhibitor JQ1 in pancreatic ductal adenocarcinoma patient-derived xenograft models (53).

Gene Network Analysis

In this study, we employed the Ingenuity Pathway Analysis (IPA) system to help us transform a list of genes into a set of relevant networks based on extensive records maintained by the Ingenuity Pathways Knowledge Base (IPKB) (25, 45). We performed a gene expression array in PANC-1 cells that were transfected with either an siRNA negative control or an siRNA targeting FOXA2 in order to understand how FOXA2 knockdown affects the biology of the cell. We wanted to identify the highly interconnected networks that were likely to represent significant biology function (119, 142, 150). Using IPA, the largest network generated contains 35 genes, which is a result of having to accommodate to several constraints including the fact that the network diagram has to be small enough to include useful information on a computer screen.

The IPA algorithm follows six steps to produce the networks (**Figure 2**). Each step attempts to display networks that are as heavily connected as possible based on the notion that highly connected genes tend to be involved in similar biological functions. The term “Focus Genes” refers to user-specified genes.

- **Step 1: Sort Focus Genes by Triangle Connectivity**
    - Genes selected as Focus Genes must be sorted in order of their interconnectedness
- **Step 2: Greedily Construct Networks of Focus Genes up to a Maximum Pre-defined Size**

- The top ranked (most connected) gene is removed from the sorted Focus Gene list prepared in step 1. Focus Genes are added to this seed gene until the maximum network size is reached (set at 35)

- **Step 3: Merge Small Networks Using Linker Genes**
  - For smaller connected components, they are combined together to make larger networks in hopes of uncovering as many relationships as possible between Focus Genes
- **Step 4: Grow Remaining Small Networks Using Available Neighborhood Genes**
  - For those networks that still have less than 35 genes, other genes are added to the periphery of the network to provide additional biological context to those focus genes
- **Step 5: Display Subgraph Defined by Network Nodes**
  - Network genes and all edges between them are pulled together into a single network with the intent that these networks contain approximately 35 genes
- **Step 6: Rank the Networks**
  - The final step is the calculation of p-scores used to rank networks. The p-scores are derived from p-values. The p-score is defined as:
  - $p - score = -\log_{10}(p\text{-value})$

In our study, gene network analysis by using the 924 differentially expressed genes in the IPA software network found the most significant (p value $=10^{-42}$) gene network

involved in cellular invasion having as central nodes PLAUR, ERK, PI3K, consistent with our gene ontology analysis (**Figure 3**). Gene network was constructed and important hubs were identified using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Mountain View, CA) based on the differentially expressed genes identified after inhibition of FOXA2 expression by siRNA FOXA2#2 in PANC-1 pancreatic cancer cell line. IPA is a robust and expertly curated database containing updated information on more than 20,000 mammalian genes and proteins, 1.4 million biological interactions, and 100 canonical pathways incorporating over 6,000 discreet gene concepts. This information is integrated with relevant databases such as Entrez-Gene and Gene Ontology. The experimental data sets were used to query the IPA and to compose a set of interactive networks taking into consideration canonical pathways, the relevant biological interactions, and the cellular and disease processes.

xCELLigence Real-Time Cell Analyzer (RTCA)

The xCELLigence System continuously and non-invasively detects cell responses throughout an experiment, without the use of exogenous labels that can disrupt the natural cell environment. The system is designed to obtain complete, continuous data profiles from cell responses generated during *in vitro* experiments (**Figure 4**). It enables real-time data collection to identify optimal time points for downstream assays. It combines real-time monitoring of cellular responses with complementary functional endpoint assays, and maximizes data quality before, during and after an experiment. The technology is based on a cellular impedance apparatus. A side view of a single well is shown before and after cells have been added (**Figure 5**). Neither the electrodes nor

the cells are drawn to scale (they have been enlarged for clarity). In the absence of cells, electric current flows freely through culture medium, completing the circuit between the electrodes. As cells adhere to and proliferate on the electrodes, current flow is impeded, providing an extremely sensitive readout of cell number, cell size/morphology, and cell-substrate attachment quality.

Wide range of applications:

- Cell invasion and migration assays
- Compound and cell mediated cytotoxicity
- Cell adhesion and cell spreading
- Cell proliferation and cell differentiation
- Receptor-mediated signaling
- Virus-mediated cytopathogenicity
- Continuous quality control of cells

CRISPR/Cas9 Technology

CRISPR consists of two components: a “guide” RNA (gRNA) and a non-specific CRISPR-associated endonuclease (Cas9) (**Figure 6**) (14). The gRNA is a short synthetic RNA composed of a “scaffold” sequence necessary for Cas9-binding and a user-defined ~20 nucleotide “spacer” or “targeting” sequence which defines the genomic target to be modified. Thus, one can change the genomic target of Cas9 by simply changing the targeting sequence present in the gRNA. CRISPR was originally employed to “knock-out” target genes in various cell types and organisms, but

modifications to the Cas9 enzyme have extended the application of CRISPR to selectively activate or repress target genes, purify specific regions of DNA, and even image DNA in live cells using fluorescence microscopy. Furthermore, the ease of generating gRNAs makes CRISPR one of the most scalable genome editing technologies and has been recently utilized for genome-wide screens.

The CRISPR/Cas9 system was employed in this study to explore the role of FOXA2 deletion in a human pancreatic cancer cell line, PANC-1. The stable FOXA2Δ pancreatic cancer cell line was used for *in vivo* studies. 5x106 cells were injected subcutaneously in the right hind-leg of immunodeficient mice.

There are many advantages of the CRISPR/Cas9 system compared to other deletion/inhibition methods:

- Affordable
- Easy to use
- Works for high-throughput, multi-gene experiments
- Permanent deletion
- Can be utilized to knock out genes and to up- or down-regulate gene transcription

Figures

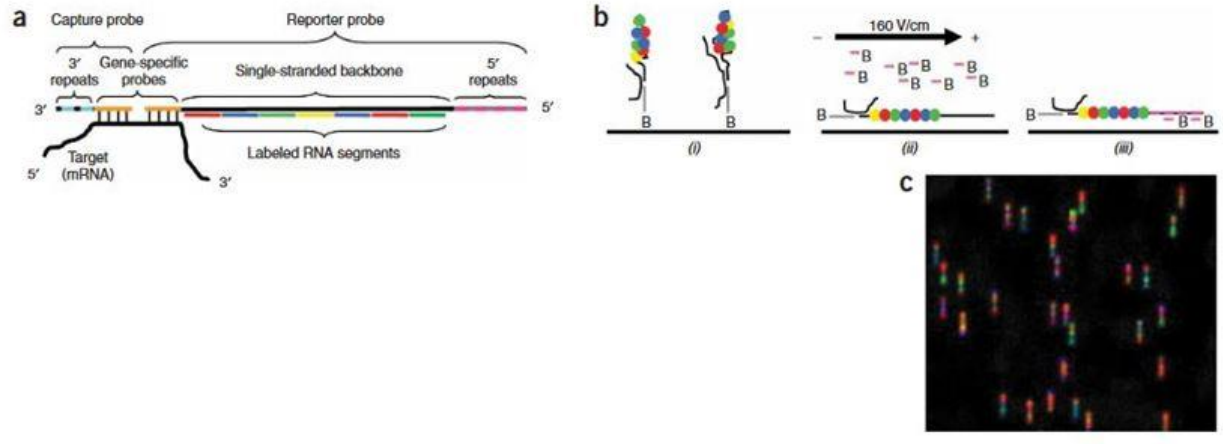

Figure 3.1. Overview of NanoString nCounter Gene Expression System.

(a) A schematic representation of the hybridized complex (not to scale). The capture probe and reporter probe hybridize to a complementary target mRNA in solution via the gene-specific sequences. After hybridization, the tripartite molecule is affinity-purified first by the 3'-repeat sequence and then by the 5'-repeat sequence to remove excess reporter and capture probes, respectively. **(b)** Schematic representation of binding, electrophoresis, and immobilization. **(i)** The purified complexes are attached to a streptavidin-coated slide via biotinylated capture probes. **(ii)** Voltage is applied to elongate and align the molecules. Biotinylated anti-5' oligonucleotides that hybridize to the 5'-repeat sequence are added. **(iii)** The stretched reporters are immobilized by the binding of the 5'-oligonucleotides to the slide surface via the biotin. Voltage is turned off and the immobilized reporters are prepared for imaging and counting. **(c)** False-color image of immobilized reporter probes. (*Adapted from Geiss et al. Nature Biotechnology 26, 317 – 325, 2008*).

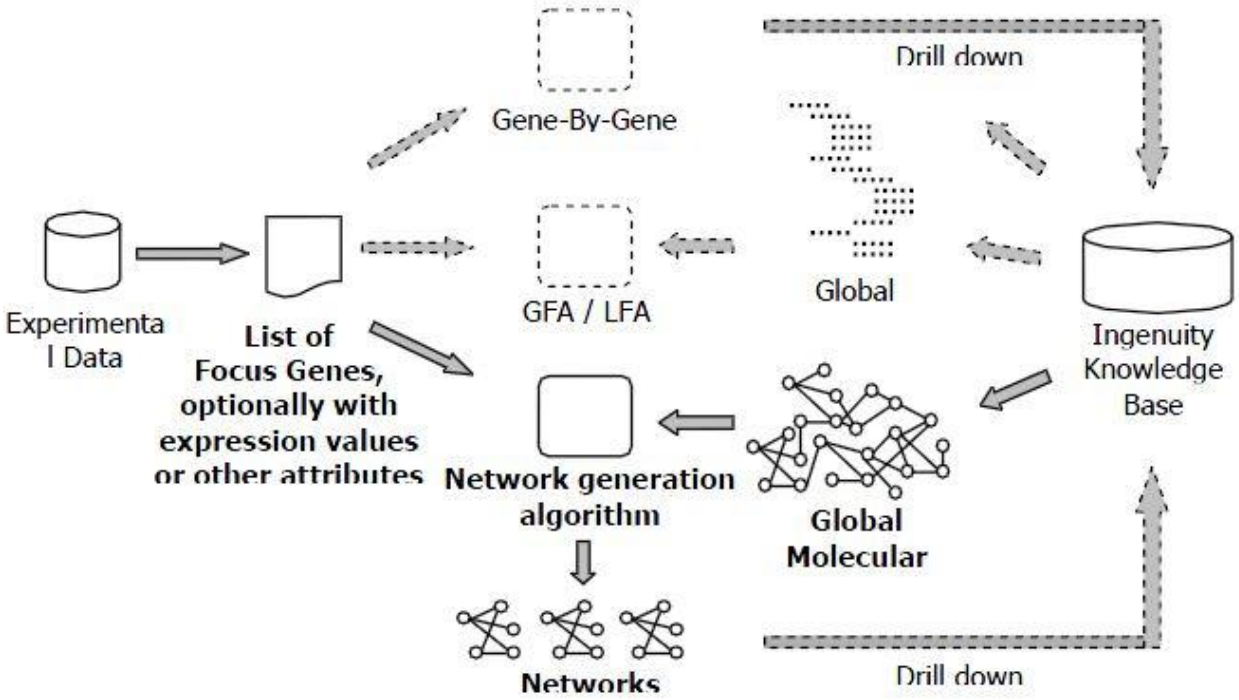

Figure 3.2. The IPA algorithm follows six steps to produce the networks. (*Adapted from IPA Network Generation Algorithm*).

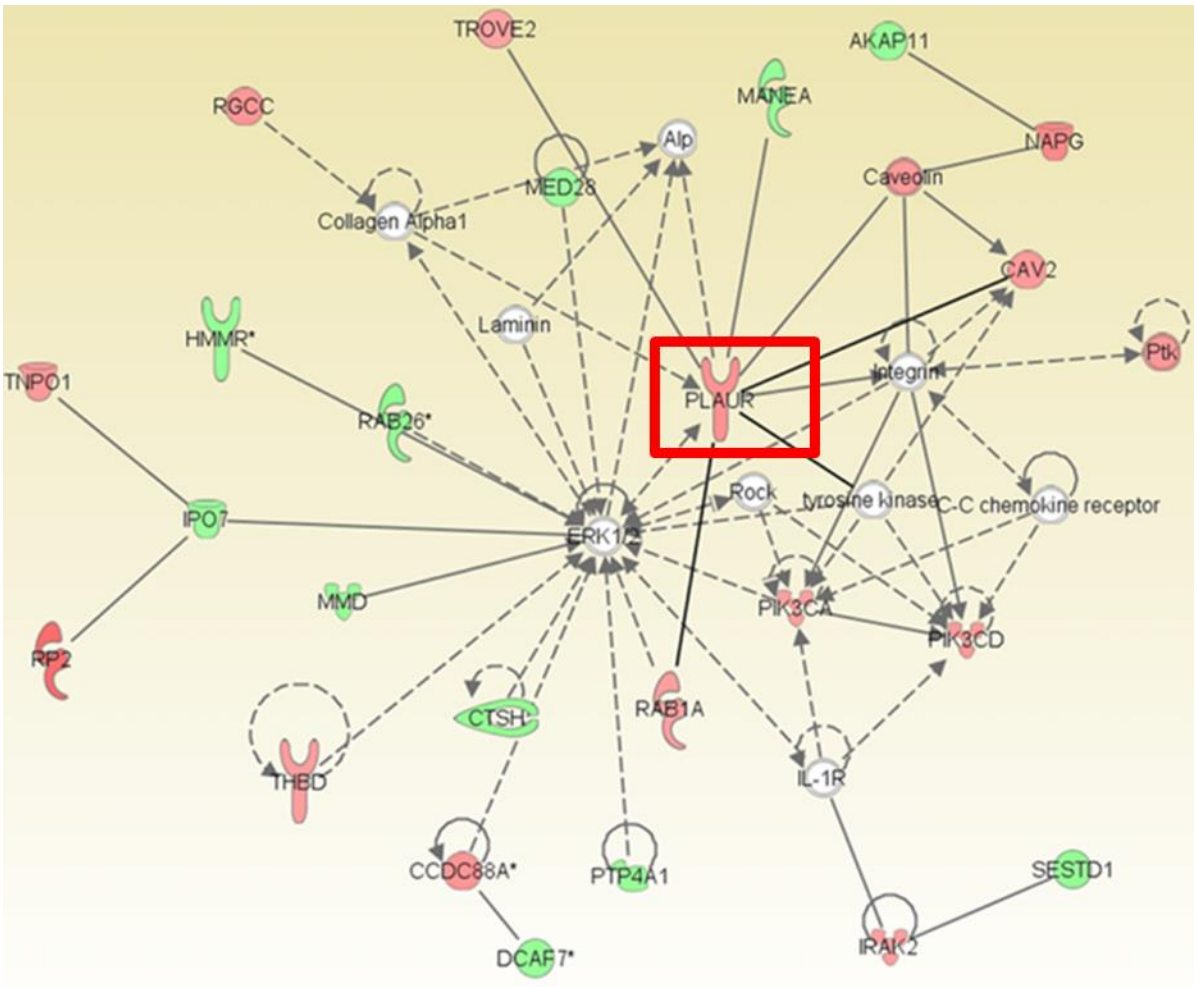

Figure 3.3. Gene Network Analysis upon FOXA2 Transient Inhibition.

Gene network analysis by using the 924 differentially expressed genes in the IPA software network found the most significant (p value =10^{-42}) gene network was involved in cellular invasion having as central nodes PLAUR, ERK, PI3K, consistent with our gene ontology analysis.

