Role of MicroRNAs in Mediating Pancreatic Cancer Response to Triptolide

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Completing my degree would not have been possible without the ongoing training and mentorship of Dr. Nameeta Mujumdar, my lab "sister." I also remember the first day we worked together, shadowing her as she was isolating protein lysates under many different conditions to deliver many positive results. I was instantly in awe of her drive for science. She trained me so well not only at the bench, but also in experimental design, data analysis, and time management. She has always been there to cheer me up

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has and will continue to benefit my career, my happiness as a person and, in the end will make us stronger together. Thank you.

Dedication

I dedicate this work to my parents and grandparents; their love and encouragement have motivated me to become an independent person, to educate myself, to do my best work, and to make a positive impact on the world.

Abstract

Pancreatic ductal adenocarcinoma (PDAC), one of the deadliest malignancies, is resistant to current chemotherapies. We previously showed that triptolide inhibits PDAC cell growth *in vitro* and blocks metastatic spread *in vivo*. Triptolide downregulates heat shock protein 70 (HSP70), a molecular chaperone upregulated in several tumor types. This study investigates the mechanism by which triptolide inhibits HSP70. As microRNAs (miRNAs) are becoming increasingly recognized as negative regulators of gene expression, we tested whether triptolide regulates HSP70 via miRNAs. Here we show that triptolide, as well as quercetin but not gemcitabine, upregulated miR-142-3p in PDAC cells (MIA PaCa-2, Capan-1, and S2-013). Ectopic expression of miR-142-3p inhibited cell proliferation, measured by Electric Cell-substrate Impedance Sensing, and decreased HSP70 expression, measured by real-time PCR and immunoblotting, compared with controls. We demonstrated that miR-142-3p directly binds to the 3'UTR of HSP70, and that this interaction is important as HSP70 overexpression rescued miR-142-3p-induced cell death. We found that miR-142-3p regulates HSP70 independently of heat shock factor 1. Furthermore, Minnelide, a water soluble prodrug of triptolide, induced the expression of miR-142-3p *in vivo*. This is the first description of an miRNA-mediated mechanism of HSP70 regulation in cancer, making miR-142-3p an attractive target for PDAC therapeutic intervention.

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Chapter 1 Introduction

Review of pancreatic cancer

Epidemiology

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States [1, 2], and about 227,000 patients succumb to the disease worldwide each year [3]. The American Cancer Society estimates that about 45,220 people (22,740 men and 22,480 women) will be diagnosed with pancreatic cancer in 2013 [4]. PDAC, comprising 95% of tumors of the exocrine pancreas, is the most common form of pancreatic cancer; tumors of the endocrine pancreas are a small minority of all pancreatic cancer cases [4]. At best, the 5-year survival rate for patients with localized PDAC, more likely candidates for surgical resection than those with metastatic disease, is 20%; however, when considering PDAC patients as a whole, including those with metastatic disease, the survival rate is only 5-6% [1, 5]. Among several cancer types, PDAC exhibits the lowest survival rates regardless of cancer stage or patient race (Figure 1-1 reprinted from reference [1]). One of the reasons why survival is so low may be that clinicians do not know enough about the environmental nor genetic risk factors responsible for PDAC.

More research needs to be done in order to identify and validate what the risk factors are for developing PDAC, but smoking and a familial history are aspects of the disease with conclusive data supporting their role in development of the disease. Clinical data support that smokers are twice as likely as non-smokers to develop PDAC, about 20% of all PDAC patients are smokers, and PDAC tumors from smokers are characterized by

more genetic mutations than non-smokers [6]. Familial history of PDAC is another risk factor for PDAC development [3], accounting for an estimated 7-10% of PDAC cases [7]. Registry-based studies suggest that first-degree relatives of individuals with familial PDAC bear a nine-fold increased risk of developing the disease [8]. This risk increases to 32-fold in those with three or more first-degree relatives afflicted with the disease [9].