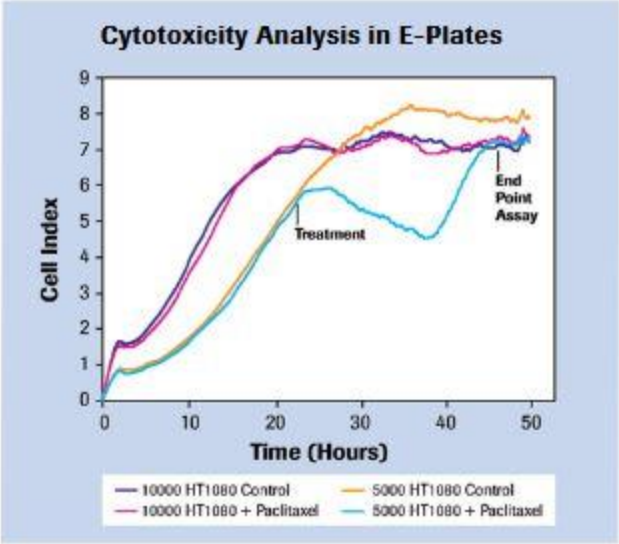

Figure 3.4. Reveal Cytotoxic Effects through Continuous Monitoring.

HT1080 cells were seeded in an E-Plate at two different densities (5,000 and 10,000 cells) and treated 24 hours later with 12.5 nM Paclitaxel, or DMSO as a control. As shown by the Cell Index profile, which reflects cell adherence, the antimitotic effect of Paclitaxel was observed in HT1080 cells that were proliferating, whereas confluent cells showed no response.

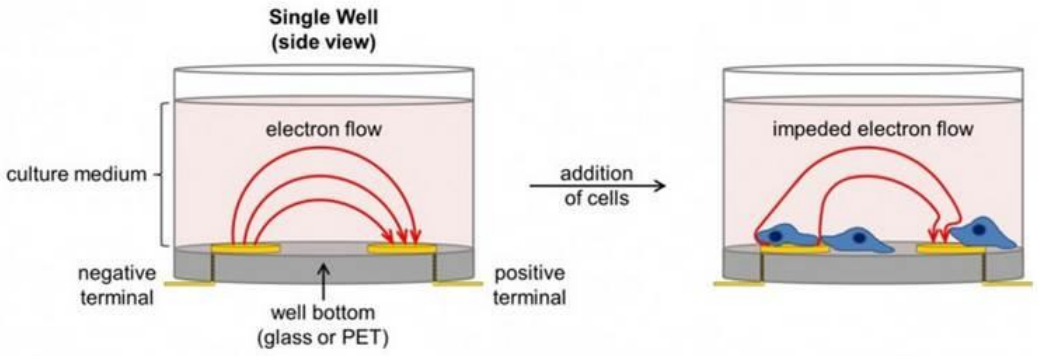

Figure 3.5. Overview of Cellular Impedance Apparatus.

A side view of a single well is shown before and after cells have been added. Neither the electrodes nor the cells are drawn to scale (they have been enlarged for clarity). In the absence of cells electric current flows freely through culture medium, completing the circuit between the electrodes. As cells adhere to and proliferate on the electrodes current flow is impeded, providing an extremely sensitive readout of cell number, cell size/morphology, and cell-substrate attachment quality.

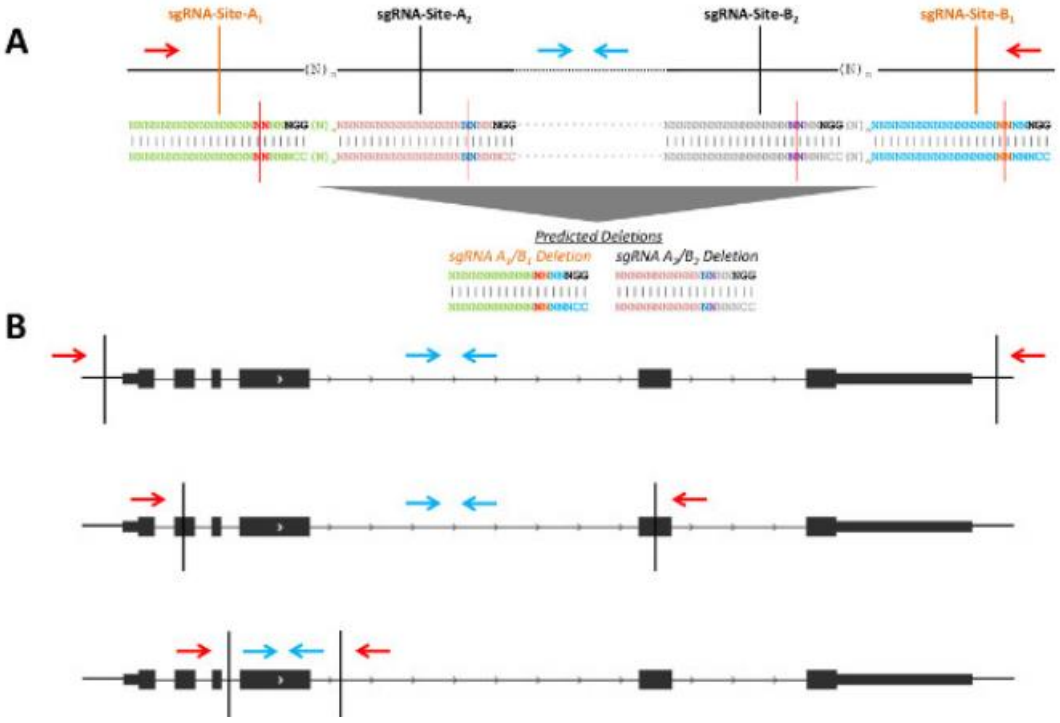

Figure 3.6. Schematic of Possible Deletion Strategies.

(A) Two example sgRNA pairs for genomic deletions (shown in black and orange, respectively). The blue arrows indicate primers to detect the non-deletion amplicon and red arrows indicate primers to detect the deletion amplicon. sgRNA positions 17 and 18 are highlighted in red and blue at sgRNA-Sites-A_1/A_2 and are highlighted in purple and orange at sgRNA-Sites- B_1/B_2 with a red line indicating the predicted Cas9 cleavage between positions 17 and 18. **(B)** CRISPR-directed cleavages are shown as vertical black lines. The blue arrows indicate primers to detect the non-deletion amplicon and red arrows indicate primers to detect the deletion amplicon. (*Figure Adapted from Bauer DE et al. Vis. Exp. (95), 2015*).

CHAPTER 4

Integration of Transcriptomic and CRISPR-Cas9 Technologies Reveals FOXA2 as a Tumor Suppressor Gene in Pancreatic Cancer

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a very aggressive cancer, with low survival rates and limited therapeutic options. Thus, the elucidation of signaling pathways involved in PDAC pathogenesis is essential to identify novel potential therapeutic gene targets. Here, we used a systems approach by integrating gene and microRNA profiling analyses together with CRISPR/Cas9 technology, to identify novel transcription factors involved in PDAC pathogenesis. FOXA2 transcription factor was found to be significantly down-regulated in PDAC relative to control pancreatic tissues. Functional experiments revealed that FOXA2 has a tumor suppressor function through inhibition of pancreatic cancer cell growth, migration, invasion and colony formation. *In situ* hybridization analysis revealed miR-199a significantly upregulated in pancreatic cancer. Bioinformatics and luciferase analyses showed that miR-199a negatively regulates directly FOXA2 expression, through binding in its 3' untranslated region (UTR). Evaluation of the functional importance of miR-199 on pancreatic cancer revealed that miR-199 acts as an inhibitor of FOXA2 expression, inducing an increase in pancreatic cancer cell proliferation, migration and invasion. Additionally, gene ontology and network analyses in PANC-1 cells treated with an siRNA against FOXA2 revealed an enrichment for cell invasion mechanisms through PLAUR and ERK activation. FOXA2 deletion (FOXA2Δ) by using two CRISPR/Cas9 vectors in PANC-1 cells, induced tumor growth *in vivo*, resulting in up-regulation of PLAUR and ERK pathways in FOXA2Δ xenograft tumors. Taken together, we have identified FOXA2 as a novel tumor suppressor in pancreatic cancer, regulated directly by miR-199a, enhancing our understanding on how microRNAs interplay with the transcription factors to affect pancreatic oncogenesis.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) accounts for >85% of all the pancreatic cancer cases. For all stages combined, the 1- and 5-year relative survival rates are 28% and 7%, respectively. More than half of patients (53%) are diagnosed at a late stage, where the 1- and 5-year survival rates reach 15% and 2%, respectively (106). Recently there are significant advances in the development of novel therapeutics, based on the rational design of targeted therapies directed at molecular alterations arising in cancer cells (185); however, PDAC remains a lethal disease. Even gemcitabine, the current standard of care chemotherapeutic, produces only a modest increase in survival in patients with PDAC (21). For metastatic disease, the standard of care is a combination four chemotherapeutic drugs, known as FOLFIRINOX (Folinic Acid, Fluorouracil, Irinotecan Hydrochloride, Oxaliplatin) (156). These treatments have limited efficacy and significant side effects, often only marginally improving the quality of life of patients (165). Therefore, there is an urgent need to identify novel therapeutic target molecules that a play a key role in pancreatic oncogenesis.

Premalignant lesions, known as pancreatic intraepithelial neoplasms (PanINs) are of ductal origin (71) and are thought to be precursors of ductal adenocarcinoma, as they progress toward increasingly atypical histological stages (93, 118, 128, 180). Multiple combinations of genetic mutations are commonly found in pancreatic adenocarcinomas (170). The *KRAS* gene, located on chromosome 12p, is one of the most frequently mutated genes in pancreatic cancer. The vast majority of mutations in this gene are at codon 12, leading to activation of the protein product of KRAS (72). *KRAS* mutations appear to occur very early in pancreatic carcinogenesis, indicating an important role in early initiation of disease (3). In addition to activating mutations, loss of function mutations in tumor suppressor genes is also commonly observed in pancreatic

carcinomas. Loss of function occurs via inactivation mutations, homozygous deletions or DNA hypermethylation of the promoter areas of tumor suppressor genes, including *p16/CDKN2A, TP53, and SMAD4* that are inactivated in more than 50% of all pancreatic cancers (2, 68, 69, 143). Other pathways, involved in PDAC include, the Notch signaling pathway (Abel, 2014), the beta-catenin signaling pathway (108) and the PI3K/AKT signaling pathway (17). Although the role of different protein signaling pathways has been examined in pancreatic oncogenesis, the role and function of several transcription factor families has not been evaluated extensively.

Transcription factors affect downstream gene transcription of signal transduction pathways triggered by genetic and epigenetic changes linked to the aggressive nature of cancer (153). For example, the constitutive activation of NF-κB, which regulates the genes involved in many cellular processes, has also been implicated in the aggressive nature of PDAC (51). Signal transducer and activator of transcription 3 (STAT3) is activated in primary pancreatic cancer and is involved in various physiologic functions, including apoptosis, cell cycle regulation, angiogenesis, and metastasis (34). Negative regulation of STAT3 at the posttranscriptional level leads to attenuation of cell proliferation and invasion of pancreatic carcinoma (181), highlighting the importance of understanding transcriptional regulation in pancreatic oncogenesis. In 2012, Xia et al. identified a transcription factor, Forkhead Box M1 (FOXM1) that is associated with poor prognosis and could be used as a prognostic molecular marker and therapeutic target for pancreatic cancer (176).

In the present study, we sought to identify key transcriptional regulators that play a functional role in the pathogenesis of pancreatic cancer by performing transcription factor expression profiling followed by functional characterization of selected

transcription factor. The aberrant expression of hepatocyte nuclear factor family of transcription factors (HNF1, HNF3, HNF4 and HNF6) have been implicated in a variety of solid tumors including lung, colorectal, hepatocellular and ovarian carcinoma (61, 104, 107, 114, 115, 188). The least studied hepatocyte nuclear factor gene family in cancer is HNF3. The hepatocyte nuclear factor 3 gene family encodes three transcription factors (HNF-3α, HNF-3β, and HNF-3γ) important in the regulation of gene expression in normal liver and lung tissue, and were first identified by their ability to bind to important promoter elements in the α_1-antitrypsin and transthyretin genes (35). Our molecular and functional analysis revealed that HNF-3β, also known as forkhead box protein A2 (FOXA2), acts as a tumor suppressor gene in pancreatic cancer by affecting pancreatic cancer cell proliferation and invasiveness through regulation of urokinase plasminogen activator surface receptor (PLAUR) gene. Furthermore, we found that FOXA2 expression is regulated directly by miR-199, while inhibition of FOXA2 expression by using the CRISPR/Cas9 (*c*lustered *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeats-CRISPR associated nuclease 9) technology increases tumor growth in pancreatic tumor xenografts. Taken together, our study revealed a novel microRNA-transcription factor signaling pathway involved in the pathogenesis of pancreatic oncogenesis.

Materials and Methods

Cell Culture

Human pancreatic cancer cell lines (AsPc-1, BxPC-3, Capan-1, Capan-2, HPAF-II and MIA PaCa-2, PANC-1) were purchased from ATCC. Human pancreatic cancer cell line PANC-1 was maintained in DMEM medium (Gibco) supplemented with 10% FBS and 10 units/ml penicillin, and 100 μg/ml streptomycin. AsPC-1 and BxPC-3 were maintained in RPMI-1640 medium (Gibco) supplemented with 10%FBS and 10 units/ml

penicillin and 100 μg/ml streptomycin. Capan-1 was maintained in ATCC-formulated Iscove's Modified Dulbecco's Medium supplemented with 10% FBS and 10 units/ml penicillin and 100 μg/ml streptomycin. Capan-2 was maintained in ATCC-formulated McCoy's 5a Medium Modified supplemented with 10% FBS and 10 units/ml penicillin and 100 μg/ml streptomycin. HPAF-II was maintained in Eagle's Minimum Essential Medium with 10% FBS and 10 units/ml penicillin and 100 μg/ml streptomycin. MIA PaCa-2 was maintained in DMEM medium (Gibco) supplemented with 10% FBS, horse serum to a final concentration of 2.5% and 10 units/ml penicillin and 100 μg/ml streptomycin.

RNA from PDAC and Control Samples

Human pancreatic tissues were obtained from consenting patients in the Department of Surgery at Stanford University and approved by the Ethics Committee of the Stanford University Medical School. RNA was extracted from 8 control (adjacent non-tumor) and 14 PDAC tissues using the TRIzol Reagent (15596-018, Life Technologies) RNA isolation method and were used for gene profiling. Nineteen control and 17 PDAC tissues were obtained from consenting patients in the Department of Surgery at the University of California, Los Angeles and approved by the UCLA Ethics Committee and were used to confirm gene expression array data.

Transcription Factor Expression Analysis

To identify transcription factors that were differentially expressed in pancreatic ductal adenocarcinoma, microarray was performed using GeneChip® Human Genome U133 Plus 2.0 Arrays. RNA was isolated from 14 PDAC and 8 control tissues. In the list of top differentially expressed genes, FOXA2 was found to be down-regulated in PDAC (cut off was 2-fold change, $p<0.05$).

Invasion Assays

We performed invasion assays in PANC-1 and HPAF-II cells at 24 hours under different transfection conditions with siRNAs or microRNAs for 24 hours. Invasion of matrigel

was conducted using standardized conditions with BD BioCoat Matrigel invasion chambers (BD Biosciences). Assays were conducted according to manufacturer's protocol, using 10% FBS as the chemoattractant. Non-invading cells on the top side of the membrane were removed, while invading cells were fixed and stained with 0.1 % crystal violet, 16 hours post-seeding. The cells that invaded through the filter were quantified by counting the entire area of each filter, using a grid and an Optech microscope at a 20X magnification. The experiment was repeated three times and the statistical significance was calculated using Student's t-test.

Migration Assays

PANC-1 and BxPC-3 pancreatic cancer cell lines were used in this assay. The migration assay was performed by starving cells overnight in media containing 0% FBS. The next day, cells were re-suspended in media with 0.5% FBS to a concentration of 5×10^5/ml. The upper chamber was loaded with 100 μL of cell suspension and the lower chamber was loaded with 500 μL medium containing 20% FBS as a chemoattractant. The cells on the bottom of each chamber were fixed with 0.1% glutaraldehyde for 30 min, rinsed briefly with PBS and stained with 0.2% crystal violet. The number of migrated cells was calculated using 20X magnification and the mean for each chamber was determined. The results were calculated as the migration rate as compared with the siRNA negative control (or miRNA-NC) cells. Each experimental condition was conducted in triplicates and the experiment was repeated three times.

Colony Formation Assays

PANC-1 cells were transfected with siRNA negative control or siFOXA2#2 for 48 hours. Triplicate samples of 10^5 cells from each cell line were mixed 4:1 (v/v) with 2.0% agarose in growth medium for a final concentration of 0.4% agarose. The cell mixture was plated on top of a solidified layer of 0.8% agarose in growth medium. Cells were fed every six to seven days with growth medium containing 0.4% agarose. The number of colonies was counted after 20 days. The experiment was repeated three times and the statistical significance was calculated using the Student's t-test.

In situ Hybridization

Double-DIG labeled Mircury LNA probes were used for the detection of hsa-miR-199a-3p (38481-15, Exiqon) with target sequence ACAGUAGUCUGCACAUUGGUUA. *In situ* hybridization protocol was used as previously described (Iliopoulos et al., 2009b) with modifications. FFPE sections of control pancreatic and PDAC were deparaffinized with xylene (3x5 min), followed by treatment with serial dilutions of ethanol (3x100%, 2x96% and 3x70%) and by two changes of DEPC-PBS. Tissues were then digested with proteinase K (15 μg/ml) for 20 min at 37ºC, rinsed with 3xDEPC-PBS. Sections were dehydrated with 3x70%, 2x96% and 2x100% ethanol, air-dried and hybridized for 1 hour with the hsa-miR-199 probe (40nM) or the double-DIG labeled U6 Control Probe (1nM) (99002-15, Exiqon) diluted in microRNA ISH buffer (90000, Exiqon) at 52ºC and 53 ºC, respectively. Following hybridization, sections were rinsed twice with 5XSSC, 2x1XSSC and 3x0.2XSSC, 5 min each, at 52ºC and PBS. The slides were incubated with blocking solution (11585762001, Roche) for 15 min and then with anti-DIG antibody (1:800) in 2% sheep serum (013-000-121, Jackson Immunoresearch) blocking solution for 1 hour at room temperature. Following three washes with PBS, 0.1% Tween-20, slides were incubated with the AP substrate buffer (NBT-BCIP tablet [11697471001, Roche] in 10 ml of 0.2mM Levamisole [31742, Fluka]) for 2 hours at 30ºC in the dark. The reaction was stopped with 2 washes of AP stop solution (50mM Tris-HCL, 150mM NaCl, 10mM KCl) and 2 washes with water. Tissues were counter stained with Nuclear Fast Red for 1 min and rinsed with water. Sections were dehydrated with 2x70%, 2x96% and 2x100% ethanol and mounted with coverslips in Eukitt mounting medium (361894G, VWR). Images were captured with a Nikon 80i Upright Microscope equipped with a Nikon Digital Sight DS-Fi1 color camera, using the NIS-Elements image acquisition software. All images were captured and processed using identical settings.

Immunohistochemistry

A pancreas disease spectrum tissue microarray of 103 cases was used (PA2081a, US Biomax, Inc.) containing 42 cases of pancreatic duct adenocarcinoma, three pancreatic

adenosquamous carcinoma, one pancreatic islet cell carcinoma, six pancreatic metastatic carcinoma, 10 pancreatic islet cell tumor, 11 pancreatic inflammation and 21 adjacent normal pancreatic tissue, duplicated cores per case. Immunohistochemical staining for FOXA2 in control and pancreatic PDACs were deparaffinized with xylene (3x5 min) followed by treatment with serial dilutions of ethanol (100%, 100%, 95% and 95%, 10 min each) and by two changes of ddH2O. Antigen unmasking was achieved by boiling the slides (95-99ºC) for 10 min, in 10 mM sodium citrate, pH 6.0. Sections were rinsed three times with ddH2O, immersed in 3% H_2O_2 for 20 minutes, washed twice with ddH2O and once with TBS-T (TBS, 0.1% Tween-20) and blocked for 1 hour with blocking solution (5% normal goat serum [5425] in TBS-T). FOXA2 (sc-6554, Santa Cruz Biotechnology) antibody was diluted 1:250 in Signal Stain antibody diluent (8112, Cell Signaling Technology) and incubated with the sections overnight at 4ºC. Staining for mouse FOXA2, antibody was diluted 1:1000 in Signal Stain antibody diluent (sc-101060 Santa Cruz Biotechnology) and incubated with the sections overnight at 4ºC. Following incubation with the antibody, sections were washed three times, 5 minutes each, with TBS-T and incubated for 1 hour at room temperature with SignalStain Boost ([HRP, Rabbit] 8114, Cell Signaling). Sections were washed three times, 5 minutes 22 each, with TBS-T, and stained with the DAB Peroxidase Substrate Kit (SK-4100, Vector Laboratories) for 30 minutes, washed and counterstained with the hematoxylin QS (H-3404, Vector Laboratories). Finally, tissues were dehydrated and mounted in Eukitt medium. Images were captured with a Nikon 80i Upright Microscope equipped with a Nikon Digital Sight DS-Fi1 color camera, using the NIS-Elements image acquisition software. All images were captured and processed using identical settings.

Real-Time PCR analysis

Quantitative real-time RT-PCR was performed to determine the expression levels of FOXA2 in 17 human PDAC tissues and 19 pancreatic control tissues for detection of miR-199a-3p. RNA was isolated using TRIzol, according to manufacturer's instructions (15596-018, Life Technologies). Real-time RT-PCR was assessed on a CFX384 detection system (BioRad) using the Exiqon PCR primer sets according to manufacturer's instructions. MicroRNA expression levels of miR-199 (204536, Exiqon)

were normalized to the levels of U6 small nuclear snRNA (203907, Exiqon) and 5S rRNA (203906, Exiqon). Reverse transcription was carried out using the Universal cDNA synthesis kit (203301, Exiqon) and ExiLENT SYBR Green for RT-PCR (203403, Exiqon). Normalized miRNA levels were quantified relative to the levels of a given control tissue. Real-time PCR was employed to determine the expression levels of FOXA2 and PLAUR. Reverse transcription was carried out using iScript cDNA synthesis Kit (1708890, Bio-Rad). Real-time PCR was carried out using the iQ SYBR Green Supermix (1708882, Bio-Rad). Gene expression levels were normalized to the levels of Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin. Normalized gene expression levels were quantified to the respective control. The sequences of the primers used are the following:

FOXA2-F: 5'-ATGCACTCGGCTTCCAGTAT-3'

FOXA2-R: 5'-GTTGCTCACGGAGGAGTAGC-3'

PLAUR-F: 5'-GCATTTCCTGTGGCTCATC-3'

PLAUR-R: 5'- CTTTGGACGCCCTTCTTCA-3'

E-Cadherin-F: 5'-GGATTGCAAATTCCTGCCATTC-3'

E-Cadherin-R: 5'-AACGTTGTCCCGGGTGTCA-3'

GAPDH-F: 5'-ATGTTCGTCATGGGTGTGAA-3'

GAPDH-R: 5'-GGTGCTAAGCAGTTGGTGGT-3'

β-actin-F: 5'-CCCAGCACAATGAAGATCAA-3'

β-actin-R: 5'-ACATCTGCTGGAAGGTGGAC-3'

IL6-F: 5'- CTCTGGGAAATCGTGGAAATGAG -3'

IL6-R: 5'-CTGTATCTCTCTGAAGGACTCTG-3'

Luciferase Assay

MIA PaCa-2 cells were transfected with the reporter vectors carrying the 3′UTR of FOXA2 (S805635, SwitchGear Genomics. The constructs harbored the seed sequence of miR-199a-3p (wildtype) or had a mutation of this sequence (miR-199 mutant). At

24 hours, the cells were transfected with miR-negative control or miR-199 mimic and at 48 h luciferase activity was measured using the Dual Luciferase Reporter Assay System (E1910, Promega).

Cell Growth Assays

PANC-1 and BxPC-3 pancreatic cancer cell lines were transfected with siFOXA2#2 or miR-199 mimic and their respective control and plated on a 96-well plate (5×10^2cells/well). Cell growth was assessed using the Cell-Titer Glo Luminescence Cell Viability Assay (G7571, Promega). The xCELLigence RTCA SP system utilizes a 96-well microtiter detection device, where the microelectrode sensor arrays are coated in 96-well microtiter plates and the microtiter plate detection device is connected to the workstation from the inside of the cell incubator. The impedance data from the selected well is exported to the computer and analyzed using RTCA software. A parameter termed cell index is used to quantify cell status based on detected cell-electrode impedance. Cell attachment and proliferation from selected wells of the plate were monitored and recovered every 15 minutes using the RTCA SP for 120h. The PANC-1 cells were transfected with miR-NC or miR-199. 24 hours post-transfection, cells were trypsinized and cells were re-suspended at 5×10^3 cells/100 µL and 5×10^3 cells were seeded into each well of the E-plate 96 in quadruplicates.

Mouse Experiments

$5x10^6$ PANC-1 control or PANC-1 FOXA2Δ cells were injected subcutaneously in the right flank of NOD/SCID mice (n= 10 mice/group). Tumor growth was monitored every seven days for a total period of 64 days. Tumor volumes were calculated by the equation V (mm^3) = $axb^2/2$, where “a” is the largest diameter and b is the perpendicular diameter. In addition, paraffin embedded tissue sections from pancreatic tissues from male 3-month and 9-month old, male, KrasG12D$^{+/-}$p48-Cre$^{+/-}$ (KC) mice, were provided by Dr. Guido Eibl’s laboratory (39). All the mouse studies were approved by the University of California Institutional Animal Care and Use Committee and conformed to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Western Blot Analysis

Protein samples were subjected to SDS PAGE and transferred to polyvinylidene difluoride membranes in 25 mM Tris, 192 mM glycine. Membranes were blocked with 5% nonfat dry milk in PBS, 0.05% Tween-20 and probed with antibodies (1:1000) followed by corresponding horseradish peroxidase-labeled secondary antibodies (1:1000). Blots were developed with ECL reagent (T) and exposed in Eastman Kodak Co. 440 Image Station.

Antibodies and Reagents

Antibodies

Two different antibodies against FOXA2 were used. One was used for western blotting experiments (8189, Cell Signaling) and the other (sc-6554, Santa Cruz Biotechnology) for immunohistochemical analysis. PLAUR antibody was used for western blotting experiments (9692, Cell Signaling). Additionally, phospho-p44/42 MAPK (Erk1/2)(Thr202/Tyr204) was used for western blotting in PANC-1 and HPAF –II cell lines (4370, Cell Signaling) along with total ERK antibody (4695, Cell Signaling), as well as total AKT (4691S, Cell Signaling) and phospho-AKT T308 (13038S, Cell Signaling) and phosphor-AKT S473 (4060S, Cell Signaling). Additionally, CREB and GAPDH antibodies were used as loading controls (9104, Cell Signaling and 5174, Cell Signaling, respectively).

Small interfering RNAs

The following siRNAs were used in this study: siRNA negative control (siNC #2, 4390847, Life Technologies) and two different siRNAs against FOXA2 (siFOXA2#1, s6691, Life Technologies) and (siFOXA2#2, s6692, Life Technologies). A single siRNA against PLAUR was siPLAUR used in this study (s10614, Life Technologies).

FOXA2 Overexpression Vector

MiaPaCa-2 cells were transfected with vector plasmids as controls (Origene, PS100001) or plasmids for overexpression of FOXA2 (Origene, RC211408) according to manufacturer's protocol.

MicroRNAs

The following microRNAs were used in this study: *miR*Vana miRNA mimic, negative control #1 (miRNC, 4464059, Life Technologies) and miR-199 *miR*Vana miRNA mimic (4464066 miRVana miRNA mimic, Life Technologies).

3'UTR FOXA2 Vector

pLightSwitch_3UTR for FOXA was purchased from SwitchGear Genomics (S805635, SwitchGear Genomics), containing the miR-199a-3p predicted binding site.

CRISPR/Cas9 system

The FOXA2 human gene knockout kit via CRISPR was ordered from OriGene (KN204066). PANC-1 clones were selected using 2μg/ml puromycin.

Statistical Analysis

All experiments were performed in triplicate unless other-wise stated. Statistical analyses were performed with the use of Origin software, version 8.6. Student's t-test was used to examine the statistical difference in FOXA2 and miR-199 expression between control and PDAC tissues. The correlation significance was determined by means of Spearman and Pearson correlation analyses. A P-value of < 0.05 was considered statistically significant (*$P < 0.05$, **$P< 0.01$, ***$P<0.001$).

Ingenuity Network Software (IPA)

Gene network was constructed and important hubs were identified using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Mountain View, CA) based on the differentially expressed genes identified after inhibition of FOXA2 expression by siRNA FOXA2#2 in PANC-1 pancreatic cancer cell line. IPA is a robust and expertly curated database containing updated information on more than 20,000 mammalian genes and

proteins, 1.4 million biological interactions, and 100 canonical pathways incorporating over 6,000 discreet gene concepts. This information is integrated with relevant databases such as Entrez-Gene and Gene Ontology. The experimental data sets were used to query the IPA and to compose a set of interactive networks taking into consideration canonical pathways, the relevant biological interactions, and the cellular and disease processes. Pathways of highly interconnected genes were identified by statistical likelihood using the following equation:

$$Score = -\log_{10}\left(1 - \sum_{i=0}^{f-1} \frac{C(G,i)C(N-G,s-i)}{C(N,s)}\right)$$

Where *N* is the number of genes in the network of which *G* are central node genes, for a pathway of *s* genes of which *f* are central node genes. *C(n,k)* is the binomial coefficient. We considered statistically significant networks those with a score greater than 5 (*p* value $<10^{-5}$).

Results

FOXA2 Transcription Factor is Down-Regulated in Human Pancreatic Cancers.

To evaluate the role of the human transcriptome in pancreatic oncogenesis, first we examined the expression levels of all the known transcription factors by performing gene profiling analysis in eight pancreatic control and fourteen PDAC tissues. This analysis revealed 43 transcription factors that were deregulated (>1.5 fold) in PDAC relative to control tissues (**Figure 1A, Table 1**). Interestingly, among the top differentially expressed transcription factors were FOXA2 (HNF-3β), HNF-1β and HNF-6, three members of the hepatocyte nuclear factor family of transcription factors (**Figure 1B**). Although the HNF family members are known to be involved in liver oncogenesis (61), their role in pancreatic oncogenesis has not been evaluated. The profiling analysis

showed FOXA2 mRNA to be highly down-regulated in PDAC relative to control tissues, suggesting a potential tumor suppressor role in PDAC. To further validate the gene expression findings, we performed quantitative real-time PCR to examine FOXA2 mRNA expression levels in 14 control and 14 PDAC tissues in a second cohort of pancreatic cancer patients. Consistent with our initial findings, FOXA2 mRNA levels were significantly downregulated in PDAC (**Figure 1C**). In addition, we performed immunohistochemical (IHC) analysis for FOXA2 in 63 human tissue sections, including 42 PDAC, 21 control pancreatic tissues and found that 31/42 (74%) of PDAC tumors had no expression of FOXA2, while FOXA2 was expressed in all of the control tissues (**Figure 1D**), further suggesting a potential tumor suppressor role of FOXA2 in PDAC. In order to investigate the role of FOXA2 expression in pancreatic oncogenesis, we performed immunohistochemical analysis for FOXA2 in 3-month and 9-month old KC mice. Consistent with the human data, expression of FOXA2 was decreased in the 9-month old mice compared to the 3-month old mice **(Figure 1E),** suggesting that FOXA2 expression is decreased during pancreatic oncogenesis. Overall, all these data show that FOXA2 mRNA and protein levels are decreased in PDAC.