Germline mutations of *BRCA2, p16/CDKN2A, STK11* and *PRSS1* alleles have all shown to play a role in hereditary PDAC development. *BRCA2* mutations, originally discovered as accountable for hereditary breast and ovarian cancer syndromes, have been shown to be associated with the highest proportion of known causes of inherited PDAC [4, 10]. Evidence suggests that germline mutations in the tumor suppressive *BRCA2* gene is associated with 5-17% of familial PDAC cases [11-13], preventing the BRCA2 protein product from functioning properly in the Fanconi DNA repair pathway. Originally associated with familial melanoma, mutations in the tumor suppressor *p16/CDKN2* have also been shown to be associated with PDAC [14]. *STK11* mutations have been shown to be associated with PDAC development in patients afflicted with Peutz-Jeghers syndrome, linked with polyps of the digestive tract and several other cancers [4, 15]. Finally, mutations in the cationic trypsinogen gene *PRSS1*, associated with hereditary pancreatitis, have also been shown to be associated with an increased risk of PDAC [16]. Despite increased recognition of familial causes of PDAC, survival rates have not improved. More research funding needs to be allocated to finding hereditary clues as to why pancreatic cancer forms.

Research Funding

Despite the fact that PDAC survival rates have remained low for many decades, National Cancer Institute (NCI)-allocated research funding for PDAC research remains very low relative to other cancer types. Figure 1-2 reprinted from reference [2] shows that PDAC research funding has comprised only one-third to one-fifth of the funds assigned for the study of the other four top cancer killers in the US (breast, colorectal, prostate and lung cancers) over the past decade [2, 17]. This is especially alarming given that epidemiological studies predict that deaths due to PDAC are on the rise and may transition from the fourth-to the second-leading cause of cancer-related death in the US by the year 2030 [18]. As with breast cancer, more funding needs to be allocated to PDAC research in order to develop better biomarkers, diagnostic strategies, and treatments. Only in this way will PDAC patient survival improve. Despite deficient funding, efforts are ongoing to understand the pathophysiology of PDAC.

Pathophysiology

The evolution of PDAC begins most commonly through intraepithelial neoplasias acquiring select genetic and epigenetic alterations (Figure 1-3 reprinted from reference [19]). PDAC may also evolve from intraductal papillary mucinous neoplasms or mucinous cystic neoplasms. In order to more fully characterize the genetic mutations most commonly associated with PDAC, the exomes of 24 PDAC samples were sequenced [20]. The most frequent genetic abnormalities in invasive pancreatic adenocarcinomas are mutational activation of the *KRAS* oncogene, inactivation of

tumoursuppressor genes including *CDKN2A*, *TP53*, *SMAD4*, and *BRCA2* [14], widespread chromosomal losses, gene amplifications [20], and telomere shortening [14, 20, 21]. In addition to the mutation of these driver genes, alterations in the expression of microRNA (miRNA) have also been shown to play a critical role in characterizing PDAC pathophysiology. Upregulation of several miRNAs in pancreatic cancers may play a role in cancer development, such as: miR-21 [22-29], miR-34 [25, 30, 31], miR-155 [32, 33] and miR-221 [27]. Although modulation of miRNA levels holds promise as therapy, understanding how current small molecule therapies modulate miRNA expression levels is an informative way to understand the mechanism of action of current therapies.

Current Adjuvant and Neoadjuvant treatments justified by Clinical Trials

Over the past several decades, PDAC survival rates have not improved due in large part to delayed detection, metastasis [10], and drug resistance of PDAC cells [34]. This underscores the importance of effective, targeted chemotherapies to treat pancreatic cancer as it grows aggressively, is highly metastatic and yet remains resistant to chemotherapy. There are currently three main FDA-approved therapies. 5-fluorouracil, a pyrimidine analog, has been used since the 1970s. Gemcitabine, a nucleoside analog, was approved in the 1996 and provides a 1.5-month survival advantage over 5-fluorouracil. Erlotinib, approved in 2006, adds a mere 10 days to the survival rate achieved by gemcitabine [4]. Erlotinib inhibits the tyrosine kinase epidermal growth factor receptor (EGFR), and its administration has shown modest benefit in clinical

trials [35-37]. Therefore, the need for effective chemotherapeutic agents exploiting novel cellular targets is urgent.