FOXA2 has Tumor Suppressor Properties in PDAC.

To study the functional role of FOXA2 in pancreatic cancer, we screened a panel of seven (PANC-1, BxPC-3, HPAF-II, Capan-1, Capan-2, AsPC-1, MiaPaCa-2) different human pancreatic cancer cell lines for FOXA2 expression. Out of the seven cell lines investigated, PANC-1, BxPC-3 and HPAF-II expressed FOXA2 mRNA and were selected to perform further molecular studies by manipulating FOXA2 expression levels. We silenced FOXA2 expression by using two different siRNAs in two pancreatic cancer cell lines that exhibited increased FOXA2 levels (PANC-1 and BxPC-3). Cell growth analysis was studied and comparisons were performed relative to the cells transfected

with an siRNA negative control (**Figure 2A**). Although siRNA#2 had a higher knockdown efficiency than siRNA#1 against FOXA2 (*data not shown*), when cells were transfected with either siRNA#1 or siRNA#2, a statistically significant increase in cell growth was observed in both PANC-1 and BxPC-3 cell lines, 48 hours post transfection (**Figure 2A**). Due to the higher knockdown efficiency, siRNA#2 was used in the follow-up experiments to manipulate FOXA2 levels *in vitro*. Specifically, FOXA2 inhibition by siRNA#2 significantly increased the ability of PANC-1 cells to form colonies in soft agar (**Figure 2B**). To further explore the functional role of FOXA2 in pancreatic cancer cell properties, we performed cell migration and invasion assays in PANC-1 (**Figure 2C and D**) and BxPC-3 cells (**Figure 2E and F**). A statistically significant higher number of migrating and invading cells were observed upon FOXA2 knockdown, suggesting that inhibition of FOXA2, promotes pancreatic oncogenesis. In order to explore the role of FOXA2 overexpression on invasion, FOXA2 was overexpressed in MiaPaCa-2 cells, a human pancreatic cancer cell line that does not express basal levels of FOXA2. There was a statistically significant difference in invasion upon FOXA2 overexpression, with a significant decrease in invasion upon FOXA2 overexpression compared to control **(Figure 2G)**.

MiR-199a Negatively Regulates FOXA2 Expression through Binding in its 3'UTR.

We were interested in identifying the molecular mechanism involved in the suppression of FOXA2 expression in pancreatic cancer. Initial DNA methylation analysis (Infinium HumanMethylation450 BeadChip assay) on 20 PDAC human tissues and 15 cancer adjacent normal tissues revealed that the FOXA2 promoter region was not differentially methylated in PDAC (*data not shown*), suggesting that DNA methylation is not the molecular mechanism responsible for FOXA2 reduced expression in pancreatic cancer. According to our previous studies, microRNAs have been found to be essential

regulators of transcription factors involved in oncogenesis (77). Bioinformatics analysis by using the TargetScan algorithm revealed that miR-199a-3p has sequence complementarity in the position of 275-81 nt of the 3'UTR of FOXA2 (**Figure 3A**). To examine the direct interaction between miR-199a and FOXA2, we performed a 3'UTR luciferase assay. MiR-199 was overexpressed in Mia PaCa-2 cells that were co-transfected with a construct harboring the 3'UTR of FOXA2 under luciferase activity. We found that miR-199a overexpression reduced FOXA2 3'UTR luciferase activity compared to control and point mutation of the miR-199a binding site in the 3'UTR FOXA2 luciferase vector abolished the suppressive effects of miR-199a (**Figure 3B**). To further validate the interaction between miR-199a and FOXA2 *in vitro*, miR-199 was overexpressed in PANC-1 cells. We examined FOXA2 mRNA and protein levels, and found that FOXA2 levels significantly decreased in miR-199a-overexpressing pancreatic cancer cells (**Figure 3C, D**). Taken together, these findings suggest that miR-199a is a direct regulator of FOXA2 expression in pancreatic cancer.

MiR-199a has an Oncogenic Function in PDAC.

Next, we were interested in investigating the relevance of miR-199a in human pancreatic cancer. We performed real-time PCR analysis in 19 control and 17 PDAC tissues and found a statistically significant up-regulation of miR-199 expression in PDAC compared to control (**Figure 4A**). In order to examine the up-regulation of miR-199a in histological tissues, we performed *in situ* hybridization on a tissue microarray containing 25 cases of pancreas adenocarcinoma with matched cancer adjacent tissue. *In situ* hybridization revealed 17/25 (68%) of adenocarcinomas highly expressed miR-199a (bottom panel), while it was not expressed in control tissues (upper panel) (**Figure 4B**). To explore the functional role of miR-199 in pancreatic oncogenesis we used the xCELLigence technology to monitor cell growth over a period of 120 hours, with a

measurement taken every 15 minutes. This assay showed that miR-199a significantly increases the growth of PANC-1 cells (**Figure 4C**). Cell growth was also performed with the same experimental samples using the CellTiter-Glo Luminescent Cell Viability assay. MiR-199a overexpression led to a 50% increase in PANC-1 cell growth compared to cells transfected with a microRNA negative control (**Figure 4C**). To further assess the functional effects of miR-199a overexpression in pancreatic cancer, we performed migration and invasion assays in PANC-1 cells and found a statistically higher number of migrating and invading cells in the miR-199a-overexpressing PANC-1 cells relative to cells transfected with the microRNA negative control (**Figure 4D and E**).

FOXA2-Regulated Gene Network in PDAC.

Our data revealed that FOXA2 has tumor suppressor properties in PDAC and its expression is regulated by miR-199a. To evaluate the molecular mechanisms that are regulated by FOXA2 suppression in PDAC and identify its downstream gene targets, we transiently knocked down FOXA2 using siFOXA2#2 in PANC-1 cells and its corresponding negative control, demonstrating an 80% inhibition of FOXA2 mRNA expression levels (**Figure 5A**). Next, we performed gene profiling analysis and found that 372 genes were up-regulated, while 552 were down-regulated (924 genes in total) in siFOXA2#2 PANC-1 cells relative to siRNA negative control by using a cut-off of $p<0.05$ and a fold change of 2 (**Figure 5B**). The Ingenuity Pathway Analysis (IPA) software was employed to perform signaling pathway analysis. The results revealed statistically significant enrichment for the cell movement/invasion pathway, cell proliferation, PI3K/AKT and MAPK signaling pathways (**Figure 5C**). To further evaluate these findings we performed gene network analysis by using the 924 differentially expressed genes in the IPA software network analysis and found that the most significant (p value = 10^{-42}) gene network was involved in cellular invasion having

PLAUR, extracellular signal-regulated kinases (ERK), and phosphoinositide 3-kinase (PI3K) as central nodes, consistent with our pathway analysis (**Figure 5D**). Consistent with IPA network analysis data, inhibition of FOXA2 in HPAF-II cells leads to activation of ERK, demonstrated by ERK phosphorylation (**Figure 5E**), suggesting that FOXA2 suppression directly or indirectly leads to ERK activation. Interestingly, PLAUR is a gene known to be related to cancer cell invasiveness and motility (36, 67, 179). To further validate the gene network findings, we examined PLAUR expression levels by real-time PCR after FOXA2 inhibition by siRNA#2. Consistent with our initial findings, FOXA2 inhibition resulted in a significant increase in PLAUR mRNA levels in PANC-1 cells (**Figure 5F**). To examine if PLAUR is mediating FOXA2 effects on pancreatic cancer cell invasiveness, we performed an invasion assay knocking down either FOXA2 or both FOXA2 and PLAUR by siRNAs in HPAF-II cells, a pancreatic cell line that expresses basal levels of both FOXA2 and PLAUR. We observed a significant increase in invasion by knockdown of FOXA2 and this increase in invasion was completely reversed when cells were transfected with both an siRNA against FOXA2 and an siRNA against PLAUR (**Figure 5G**), suggesting that PLAUR is a major mediator of FOXA2 effects on pancreatic cell invasiveness. Taken together, these data suggest that FOXA2 regulates pancreatic cell invasiveness through regulation of PLAUR expression levels.

Furthermore, it is known that microRNAs have multiple downstream gene targets and recent studies have shown that the NF-kB pathway, which is affected by miR-199a, cross-talks with the FOXA2 signaling pathway (114). To shed some light on the potential cross talk between FOXA2 and other common oncogenic pathways like nuclear factor-κB (NF-κB), we looked at the expression of IL6, a downstream target of NF-kB, upon transient inhibition of FOXA2 in the BxPC-3 cell line. Upon knockdown of

FOXA2 with siRNA#2, there is a significant increase in IL6 levels **(Figure 5H)**, indicating activation of the NF-kB pathway.

Generating a FOXA2Δ Pancreatic Cell Line Using the *CRISPR/Cas9 System.*

We observed the effects of FOXA2 inhibition of expression *in vitro* through a series of functional and gene expression assays. In order to study the effects of FOXA2 deletion *in vivo*, we developed a cell line with a permanent knock-out of FOXA2 at the chromosomal level (FOXA2Δ). We used the CRISPR/Cas9 system, where we co-transfected PANC-1 cells with two FOXA2 gRNA vectors, containing two different target sequences (**Figure 6A**) and the corresponding donor control vector. After clonal selection in puromycin, we validated FOXA2Δ at the protein level (**Figure 6B**) and also found a significant increase in PLAUR mRNA levels in FOXA2Δ compared to control (**Figure 6C**), consistent with our siRNA experimental setting. Next, we examined the phosphorylation levels of ERK and AKT by western blot and found both kinases to be activated in FOXA2Δ compared to control, consistent with our gene network analysis (**Figure 6D and E**). Conclusively, this data demonstrates the high efficiency of the CRISPR/Cas9 system, its consistency with the siRNA system, providing us with a powerful tool to study the role of FOXA2 *in vivo*.

CRISPR/Cas9 FOXA2 Inhibition Increases Pancreatic Tumor Growth in vivo.

To further support the role of FOXA2 as a tumor suppressor gene in pancreatic cancer, we wanted to test its properties *in vivo*. We performed subcutaneous injections in NOD/SCID mice with either FOXA2Δ PANC-1 ($5x10^5$ cells) or its corresponding PANC-1 control cell line (n=10/group). On day 64, mice were sacrificed and tumors were isolated. The FOXA2Δ tumor volumes (mm^3) and weight (g) were significantly larger than the PANC-1 control tumors (**Figure 7A, B and C**). On day 64, RNA was isolated

from each tumor and quantitative real-time PCR showed that FOXA2 was not expressed in the FOXA2Δ tumors relative to controls (**Figure 7D**). Furthermore, in accordance with our *in vitro* findings, FOXA2Δ tumors showed increased PLAUR mRNA levels (**Figure 7E**). Moreover, E-cadherin levels decreased in FOXA2Δ tumors, indicating FOXA2 may also regulate cellular motility (**Figure 7F**). Taken together, the *in vivo* data suggest that inhibition of FOXA2 increases the pancreatic tumorigenicity and aggressiveness.

Discussion

Our study revealed FOXA2 as a novel tumor suppressor gene in pancreatic cancer. FOXA2 is a 455-amino acid member of the forkhead class of DNA-binding proteins and contains a highly conserved winged-helix DNA-binding domain (135). FOXA2 is a transcription factor that was initially identified in hepatocytes, where it binds in the promoter areas of important liver-enriched genes transthyretin, alpha 1-antitrypsin and albumin (35, 49, 65). It is required for the formation of the node, notochord, nervous system, and endoderm-derived structures (49, 84). In adulthood, FOXA2 has been shown to control metabolic homeostasis and to contribute to insulin resistance (174).

In the last decade, several studies have implicated the role of FOXA2 in solid tumors. FOXA2 has been found to be expressed in all types of neuroendocrine lung tumors (90) and shown to be a key regulator in colorectal liver metastases (104). We found that FOXA2 inhibition induces cancer cell invasiveness, consistent with its function in other cancers. Specifically, in human lung cancer cells, upon TGF-β1 treatment, FOXA2 levels are decreased, leading to activation of Slug transcription, thus inducing epithelial-mesenchymal transition (EMT) and promoting invasion (155). More recently, Liu et al.

demonstrated FOXA2 phosphorylation by TNFα-induced IKKα stimulates the NOTCH1 pathway to promote liver cell proliferation and growth, indicating FOXA2 suppression by phosphorylation plays an important role in TNFα mediated tumorigenesis (114).

Although dysregulation of FOXA2 has been directly linked to the progression of certain cancers, this class of transcription factors can paradoxically serve as both tumor suppressors and oncogenes (101). Very little is known about the roles of FOXA2 in invasion and tumor metastasis in pancreatic cancer. Our study identifies the transcription factors differentially expressed in PDAC and shows that FOXA2, and other hepatocyte nuclear factors, are significantly downregulated in human PDAC. Knockdown of FOXA2 led to a significant increase in cellular growth, migration, invasion and colony formation, indicating that FOXA2 harbors tumor suppressive properties.

Recent advances in pancreatic cancer biology have emerged important roles for microRNAs (miRNAs) in regulating tumor responses. MiRNAs, a class of non-coding RNAs, have emerged as critical players in cancer initiation and progression by modulating many pathological aspects related to tumor development, growth, metastasis, and drug resistance (113). Studies have found that miRNAs control many cellular processes through involvement in development, proliferation, the stress response, apoptosis, cell cycle progression, and differentiation (4, 11, 12, 43, 112). The major function of miRNAs is to post-transcriptionally regulate gene expression depending on recognition of complementary sequence residing in target mRNAs. Several key oncogenic miRNAs have been identified in pancreatic cancer, including miR-483-3p, miR-155, miR-21/miR-221, miR-27a, miR-371-5p and miR-21/miR-23a/miR-27a. Inhibition of oncogenic miRNAs reduces functional properties of pancreatic oncogenesis (48, 56, 60, 62, 120, 136). Our data indicate that miR-199a-3p

plays an oncogenic role, with a significant increase in expression in PDAC compared to control. In the last decade, investigations have revealed that the expression of miRNA-199 is altered in several human cancers (54, 102, 183). Specifically, the expression of miRNA-199 is increased in ovarian cancer cells and cervical carcinomas (54, 183) in accordance to our data in PDAC. Specifically, overexpression of miR-199 in pancreatic cancer cells led to an increase in pancreatic cell growth, migration and invasion *in vitro*, demonstrating miR-199 oncogenic properties in pancreatic cancer.

We found that miR-199a-3p directly regulates FOXA2 mRNA and protein expression, through binding in its 3'UTR. Furthermore, recent studies have identified additional downstream targets of miR-199 in other cancer types. For example, miR-199 targets Frizzled type 7 receptor (FZD7), one of the most important Wnt receptors involved in cancer development and progression (149). Additionally, mTOR, c-MET, MET proto-oncogene and CD44 have also been identified as direct targets of miR-199, playing a major role in cancer initiation and progression in different types of cancer (31, 47, 52, 64, 92).

Conventionally, loss of function genetic screens in cultured cells is mainly conducted with the aid of RNA interference (RNAi) libraries (19, 184). However, RNAi could only partially and temporary suppress gene expression and thus its application is limited to knockdown screens (19, 125). Moreover, due to the endogenous nature of the RNAi pathway, it often incurs pervasive off-target events because of the extensive endogenous interactions. These off-target effects may confound the interpretation of screen results (78). Recently, the emergence of CRISPR/Cas9 technique offers a novel and versatile platform for genetic screen studies (10, 33, 122). For these reasons, we chose the highly efficient CRISPR/Cas9 deletion system to permanently knock-out

FOXA2 in a pancreatic cancer cell line to study its effects *in vivo*. In addition, inhibition of FOXA2 expression levels by CRISPR/Cas9 *in vitro*, led to the activation of PLAUR gene, which is known to be involved in cancer invasiveness (91). Importantly, these findings were consistent with our data where FOXA2 expression was suppressed by siRNA, suggesting that the CRISPR/Cas9 system is very effective to block gene expression in cancer cells. Taken together, our study has revealed a novel signaling pathway, consisting of the miR-199 and FOXA2 tumor suppressor gene involved in pancreatic oncogenesis.

Figures

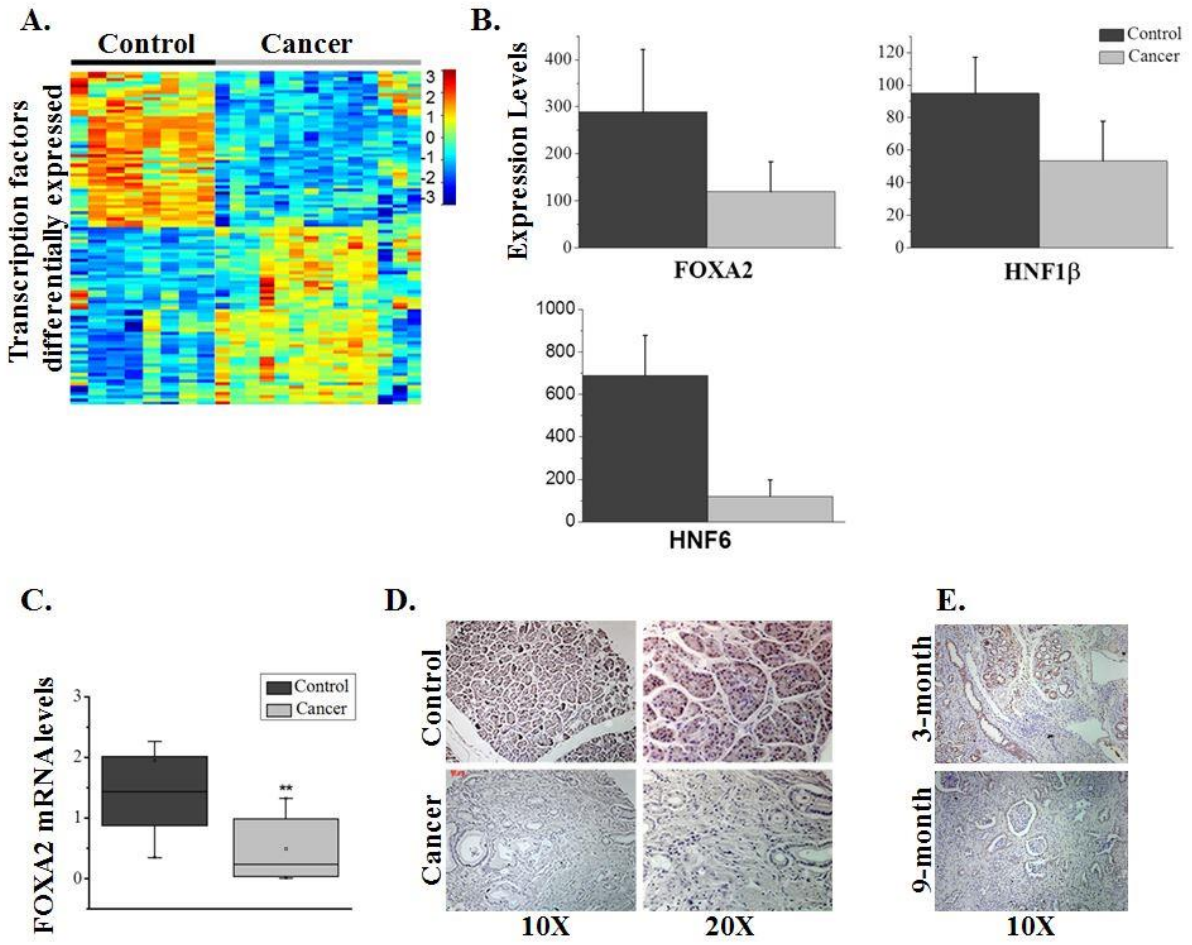

Figure 4.1. FOXA2 transcription factor is down-regulated in human pancreatic cancers.

A. Pancreatic cancer transcription factor transcriptome. Heatmap showing unsupervised clustering of expression Z-scores of mRNA expression of 105 probes from 43 transcription factor genes in 22 human pancreatic tissue (control =7 and cancer =15). *B.* Expression levels of hepatocyte nuclear factor family transcription factors (FOXA2, HNF-1β and HNF-6) from the list of 43 transcription factors differentially expressed in

PDAC. *C.* FOXA2 mRNA levels by real-time PCR in 28 human pancreatic tissue (control =14 and cancer =14). *D.* Immunohistochemical staining for FOXA2 in control and PDAC tissue under 10X (left column) and 20X (right column) magnification. *E.* Immunohistochemical staining for mouse FOXA2 in 3-month old (top panel) and 9-month old KrasG12D$^{+/-}$p48-Cre$^{+/-}$ (KC) mice (bottom panel). The experiments have been performed in triplicate and all data are represented as mean ± SD. ***P<0.001, **P<0.01, *P<0.05.

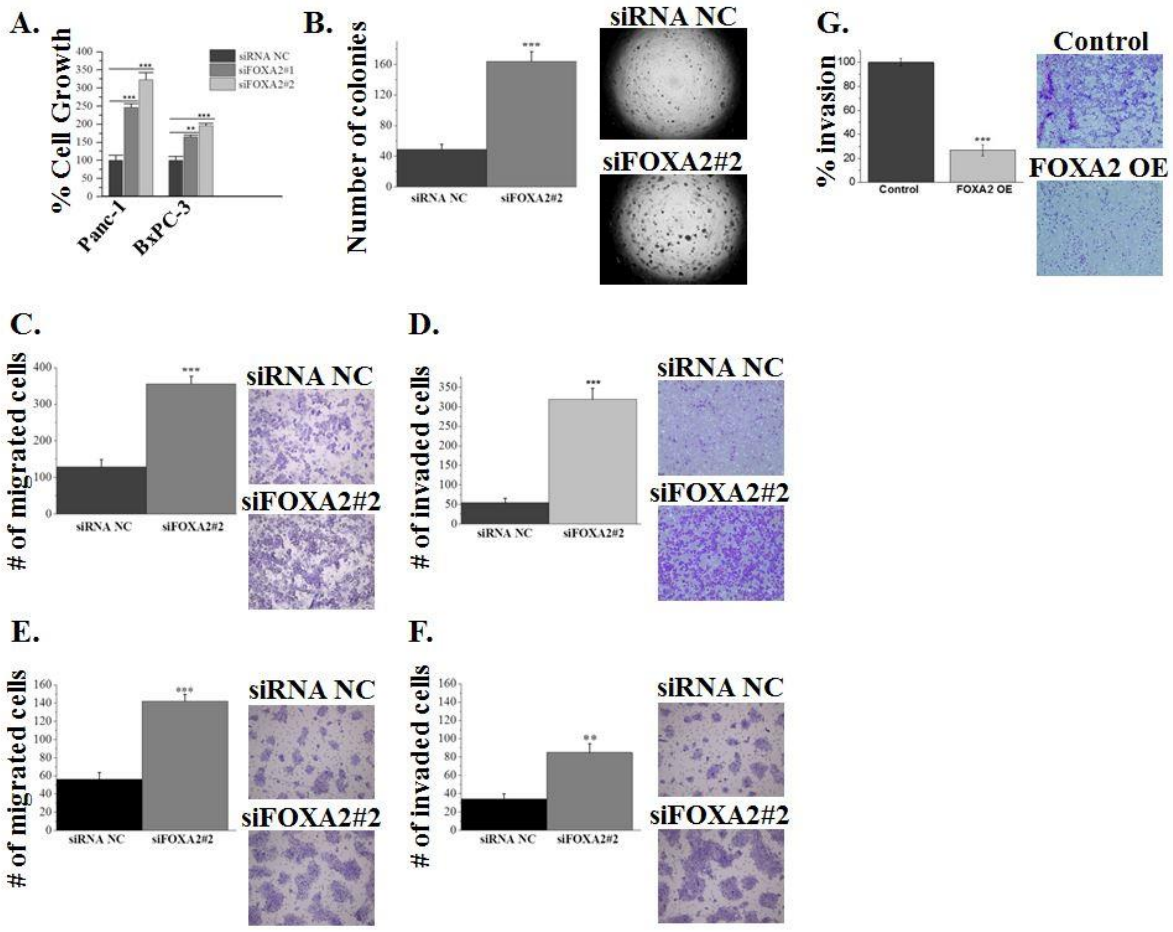

Figure 4.2. FOXA2 has tumor suppressor gene properties in PDAC.

A. Relative percent cell growth measured in PANC-1 and BxPC-3 treated for 48 h with siRNA negative control (siRNA NC) or two different siRNAs against FOXA2 (siFOXA2#1 and siFOXA2#2) using the Cell-Titer Glo Luminescence Cell Viability Assay. *B.* Soft agar colony formation assay of PANC-1 cells treated for 48 h with siRNA NC or siFOXA2#2. Colonies (mean ± SD) 50 mm were counted using a microscope 20 days later. *C.* Transwell cell migration assay in PANC-1 cells transfected with siRNA NC or siFOXA2#2, migrating across 8 mm micropore membranes. *D.* Invasion through matrigel-coated transwell inserts in PANC-1 cells transfected with siRNA NC or

siFOXA2#2. *E*. Transwell cell migration assay in BxPC-3 cells transfected with siRNA NC or siFOXA2#2, migrating across 8 mm micropore membranes. *F.* Invasion through matrigel-coated transwell inserts in BxPC-3 cells transfected with siRNA NC or siFOXA2#2. *G.* Invasion through matrigel-coated transwell inserts in MiaPaCa-2 cells transfected with control vector (control) or FOXA2 overexpression vector (FOXA2 OE). The experiments have been performed in triplicate and all data are represented as mean ± SD. ***P<0.001, **P<0.01, *P<0.05.

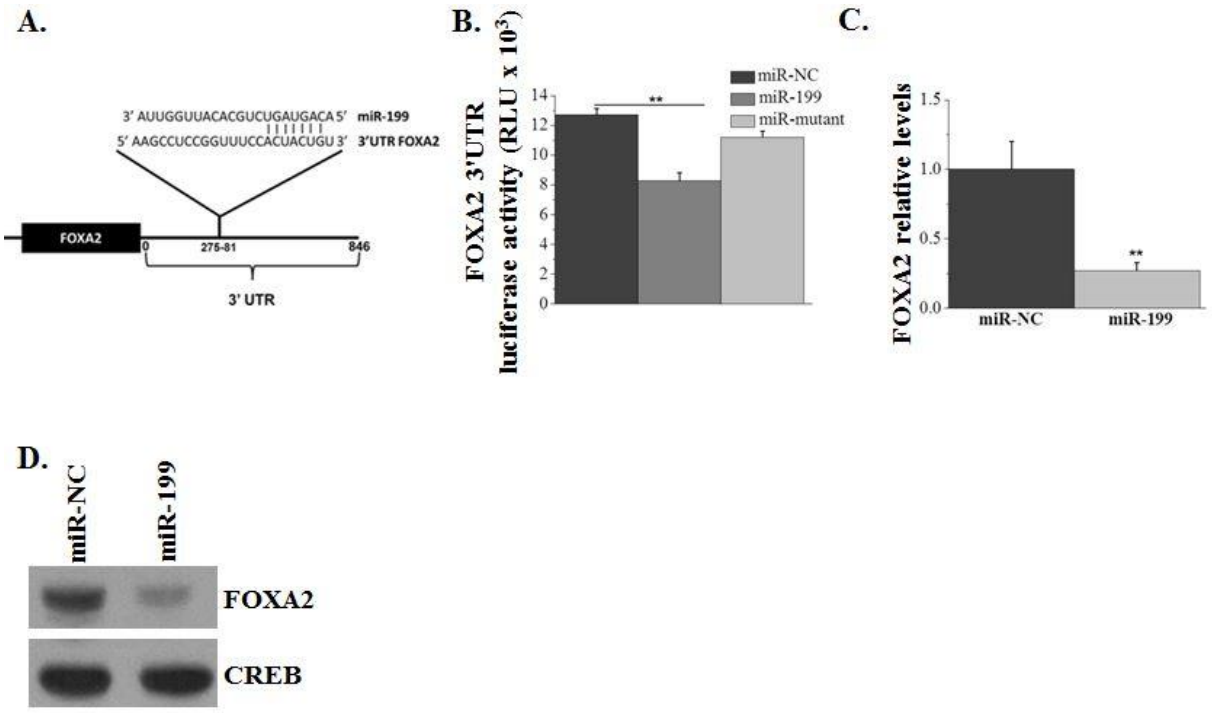

Figure 4.3. FOXA2 as a direct target of miR-199a-3p in PDAC.

A. Sequence complementarity between miR-199a-3p seed sequence and the 3'UTR of FOXA2. *B*. FOXA2 3'UTR luciferase activity in MIA PaCa-2 cells transfected with miR-NC or miR-199, 48 h post transfection. MiR-199 sequence was wildtype (miR 199) or mutated (miR mutant). *C*. FOXA2 relative mRNA levels in PANC-1 cell line 24 h post transfection with miR-199 mimic. *D.* Western blot showing FOXA2 protein levels in PANC-1 cell line 72 h post transfection with miR-199 mimic. The experiments have been performed in triplicate and all data are represented as mean ± SD. ***P<0.001, **P<0.01, *P<0.05.