Chemotherpeutic agents may be administered at any stage of pancreatic cancer, but they are most commonly employed when PDAC is advanced and cannot be completely resected with surgery. Chemotherapy may also be used after the cancer has been removed with surgery to eliminate any macroscopically invisible cancer cells. This type of treatment is called *adjuvant* treatment. Adjuvant treatment is recommended for those patients who are undergoing PDAC resection with a curative, as opposed to palliative , goal in mind [38]. Adjuvant treatment has been shown lower PDAC recurrence as justified in randomized controlled clinical trials [39, 40] and retrospective studies [41-44]. When chemotherapy is administered with radiation, it can help to made the radiation more effective in decreasing tumor size. This, known as *chemoradiation* or *chemoradiotherapy*, has more severe side effects. Chemotherapy can also be given before surgery to try to decrease tumor burden. This is known as *neoadjuvant* treatment. There are no randomized clinical trials demonstrating that neoadjuvant therapy increases the number of patients who may undergo surgical resection of PDAC, but one meta-analysis suggests that the number of patietns eligible for resection is similar whether or not neoadjuvant chemotharpy is administered [45]; however, neoadjuvant therapy is advised in patients who are on the cusp of being eligible for PDAC resection [46]. Although neoadjuvant therapy may improve PDAC patients' eligibility for surgery, it can improve the likelihood that surgical candidates may undergo curative resection. Despite modest advances in understanding the benefits of adjuvant and

neoadjuvant therapies, novel therapies exploiting different characteristics of PDAC are urgently needed. Two clinical trials that have been successful include recent studies with FOLFIRINOX [47] and nab-paclitaxel [48]. FOLFIRINOX is a combination therapy including 5FU, a derivative of folic acid, a topoisomerase inhibitor, and a DNA-crosslinking agent which extends survival from about 6 to 11 months, but it is highly toxic [47]. Therapies targeting heat shock proteins are not exploited in treating PDAC, and this would be worthy endeavor to pursue.

Heat shock proteins

Role of heat shock proteins in cancer

Stress-protective heat shock proteins play important protective roles in eukaryotic cells as regulators of protein conformation and stress sensors. Inducible heat shock protein 70 (HSP70) is important aspects of ensuring proper protein orchestration within cells: aiding in the folding of newly synthesized proteins, transporting them in and out of the nucleus, and shuttling them between other organelles (e.g. endoplasmic reticulum, mitochondria and lysosomes) within cells (Figure 1-4 reprinted from reference [49]). Aside from its normal functions in protecting eukaryotic cells undergoing stressful conditions, HSP70 has gained recognition as an attractive cancer drug target as it inhibits multiple steps in the apoptotic cascade, such as: rise in intracellular calcium concentration, loss of lysosomal membrane permeability, and release of cytochrome *C* from the mitochondria (Figure 1-5 reprinted from reference [50]). HSP70 is overexpressed in many types of cancer as compared to non-transformed tissue [51], and

inhibition of HSP70 via siRNA technology causes apoptosis of pancreatic cancer cells [52]. Derived from a Chinese herb, triptolide has been identified as an inhibitor of HSP70 expression via inhibition of the transactivation of the heat shock transcription factor-1 (HSF1) [53]. Our lab has shown that triptolide selectively induces apoptosis of cancerous cells, sparing non-transformed pancreatic ductal cells, *in vitro* and selectively triggers apoptosis of tumor tissue, without being toxic to laboratory animals at therapeutic doses, *in vivo.* Our lab has shown that a major mechanism by which triptolide triggers apoptosis is via inhibition of HSP70 expression [54, 55]. Inhibiting total HSP70 mRNA in cancerous cells is one arm of anti-HSP70 therapy, there are also several other ways in which HSP70 may be inhibited as well.

Therapeutic modulation of heat shock proteins in cancer

Besides globally inhibiting the transcription of inducible HSP70, several other studies have evaluated the antitumor effectiveness of blocking HSP70 activity, notably by blocking one of three functions: its protein-binding domain, its ATP-binding domain or its interaction with other heat shock chaperones. Targeting the protein-binding domain (PBD) of HSP70 with 2-phenylethynesulfonamide (PES) has shown to be a promising avenue in cancer therapy. PES has been shown to interact with the C-terminal PBD of HSP70 and works by disrupting the association between HSP70, its cofactor heat shock protein 40, and its client proteins such as APAF-1 and p53 [56]. Further, PES selectively induced caspase-dependedn apoptosis in several tumor types involving increased protein aggregation, lysosomal impairment, and blockage of autophagy [56].