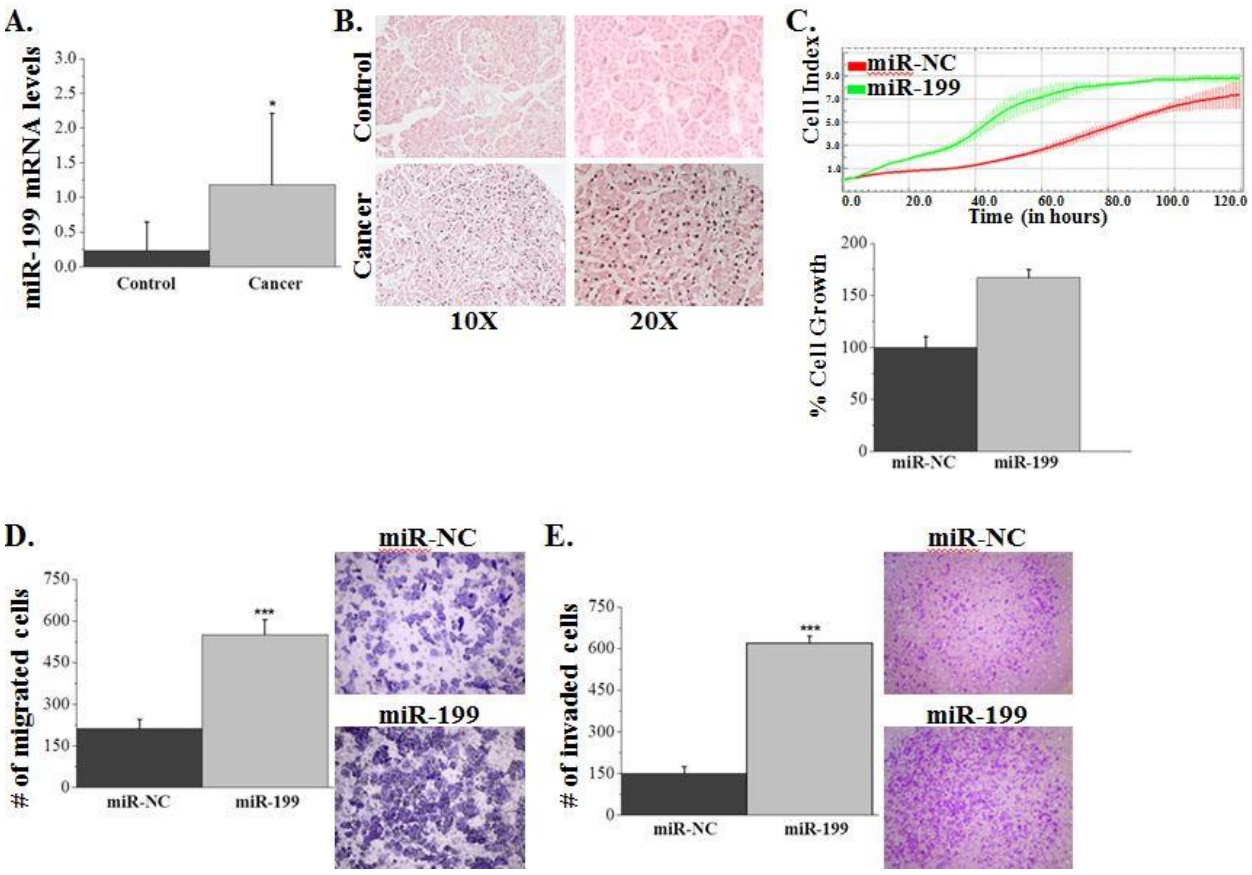

Figure 4.4. miR-199 has an oncogenic function in PDAC.

A. MiR-199 mRNA levels in human pancreatic control (n=19) and cancer tissue (n=17). *B. In situ* hybridization miR-199 in human pancreatic control and cancer tissue under 10X and 20X magnification. *C*. Cell proliferation in Panc-1 cells 24 h post transfection with miR negative control (miR-NC) or miR-199 mimic (miR-199) using the xCELLigence system. PANC-1 cells were seeded at a density of $5x10^3$ cells/well in 96-well E-plates and monitored for 120 h. *D.* Percentage cell growth measured in BxPC-3 cells treated with miR-NC or miR-199 for 24 h then plated and measured 48 h later

using the Cell-Titer Glo Luminescence Cell Viability Assay. *E*. Transwell cell migration assay in PANC-1 cells transfected with miR-NC or miR-199. *F*. Invasion through matrigel-coated transwell inserts in PANC-1 cells transfected with miR-NC or miR-199. The experiments have been performed in triplicate and all data are represented as mean ± SD. ***P<0.001, **P<0.01, *P<0.05.

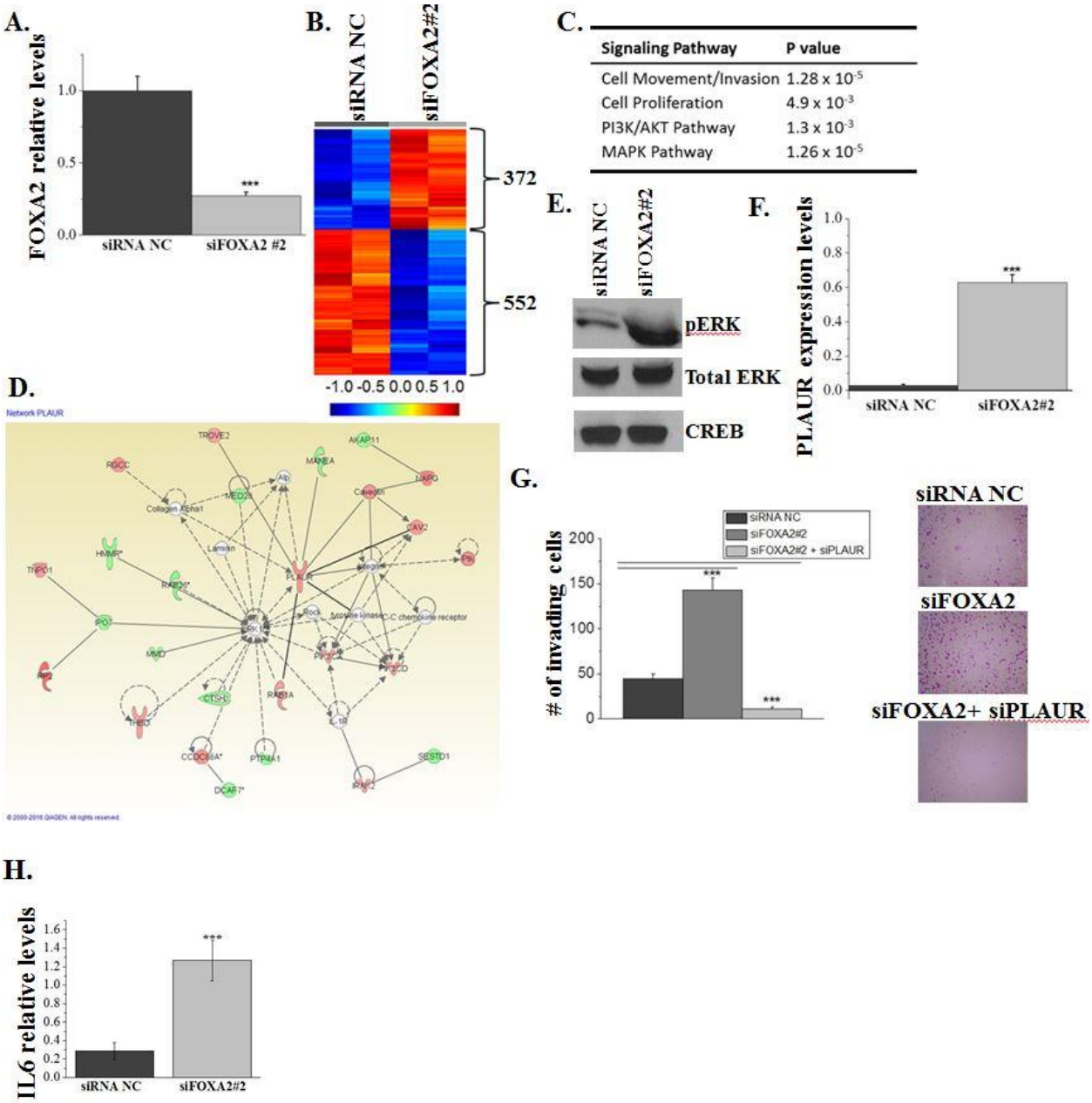

Figure 4.5. FOXA2-regulated gene network in PDAC.

A. Relative FOXA2 mRNA levels in PANC-1 cells transfected with siRNA NC or siFOXA2#2 for gene profiling studies, duplicate experimental samples were performed. *B.* Heatmap indicating expression levels of 372 genes up-regulated and 552 genes down-regulated in siRNA NC compared to siFOXA2#2 samples in PANC-1 cell line. *C*.

Ingenuity Pathway Analysis (IPA) reveals statistically significant enrichment for the cell movement/invasion pathway, cell proliferation, PI3K/AKT and MAPK signaling pathways. *D.* Gene network analysis by using the 924 differentially expressed genes in the IPA software network found the most significant (p value =10^{-42}) gene network was involved in cellular invasion having as central nodes PLAUR, ERK, PI3K, consistent with our gene ontology analysis. *E*. Western blot indicating phosphorylation of ERK, total ERK and CREB in PANC-1 cells treated with siRNA NC or siFOXA2#2. *F*. PLAUR mRNA levels in HPAF-II cells treated with siRNA NC or siFOXA2#2. *G.* Invasion through matrigel-coated transwell inserts in HPAF-II cells transfected with siRNA NC, siFOXA2#2 or both siFOXA2#2 and siPLAUR. *H.* IL6 mRNA levels with BxPC-3 cells transfected with siRNA NC or siFOXA2#2. The experiments have been performed in triplicate and all data are represented as mean ± SD. ***P<0.001, **P<0.01, *P<0.05.

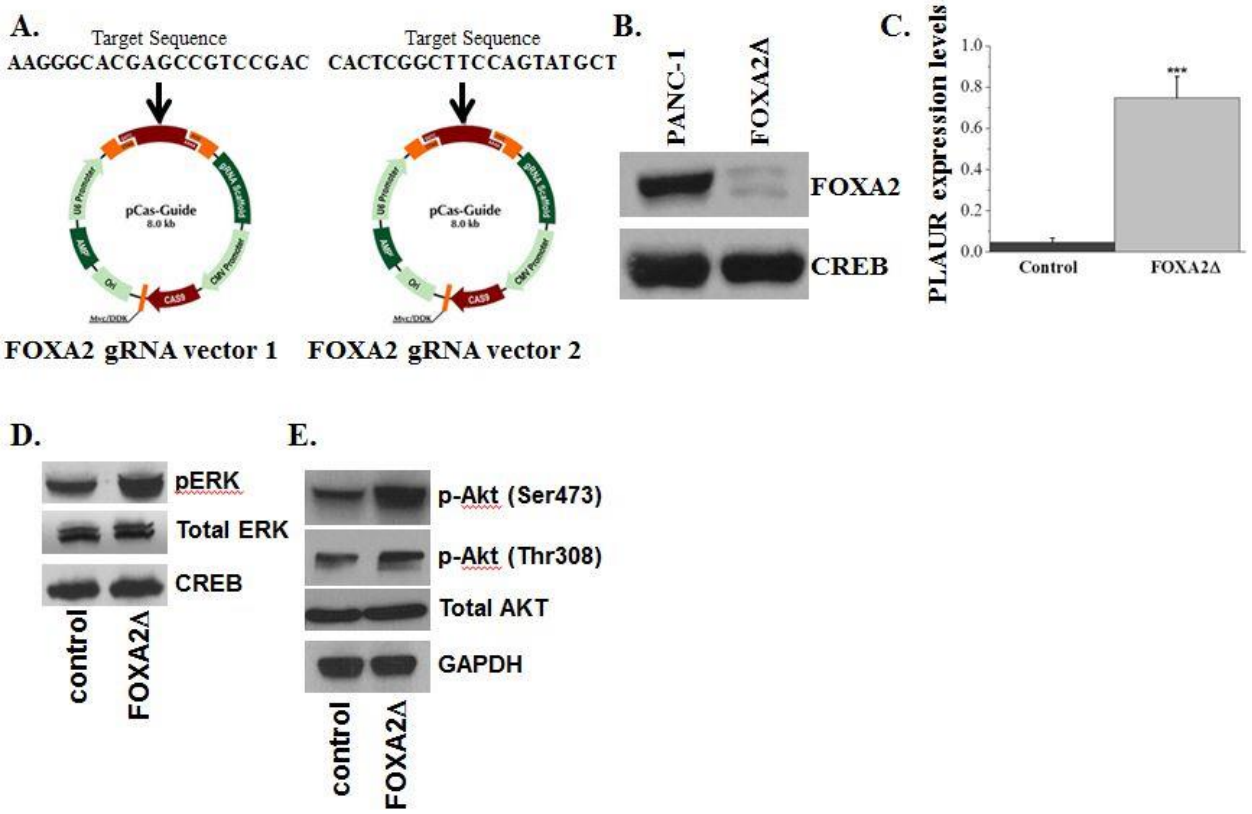

Figure 4.6. Generating a FOXA2Δ pancreatic cell line using the CRISPR/Cas9 system.

A. Sequences of FOXA2 gRNA vectors. PANC-1 cells were transfected with either 1.) two gRNA vectors and donor vector (donor vector not shown) referred to as FOXA2Δ or 2.) a scramble vector and a donor vector (scramble vector and donor vector not shown) referred to as PANC-1 control. *B*. Western blot for PANC-1 control and FOXA2Δ generated cell lines. *C.* PLAUR mRNA expression levels in PANC-1 control and FOXA2Δ cell lines. *D*. Western blot indicating phosphorylation of ERK and total ERK plus loading control in PANC-1 control and FOXA2Δ cell lines. *E*. Western blot indicating phosphorylation of AKT at two phosphorylation sites (Ser473 and Thr308) and total AKT plus loading control in PANC-1 control and FOXA2Δ cell lines. The

experiments have been performed in triplicate and all data are represented as mean ± SD. ***P<0.001, **P<0.01, *P<0.05.

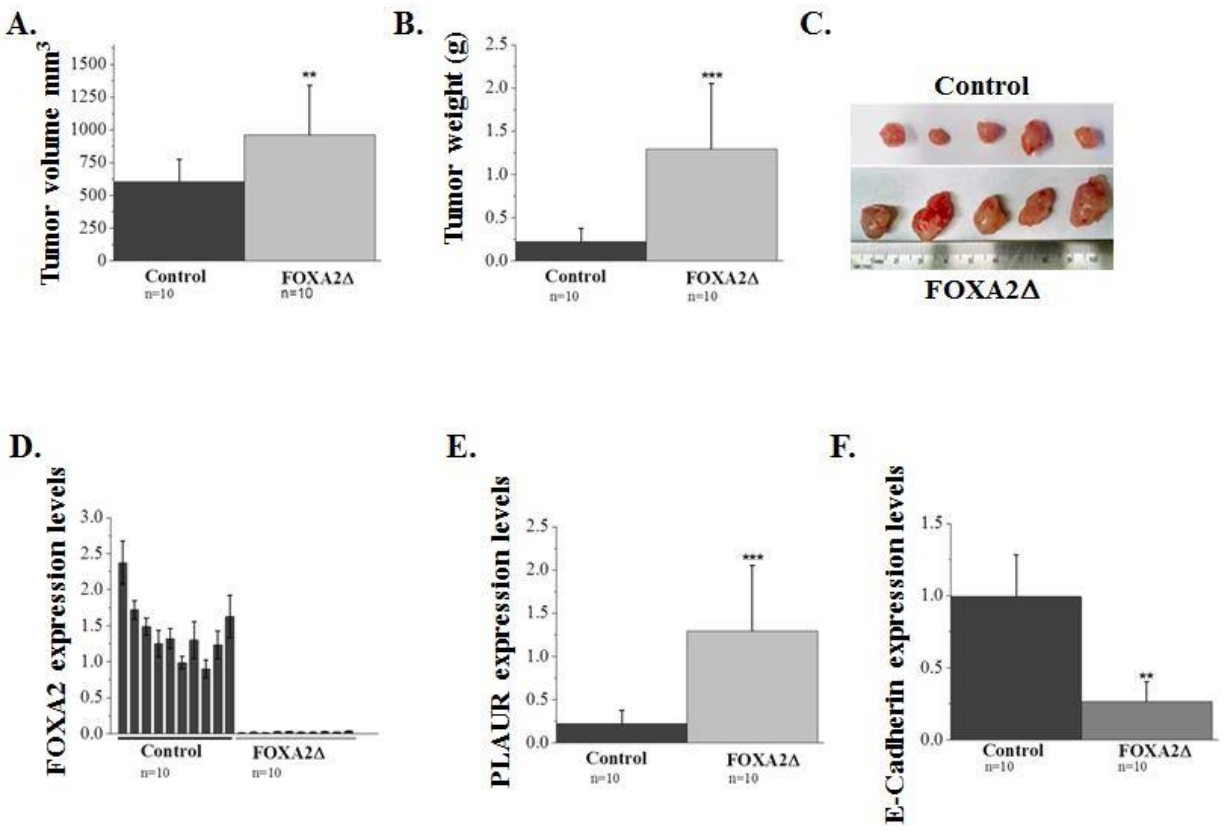

Figure 4.7. CRISPR/Cas9 FOXA2 Inhibition suppresses pancreatic tumor growth *in vivo*.

A. At day 64, tumor volumes (mm^3) were measured in PANC-1 control and FOXA2Δ (n=10/group) tumors. *B*. At day 64, tumors were excised and tumor weight (g) was measured in PANC-1 control and FOXA2Δ tumors. *C*. At day 64, PANC-1 control and FOXA2 tumors were excised and photographed, pictured with ruler (mm). *D*. At day 64, RNA was isolated from tumors and FOXA2 mRNA levels were examined in PANC-1 control and FOXA2Δ tumors. *E.* PLAUR mRNA levels were examined in PANC-1 control and FOXA2Δ tumors. *F.* Relative E-cadherin mRNA levels in PANC-1 control and FOXA2Δ tumors (n=10/group). The experiments have been performed in triplicate and all data are represented as mean ± SD. ***P<0.001, **P<0.01, *P<0.05.

CHAPTER 5

miRNAs as Therapeutic Strategies & Future Perspectives

MiRNAs Related to Chemotherapy Treatment in PDAC

The poor prognosis of PDAC is mainly due to its propensity to acquire resistance to chemotherapeutic agents and metastasize (165). The combination of chemotherapy and radiation is typically not curative and provides only minor increases in survival rates in most cases. Chemotherapy drugs such as gemcitabine, albumin-bound paclitaxel nanoparticles (Abraxane) or the combination of four chemotherapy drugs, known as FOLFIRINOX (Folinic Acid, Fluorouracil, Irinotecan Hydrochloride, Oxaliplatin) are the standards of care for metastatic disease (156). These treatments have limited efficacy and significant side effects, often only improving the quality of life of patients for a period of time rather than curing the disease itself. Consequently, there is an urgent need to develop a better understanding of the molecular drivers of PDAC progression and how they may relate to chemotherapy responses.

It is of note that, miRNA-targeting approaches have been shown to induce changes in the chemosensitivity or radiosensitivity of PDAC cells in a variety of settings. Several studies reported that antisense targeting of miR-21 and miR-221 could improve the chemosensitivity of gemcitabine resulting in significant cell killing under various conditions (136). Consistently, anti-miR-21 transfection rendered the pancreatic cancer cells more susceptible to the cytotoxic effects of gemcitabine treatment and increased the expression of Fas Ligand (FasL). The latter proved to serve as a direct target of miR-21. Importantly, an inverse correlation between the expression of miR-21 and FasL during gemcitabine treatment was observed (163). MiR-181b transfection has been shown to sensitize PDAC cells to gemcitabine treatment *in vivo* and *in vitro*, as

evidenced by higher levels of apoptosis (22). Nagano and colleagues suggested that activation of the Wnt/beta-catenin signaling pathway mediates the miR-29a-induced resistance to gemcitabine in PDAC cells (129). In another study, transfection of PANC-1 and BxPC-3 cells with a miR-17-5p inhibitor showed growth inhibition, spontaneous apoptosis, higher caspase-3 activation and increased chemosensitivity to gemcitabine (182). Additionally, miR-205 was found to be consistently downregulated across pancreatic cancer specimens, making it a suitable therapeutic target. Treatment of PDAC cells with miR-205 mimic resulted in the restoration of chemosensitivity to gemcitabine, with decreased expression of stem cell markers OCT3/4 and CD44 and chemoresistance marker class III b-tubulin (147).

Furthermore, it has been demonstrated that miR-23b overexpression inhibits radiation-induced autophagy and sensitizes PDAC cells to radiation. The authors proposed that reduced miR-23b levels increase levels of its target AGT12 and autophagy to promote radioresistance (162). Treatment of cells with a combination of the tumor suppressor miR-205 and gemcitabine micelles reduced cell invasion and restored the gemcitabine chemosensitivity, while intratumoral injection of the combinatorial treatment in mice bearing gemcitabine resistant xenografts potently arrested tumor growth (124).

The desmoplastic microenvironment promoting tumor growth and metastasis forms a barrier to chemotherapy. Hedgehog (Hh) signaling, that is implicated in initiation and progression of PDAC, also contributes to desmoplasia (74). A combination therapy comprising of GDC-0449 (inhibitor of Hh pathway) and the miR-let7b mimic, effectively

inhibited tumor growth when injected to athymic nude mice bearing ectopic tumors, compared to micelles carrying GDC-0449 or miR-let7b alone. Immunohistochemical analysis revealed attenuated tumor cell proliferation with augmented apoptosis in the animals treated with miR-let7b and GDC-0449 combination (96). Based upon this evidence, rational design of combined therapeutic approaches consisting of anticancer agents and miRNA modulators that act synergistically, will potentially improve therapeutic response.

Manipulation of miRNA Expression Levels as a Therapeutic Strategy in PDAC

miRNA Replacement Therapy

After being widely demonstrated to be deregulated in cancer cells, researchers are now exploring therapeutic strategies based on modulation of miRNA activity. Molecular approaches are being applied that reverse the aberrant miRNA expression levels. The therapeutic application of miRNAs involves direct administration of a) miRNA formulations (miRNA mimics) naked, coupled to a carrier or delivered via a viral vector in order to boost miRNA endogenous levels or b) single-stranded oligonucleotides with miRNA complementary sequences (antisense miRNAs) either small molecules so as to diminish miRNA functions.

MiRNA mimics are double-stranded synthetic miRNA oligonucleotides, used to restore miRNAs that show a loss of function. When transfected into cells, miRNA mimics are processed into a single-strand form and regulate protein-coding genes in a miRNA-like manner. This approach, also known as miRNA replacement therapy, has gained a lot of

attention as it enables therapeutic exploitation of tumor suppressors (7). Using *in vitro* and *in vivo* models based on pre-miRNA precursors (synthetic miRNA mimics), lentiviral-mediated stable miRNA overexpression or vector-mediated miRNA transient overexpression in pancreatic carcinoma cell lines, the oncogenic or tumor-suppressive role of certain miRNAs has been validated in PDAC (summarized in **Table 1**).

Restoration of let-7 levels in cancer-derived cell lines strongly inhibits cell proliferation, K-Ras expression and Mitogen-activated protein kinases-activation, but fails to impede tumor growth progression after intratumoral gene transfer or after implantation of Capan-1 cells stably overexpressing let-7 miRNA (157). A preclinical *in vivo* experiment has been described, according to which PDAC cells were directly injected with miR-217-expressing plasmids using in vivo-jet PEI. The results from these assays indicated that miR-217 overexpression sufficiently suppresses tumor cell growth (196). In another study, stable lentivirus-mediated overexpression of miR-148a in the IMIM-PC2 pancreatic cancer cell line was shown to inhibit tumor growth and colony formation possibly via targeting of Cell Division Cycle 25B gene (111). As evidenced by miR-126 overexpression and silencing of its downstream target ADAM Metallopeptidase Domain 9 (ADAM9), the miR-126/ADAM9 axis controls pancreatic cancer cell invasive growth (59). It has been demonstrated that overexpression of miR-148b dramatically suppresses growth, which is attributable to induction of apoptosis and cell-cycle arrest at S phase, remarkably inhibits invasion and enhances chemosensitivity of pancreatic cancer cells. Moreover, ectopic expression of miR-148b was able to inhibit tumorigenicity in nude mice (194).

As previously described, overexpression of miR-204 either by a miR-204 mimic or by triptolide treatment, downregulates MCL1 by directly binding to the 3′ UTR of the gene and causes a subsequent decrease in cell viability and pancreatic cancer cell death (29). In addition, transfection of lentivirus containing miR-137 mimic inhibits cancer cell invasion, increases sensitivity to Fluorouracil and suppresses tumor growth *in vivo* (177), while miR-216a overexpression markedly inhibits the JAK2/STAT3 signaling pathway and xenograft tumor growth *in vivo* (164). Furthermore, stably miR-135a over-expressing cells display reduced proliferation and clonogenicity, at least in part via the regulation of Bmi1 (38). Restoring the expression of miR-218 in pancreatic cancer resulted in downregulation of ROBO1 and efficient attenuation of cell migration and invasion (63). A recent study refers to stably miR-663 overexpression exerting anti-proliferative, anti-invasive and pro-apoptotic effects on pancreatic cancer cells by targeting Eukaryotic elongation factor 1-a (190).

Novel miRNA Delivery Approaches

More recently, novel miRNA delivery approaches have been described. Systemic administration of a miR-34a delivery system consisting of nano-complexes with a tumor-targeting and -penetrating bifunctional CC9 peptide in a pancreatic xenograft cancer model seems to significantly inhibit tumor growth and induce cancer cell apoptosis (73). In the same line, Pramanic and colleagues, synthesized a lipid-based nanoparticle for systemic delivery of miRNA expression vectors to cancer cells and demonstrated the significant therapeutic efficacy of restituting either miR-34a or miR-143/145 expression

in subcutaneous and orthotropic pancreatic cancer xenograft models (138). These approaches affirm the usefulness of miRNA-uptake delivery systems in therapeutic intervention for the disease.

Furthermore, small vehicles derived from early endosomes, which fuse to multi-vesicular bodies called exosomes, are being discussed as possibly the most potent gene delivery system. Due to their ubiquitous presence, their particular protein profile, their equipment with mRNA and miRNA, as well as their most efficient transfer in target cells, the use of exosomes as therapeutics holds great promise (158). Interestingly, it was recently shown that tumor exosome miRNA uptake from a metastasizing rat pancreatic adenocarcinoma affected premetastatic organ stroma cells towards supporting tumor cell hosting. Analysis of the exosomal miRNA-modulated gene expression in target cells showed increased protease activity, pronounced adhesion molecule and chemokine ligand expression, and upregulation of cell cycle- and angiogenesis-promoting genes, as well as of genes engaged in oxidative stress response, all fitting the demands of metastasizing tumor cells for settlement and growth (141). These findings could potentially offer a means to interfere with tumor exosome promoted metastasis, a major target in pancreatic cancer therapy. Issues of elucidation of mechanism of action, selective uptake and side effects need to be addressed and further experimental studies are necessary in order to possibly place exosomes into therapeutic settings.

On the other hand, for strategies that block miRNA functions, both oligonucleotide-based and small-molecule-based approaches are being explored. It has been shown that ASOs can competitively inhibit upregulated oncogenic miRNAs in tumors in a specific, efficient, and long-lasting manner (44). Preclinical studies indicate potent activity of AEG35156 (targets X-linked inhibitor of apoptosis mRNA) in combination with gemcitabine in PDAC (121). These data imply that targeting miRNAs with ASOs could be a potential new therapeutic strategy for PDAC (summarized in **Table 1**). Chemical modifications to the backbones of these nucleotides led to the development of miRNA antagonists (also known as anti-miRs), including antagomiRs and Locked Nucleic Acids (LNAs). AntagomiRs represent often cholesterol-conjugated, single-stranded RNA molecules about 21-23 nucleotides in length and complementary to mature target miRNAs. The introduction of 2'-O-methyl groups or 2'-O-methoxyethyl groups contributes to nuclease resistance, as well as improved affinity and specificity towards the endogenous miRNA that is further unable to be processed by RISC (168)-(75). LNAs are a class of nucleic acid analogues in which the ribose ring is “locked” by a methylene bridge connecting the 2’-O atom and the 4’-C atom. By “locking” the molecule with the methylene bridge, LNA oligonucleotides display remarkable hybridization affinity toward complementary single-stranded RNA (159). MiRNA antagonists specifically silence the expression of miRNA leading to upregulation of miRNA’s downstream gene targets (89).

In addition, multiple studies indicate the therapeutic potential of anti-miRs in pancreatic cancer. Specifically, transfection of Capan-2 cells with an oligonucleotide able to inhibit

the miR-155 activity caused TP53INP1 re-expression, as well as a significant increase in apoptosis (56), while inhibition of miR-27a was shown to inhibit growth, colony formation and migration of pancreatic cancer cells (120). Likewise, anti-miR-483-3p transfection in SW1990 and PANC1 cells significantly reduced cell proliferation and colony formation (60). Another study showed that anti-miR-371-5p treatment causes proliferative inhibition of pancreatic cancer cells, which is partially due to a G1-phase arrest (62). By analyzing the combined effects of altered activities of miRNAs in PDAC cell lines and in PDAC samples from patients, a combination of 3 miRNAs (miR-21, miR-23a, and miR-27a) that cooperatively promote tumor growth, was identified. Importantly, inhibition of miR-21, miR-23a, and miR-27a had synergistic effects in reducing proliferation of PDAC cells in culture and growth of xenograft tumors in mice. These findings provide evidence that the co-inhibition of multiple miRNAs holds promise for the development of novel cancer therapies (48).

The formation of stem loops in pre-miRNAs and bulges in miRNAs found in their secondary structure reinforced the idea of small-molecule targeting. Such structural features provide specificity basis for structure-based drug design. Small molecule inhibitors strongly interact with the surface of miRNAs, thereby interfering with the processing and the biological functions of the latter (191). Several compounds, including isoflavone, have been shown to significantly downregulate miR-200b, miR-200c, Let-7b, Let-7c, Let-7d, and Let-7e in gemcitabine-resistant cancer cells (109). A naturally occurring flavonoid, curcumin (diferuloylmethane) has been shown to have dramatic effects on the expression profiles of miRNAs in pancreatic cancer models, causing

upregulation of miR-22 and downregulation of miR-199a, concomitantly regulating Sp1 and estrogen receptor 1, which are involved in cell growth, metastasis, and apoptosis (152).

Strategies Blocking miRNA Functions

Ribonucleic acid interference (RNAi) is possibly one of the most anticipated tools for modern day therapeutics. RNAi is showing huge potential for the treatment of fatal diseases that currently have no appropriate treatment or cure. RNA interference is a potent methodology for reducing the expression of endogenously expressed proteins. Innovative RNAi therapeutics is being developed by silencing the mRNA encoding gene or by using pathogenic proteins. A number of candidates including delivery systems and other RNAi tools are currently being developed and are in various stages of clinical trials. RNAi therapeutics, as well as RNAi application in diagnostics and agriculture, is expected to hold great potential with companies bringing out various innovative delivery mechanisms as well as other modified RNAi reagents (siRNA, shRNA, etc.).

Andrew Fire and Craig Mello made the discovery of the RNAi phenomenon in 1998. This event assured the enabling of gene knockdown activity by double-stranded RNAs (dsRNAs), and various RNAi-based methods are further being developed for therapeutic as well as for biological research purposes. Furthermore, the significance of RNAi mechanisms in functional genomics was revealed with the identification of genome sequences for various organisms including *C. elegans*, Drosophila (fruit flies), mice, rats and humans. These genome-wide genomic resources allow reverse genetic

screens in tissue culture as well as *in vivo*. The development of RNAi therapeutics goes through defined stages through the commercialization of candidates. The technique is still in its evolutionary phase and no approved RNAi therapeutics is available to date. Thus, the research process, the delivery process and the exploratory phases require a significant amount of time as well as significant funding. Broadly speaking, the stages include:

- Exploratory phase (proof of concept/research)
- Pre-clinical
- New drug approval
- Clinical trials
- Commercial approvals
- Post-commercial monitoring

The demand for therapeutics for diseases like cancer, neurodegenerative diseases as well as rare and infectious diseases are mounting and patients are left with no curative options. RNAi has shown to offer superior specificity and flexibility compared to traditional biologics or small molecule drugs used to silence gene expression. There are no RNAi therapeutics or other gene silencing products available commercially. However, many candidates are in later stages of their development; therefore, organizations have filed their patents for protecting their inventions associated with RNAi drugs for diseases with no available treatment.