Besides blocking the PBD of HSP70, another demonstrated way of disrupting HSP70 function is by blocking its ability to exchange ADP for ATP, or inhibiting the ATP-binding domain (ABD) of HSP70. One adenosine-derived compound, VER-155008, has shown to be particularly promising as an inducer of caspase-dependent apoptosis in breast cancer cells and caspase-independent cell death in colon cancer cells [57]. This compound is likely to be a non-selective inhibitor of the ABD of similar proteins of the HSP70 family, as it has also been shown to block the activity of glucose regulated protein (GRP78) involved in regulating endoplasmic reticulum stress [58]. Although VER-155008 has not yet been tested *in vivo*, it holds promise because it sensitized colon cancer cells to HSP90 inhibitors [57]. Finally, aside from blocking the PBD or ABD domains of HSP70, another strategy is to block the ability of HSP70 to bind to its co-chaperones, such as HSP40. The ability of MAL2-11B to interfere with HSP70/HSP40 binding is well documented. This inference prevents HSP40 from stimulating HSP70 ATPase activity, thereby compromising the cellular functions of HSP70 [59]. This mechanism was only validated in blocking polyomavirus replication, it would be of interest to evaluate the effectiveness of MAL2-11B in a pancreatic cancer cells. It would be interesting to contrast the effectiveness of blocking the PBD of HSP70, blocking the ABP of HSP70, blocking the HSP70/HSP40 interaction as opposed to total inhibition of HSP70 transcription with compounds such as triptolide.

Triptolide

Role of triptolide in cancer

An important part of this process is to understand the mechanism of action of potential chemotherapeutic agents. Triptolide, a diterpene triepoxide extract from the Chinese herb *Tripterygium wilfordii*, has been shown to inhibit PDAC cell viability *in vitro* [54, 60] and to block growth and metastatic spread *in vivo* [54, 61]. To date, *in vivo* studies have shown that triptolide inhibits the growth of cholangiocarcinoma cells in hamsters [62] and xenografts of human melanoma, breast cancer, bladder cancer, and gastric carcinoma in nude mice [63]. Previous data from our laboratory have shown that triptolide inhibits the growth of neuroblastoma cells *in vitro* and prevents tumor growth *in vivo* [64]. Prior to triptolide, the bioflavonoid quercetin was shown to inhibit the induction of HSP70 at the mRNA level [51, 65]. Quercetin had to be used as micromolar concentrations in order to observe a therapeutic effect in cancer cells, whereas the diterpene triepoxide triptolide may be used in nanomolar concentrations to achieve the same effects [51, 54]. Although triptolide action is promising *in vitro*, its clinical potential is limited by its solubility in organic solvents. To address this, our laboratory developed and patented Minnelide, a water-soluble prodrug of triptolide. The preclinical effectiveness of Minnelide in decreasing PDAC tumor burden has been established [61]. Another prodrug of triptolide, 14-succinyl triptolide sodium salt PG490-88, entered clinical trials in Europe for the treatment of solid tumors, but it was withdrawn from clinical trials due to toxic effects [66]; however, in a murine models of

PDAC Minnelide has been shown to be decrease tumor burden no overt signs of toxicity when administered for 385 days at a dose of 0.42 mg/kg [61]. Our laboratory remains hopeful that Minnelide will be tolerated equally well in PDAC patients enrolling in upcoming phase I clinical trials. Thus the effectiveness of triptolide in cancer is becoming more and more established, but there are many proposed mechanisms of triptolide action in cancer.

Besides studies in our laboratory demonstrating HSP70 as a downstream target of triptolide, triptolide has also been shown to target other molecules such as: XPB, nuclear factor-κB (NF- κB), and activator protein-1 (AP-1) [67]. The studies showing that triptolide globally blocks transcription via targeting XPB, also known as ERCC3 and a subunit of the transcription factor TFIIH, offers a reasonable explanation for the commonly observed phenomenon that triptolide downregulates mRNA targets; however, these studies employed micromolar concentrations of triptolide, which are speculated to show toxic effects *in vivo* [68]. For several decades, triptolide has been hypothesized to inhibit the activity of certain transcription factors such as NF- κB and AP-1; interestingly, some studies support that wild type p53 is required for NF- κB and AP-1 dependent-apoptosis in gastric cells [69]. The effect of triptolide on blocking the transactivation of NF- κB is well established in T-cell leukemic cells [70], and its role in blocking the transactivation of NF- κB as a mechanism of apoptosis is shown in in other solid tumor types, such as: non-small cell lung cancer, breast cancer and fibrosarcoma cells [71]. Triptolide has been shown in multiple cancer cell types to block

the function of several transcription factors, resulting commonly in downregulated transcription.