In regard to RNAi therapeutics, companies are now mostly emphasizing successful delivery systems of the RNAi reagents into the target cell. After target identification and validation, delivery of the RNAi reagents successfully into the target cell for gene knockdown is the most challenging task. This is due to degradation of siRNAs, off-target effects, stability issues and other factors. Thus, manufactures of RNAi technologies are emphasizing robust, flexible and effective delivery technologies such as systems that can be customized for diverse targets, conjugate systems with flexible modes of administration (subcutaneously, intravenous, etc.) and lipid/polymer/nanoparticle-based delivery systems with improved cellular uptake and various other elements.

Detailed pipelines for RNAi candidates as well as RNAi delivery methods and other technologies are published by the companies developing them along with their respective stages and phases of development. **Table 2** below presents a list of the RNAi candidates and their technologies in various stages of developments. Information was obtained from respective company websites.

Future Perspectives

The number of studies on miRNAs in a PDAC setting is increasing at an exponential rate in recent years. Multiple deregulated microRNAs and genes in different steps of pancreatic oncogenesis have been described, supporting the link of miRNAs expression with cancer pathogenesis. Importantly, several studies provide evidence of miRNA's functional significance as mediators of important molecular drivers of PDAC. Identification of the role of miRNAs in the regulation of cell signaling pathways involved

in pancreatic cancer development and progression is needed in order to broaden our understanding of the molecular background of the disease. Notably, combinatorial treatments consisting of miRNA-intervening approaches and chemotherapeutic agents regulate tumor responses more efficiently.

From a clinical point of view, the perspective that introduction of miRNA mimics may contribute to pancreatic cancer control provides a novel method for pancreatic cancer therapy. Given that several reports about restoring miRNA function based on *in vitro* but also *in vivo* models have yielded significant results in repressing pancreatic cancer development, efficient reconstitution of miRNAs through *in vivo* delivery of pre-miRNA precursors is a crucial factor for the development of successful miRNA-based treatment modalities. However, the *in vivo* delivery to target sites, its penetration into tumor tissues, stability and pharmacokinetics, remain great challenges. Establishment of effective delivery systems is necessary to improve the stability and uptake of miRNAs. Towards this direction, novel miRNA-delivery systems such as nanocomplexes or exosomes should gain further attention as potential means of miRNA-based PDAC therapeutics.

Silencing of miRNAs using intravenously administered chemically engineered oligonucleotides has been successfully performed in many solid organs and *in vivo* models (95). So far, efficient delivery of antagomiRs via systemic administration to the pancreas and hypovascular PDACs has not been demonstrated. A major disadvantage of miRNA antagonists is that they possibly exert side effects due to non-selective distribution to non-target organs. Conjugating the anti-miR oligonucleotide with ligands for target organ specific cell surface receptors could contribute in overcoming the latter

issue. Furthermore, development of small-molecule drugs seems to be a promising prospect on the miRNA-targeted drug discovery.

Taken together, there is increasing evidence that modulating miRNA levels and functions in pancreatic cancer could be potentially exploited for therapeutic gain. As discussed above, each miRNA can have different targets and could also possibly display bivalent behavior depending on the cellular environment. Therefore, therapeutic strategies directed towards targeting of a single miRNA may yield dramatic alterations in several cellular processes and also in a cell-type specific manner. These features of miRNA activity could be utilized for cancer therapy where the targeting of multiple pathways might be desirable. Since the miRNA mimics and antagonists may also affect non-target tissue when administered, be susceptible to nuclease degradation and be targeted by the innate immune system, the aforementioned methods have certain limitations with regard to their therapeutic applications. Despite these disadvantages, miRNA-based approaches remain a promising option for effectively targeting endogenous miRNAs. As soon as the above mentioned impediments are overcome, rapid progress in clinical translation can be expected.

Conclusions

In the present study, we have identified a novel role of FOXA2 in pancreatic ductal adenocarcinoma. FOXA2 contributes to the invasiveness of PDAC by targeting PLAUR, a glycoprotein involved in extracellular matrix degradation, contributing to cell invasion and metastasis. It would be interesting to assess the effects of miR-199a-3p inhibition *in vitro* and *in vivo* and investigate the potential therapeutic intervention. Furthermore, it would be interesting to examine upstream regulators of miR-199a-3p in PDAC and

identify transcription factor binding sites in the miR-199a-3p promoter area to understand the players contributing to miR-199a-3p regulation.

Oncogenic transcription factors are valuable therapeutic targets because direct inhibition of transcription factor expression (e.g., with RNA interference or microRNAs) has demonstrated antitumor responses with minimal side-effects. FOXA2, however, is a tumor suppressor transcription factor and is not an ideal candidate for therapeutic intervention. Nevertheless, it is important to further explore the role of FOXA2 overexpression *in vivo* and *in vitro* in order to assess the impact of FOXA2 on pancreatic oncogenesis.

Figures

Table 1. miRNA-based therapeutic approaches in pancreatic cancer.

miRNA target	Role	Targeting approach	Functional outcome of miRNA-based targeting
miR-483-3p	Oncogenic	Anti-miRNA	Reduces cell proliferation and colony formation
miR-155	Oncogenic	Anti-miRNA	Significant increase in cell apoptosis
miR-21/mir-221	Oncogenic	ASO	Increases cell apoptosis/cell cycle arrest
miR-21	Oncogenic	HIV-1-based lentiviral vectors	Inhibits pancreatic cancer tumor growth both *in vitro* and *in vivo*
miR-27a	Oncogenic	Anti-miRNA	Suppresses growth, colony formation and migration
miR-371-5p	Oncogenic	Anti-miRNA	Proliferative inhibition
miR-21/miR-23a/miR-27a	Oncogenic	Anti-miRNA	Synergistic effects in reducing cell proliferation both *in vivo* and *in vitro*
miR-200 and let-7 families	Downregulated in gemcitabine-resistant cells	miRNA mimic or isoflavone	Reversal of EMT phenotype leading to epithelial morphology
↑ miR-22 and ↓ miR-199a	–	miRNA mimic or curcumin	Suppressed expression SP1 transcription factor and ESR1
Let-7	Tumor suppressor	Plasmid-based synthetic miRNAs or by lentiviral transduction	Strongly diminishes cell proliferation
miR-34a	Tumor suppressor	Lentiviral system	Inhibits clonogenic cell growth and invasion and induces apoptosis and cell cycle arrest at G1 and G2/M phase
miR-217	Tumor suppressor	miRNA-expressing plasmids	Suppresses tumor cell growth *in vivo*
miR-20a	Tumor suppressor	Lentiviral system	Inhibits proliferation and metastasis
miR-96	Tumor suppressor	miRNA mimic and miRNA-expressing plasmid	Reduces proliferation and invasion capacity *in vitro* and *in vivo*
miR-148a	Tumor suppressor	miRNA mimic	Drastic inhibition of the invasive properties of HCC
miR-148a	Tumor suppressor	miRNA mimic and miRNA-expressing vector	Suppresses the EMT and metastasis of HCC
miR-148a	Tumor suppressor	Lentiviral system	Inhibits tumor cell growth and colony formation
miR-126	Tumor suppressor	miRNA mimic	Inhibition of invasive growth
miR-148b	Tumor suppressor	miRNA mimic and lentiviral system	Remarkably suppresses growth and invasion and enhances chemosensitivity/inhibits tumorigenicity in nude mice
miR-204	Tumor suppressor	miRNA mimic or triptolide treatment	Decrease in cell viability and cell death
miR-137	Tumor suppressor	Lentiviral system	Inhibits cell invasion/increases sensitivity to Fluorouracil/suppresses tumor growth *in vivo*
miR-216a	Tumor suppressor	miRNA mimic	Inhibition of the JAK2/STAT3 signaling pathway and xenograft tumor growth *in vivo*
miR-135a	Tumor suppressor	Lentiviral system	Reduced proliferation and clonogenicity/ induced G1 arrest and apoptosis
miR-218	Tumor suppressor	miRNA mimic and lentiviral system	Attenuation of cell migration/invasion
miR-663	Tumor suppressor	Lentiviral system	Antiproliferative, anti-invasive and pro-apoptotic effects *in vivo* and *in vitro*

The oncogenic or tumor-suppressive effect of miRNA-based targeting is denoted in several pancreatic adenocarcinoma settings.
ASO: Antisense oligonucleotide; EMT: Epithelial–mesenchymal transition; HCC: Hepatocellular carcinoma cell.

Table 5.1. miRNA-based therapeutic approaches in pancreatic cancer.

The oncogenic or tumor-suppressive effect of miRNA-based targeting is denoted in several PDAC settings.

Company	RNAi	Phase/ Stage	Target Indication
Alnylam Pharmaceuticals Inc.	Patisiran (ALN-TTR02)	Phase III	TTR-mediated amyloidosis (familial amyloidotic polyneuropathy-FAP).
	Revusiran (ALN-TTRsc)	Phase III	TTR-mediated amyloidosis (familial amyloidotic cardiomyopathy-FAC).
	ALN-AT3	Phase I/II	Hemophilia and rare bleeding disorders.
	ALN-CC5	Phase I/II	Complement-mediated diseases.
	ALN-AS1	Phase I	Hepatic porphyrias.
	ALN-AAT	Phase I	Alpha-1 antitrypsin deficiency.
	ALN-GO1	Development	Primary hyperoxaluria.
Alnylam Pharmaceuticals Inc	ALN-TMP	Development	Beta-thalassemia /iron overload disorders.
	ALN-AC3	Development	Hypertriglyceridemia.
	ALN-ANG	Development	Mixed hyperlipidemia and hypertriglyceridemia.
	ALN-AGT	Development	Hypertension /preeclampsia.
	ALN-HBV	Development	Hepatitis B virus infection.
	ALN-HDV	Development	Hepatitis D virus infection.
	ALN-PDL	Development	Chronic liver infection.

Table 5.2. New Developments in the Research Pipeline of RNAi Therapeutics and Technologies as of September 2015. (*Source: BCC Research Report: RNAi Technologies and Global Markets, January 2016*).

Apeiron Biologics AG	APN401	Phase I	Melanoma, kidney cancer, pancreatic cancer or other solid tumors.
Arbutus Biopharma Corp. (formerly Tekmira Pharmaceutical Corp.)	TKM-HBV	Phase 1	Hepatitis B virus (HBV) infection.
	TKM-PLK1	Phase 1	HBV infection.
	TKM-PLK1	Phase IIb	Neuroendocrine tumor G1 (NET G1).
	TKM-PLK1	Phase IIb	Adrenocortical carcinoma (ACC).
	TKM-PLK1	Phase IIb	Hepatocellular carcinoma (HCC).
	TKM-ALDH	Pre-clinical	Alcohol use disorder.
	TKM-HTG	Pre-clinical	Hypertriglyceridemia (HTG).
Arcturus Therapeutics Inc.	LUNAR-101	Pre-clinical	Cardiomyopathy.
	LUNAR-102	Research	Polyneuropathy.
Arrowhead Research Corp.	ARC-520	Phase II	Hepatitis B.
	ARC-AAT	Phase I	Alpha-1 antitrypsin deficiency.
		Pre-IND	Liver targets.
		Pre-clinical	Liver targets.
Ascletis Inc.	ASC06 (ALN-VSP)	Phase I	Liver cancers.
Benitec Biopharma Ltd.	TT-034	Phase I/IIa	Hepatitis C.
	Hepbarna	*In vitro*	Hepatitis B.
	TT-211	*In vivo*	Age-related macular degeneration (AMD).
	Tribetarna	–	Drug-resistance non-small cell lung cancer.
	Pabparna	–	Oculopharyngeal muscular dystrophy (OPMD).
Bioneer Corp.	Solid Cancer (SAMiRNA-Survivin) program	Pre-clinical	Cancer
	COPD/IPF program	Pre-clinical	Chronic obstructive pulmonary disease (COPD)/idiopathic pulmonary fibrosis (IPF).
	Liver fibrosis program	Discovery	Liver fibrosis.
	Antiviral	Discovery	Dengue.
Bioneer Corp.	Liver cancer program (in conjunction with Sanofi)	Development	Liver cancer.
Cerulean Pharma Inc.	CRLX101	Phase II	Relapsed renal cell carcinoma.
		Phase I	Relapsed ovarian cancer.
		Phase II	Non-metastatic rectal cancer.
	CRLX103	Phase I	Solid tumors malignancy.

Source: BCC Research Report: RNAi Technologies and Global Markets, January 2016.

Dicerna Pharmaceuticals Inc.	DCR-PH1	Pre-clinical	Primary hyperoxaluria Type 1 (PH1) in liver disorder.
	DCR-MYC	Phase Ib/2	Hepatocellular carcinoma (HCC).
	DCR-MYC	Phase I	Solid tumors, multiple myeloma non-Hodgkin's lymphoma.
Marina Biotech Inc.	SMARTICLES	–	Nucleic acid delivery system.
	tkRNAi	–	RNAi delivery systems.
	CEQ508	Phase I	Familial adenomatous polyposis (FAP).
Nitto Denko Corp.	ND L02-s0201	Phase I	Liver cirrhosis.
PeptiMed Inc.	PEP0101	Pre-IND	Various cancers including breast, lung, prostate etc.
	PEP0201	Pre-IND	Various cancers including breast, lung, prostate, etc.
	PEP0102	Proof of concept	Pancreatic cancer.
	PEP0202	Proof of concept	Pancreatic cancer.
Quark Pharmaceuticals Inc.	QPI-1002	Phase III	Delayed graft function.
	QPI-1002	Phase II	Acute kidney injury.
	QPI-1007	Phase III	Non-arteritic anterior ischemic optic neuropathy.
	QPI-1007	Phase II	Acute primary angle closure glaucoma.
	PF-655	Phase IIb	Diabetic macular edema.
	PF-655	Phase II	Age-related macular degeneration.
	QP-LI1	Development	Lung transplantation, primary graft dysfunction.
	QP-LI2	Discovery	Acute lung injury.
	QP-CO1	Discovery	Chronic obstructive pulmonary disease.
	QP-HL1	Discovery	Otoprotection.
	QP-HL2	Development	Ménière's disease.
	QP-HL3	Discovery	Hearing regeneration.
	QP-CP1	Discovery	Myocardial infarction.
	QP-AL1	Discovery	Chemotherapy-induced alopecia.
	QP-AL2	Discovery	Alopecia.
	QP-NR1	Discovery	Allodynia.
	QP-EN1	Discovery	Sarcoma.
	QP-EN2	Discovery	Cancer.
RXi Pharmaceuticals Corp.	RXI-109	Phase II	Hypertrophic scars and keloids.

Source: BCC Research Report: RNAi Technologies and Global Markets, January 2016.

Silence Therapeutics Plc.	Atu027	Phase II	Pancreatic cancer.
	Atu027	Pre-clinical	Head and neck cancer.
	Atu111	Pre-clinical	Lung indications.
Silenseed Ltd.	siG12D-LODER	Phase IIb	Advanced pancreatic cancer.
	si-PT-LODER	Pre-clinical	Prostate cancer.
	si-GBMT- LODER	Research	Brain cancer.
Solstice Biologics	Short interfering ribonucleic neutrals (siRNNs)	–	For diverse cell types.
SomaGenics	Synthetic short/small hairpin RNA (sshRNAs)	Pre-clinical	Hepatitis delta virus (HDV) infection.
University of Texas MD Anderson Cancer Center	siRNA-EphA2-DOPC	Phase I	Advanced cancer/solid tumors (ovarian cancer).
Zeltia Group S.A. (Sylentis)	SYL040012	Phase II	Glaucoma.
	SYL1001	Phase II	Ocular pain.

Source: BCC Research Report: RNAi Technologies and Global Markets, January 2016.

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