Role of triptolide in blocking heat shock protein 70 (HSP70)

Because triptolide was identified in a small molecule screen to inhibit tumorigenic molecular chaperone heat shock protein gene transcription [53], our laboratory has continued to demonstrate that triptolide likewise inhibits cancer cell proliferation while concurrently inhibiting heat shock protein 70 (HSP70) expression in PDAC cells [52, 54, 60, 61] or neuroblastoma cells [55]. Studies show that triptolide inhibits the transactivation of transcription factor heat shock factor 1 (HSF1) and in this way suppresses HSP70 transcription [53]. Blocking HSF1 may non-specifically decrease the transcription of all heat shock proteins, non-specifically blocking the ability normal housekeeping heat shock proteins. Because of this potential disadvantage, triptolide toxicity will need to be monitored very closely. In a murine models of PDAC, however, Minnelide, the soluble pro-drug of triptolide developed by our laboratory, has been shown to be decrease tumor burden no overt signs of toxicity when administered for 385 days at a dose of 0.42 mg/kg [61]. One common observation in all the studies of triptolide inhibition of transcription factors HSF1, NF- κB, and AP-1 is that triptolide blocks the transactivation, presumably defined as the recruitment of cofactors, of each of these transcription factors without affecting their DNA-binding activity [70, 72], as shown for HSF1 in Figure 1- reprinted from reference [53]. Since this schematic of how triptolide blocks the transactivation of HSF1 was published, recent studies have

demonstrated that heat shock factor 2 (HSF2) plays a role in heterotrimerizing with HSF1 and in this way increases the transcription of downstream targets such as HSP70 [73, 74]. Given this new information, it would be interesting to test whether triptolide specifically disrupts the transactivation of HSF1 homotrimers, HSF1 homotrimers or HSF1/2 heterotrimers. We sought to explore whether triptolide may alter HSP70 expression via a mechanism independent of the HSF1 pathway. As microRNAs (miRNAs) become increasingly recognized as major negative regulators of gene expression, we asked whether triptolide may regulate HSP70 expression via miRNAs.

MicroRNAs

Role of miRNAs in cancer

In cancer cells, the dysregulation of microRNA (miRNA) expression serves as an efficient means to rewire the cell gene expression map and generate a cancer phenotype. In particular, the loss of tumor-suppressive miRNAs upregulates oncogenic targets [75]. As small non-coding RNAs that negatively regulate gene expression, miRNAs are now recognized as powerful gatekeepers of many cellular processes, including apoptosis and proliferation [76]. MicroRNAs have shown to be valuable in classifying tissue and cancer types [77], which is not surprising in light of recent studies indicating that >60% of mRNAs are targeted by miRNAs [78]. The ability of miRNAs to alter entire networks of proliferation or apoptosis gene targets is what makes them an especially appealing target for cancer therapy (Figure 1- reprinted from reference [79]). The biogenesis of miRNAs is comprised of several steps including: transcription into a

primary transcript, cleavage into pre-miRNA, export from the nucleus to the cytoplasm, processing to create a transient duplex that is loaded onto the RISC (RNA-induced silencing complex) and negative regulation of gene expression either by mRNA cleavage or translational repression (Figure 1- reprinted from reference [32]). As the study of miRNAs in cancer has matured, certain miRNAs may be classified as either oncogenic, "oncomirs," or as tumor suppressive (Figure 1- reprinted from reference [80]). An oncomir is characterized as an miRNA whose expression is upregulated in cancer relative to normal tissue and which targets tumor suppressive genes. A tumor suppressor miRNA, in contrast, is characterized as an miRNA whose expression is downregulated in cancer relative to normal tissue and which targets oncogenic genes. Understanding how small molecules affect the miRNAome of cancer cells is an increasingly evolving field.

Role of miRNAs in mediating therapeutic action of small molecules

Evaluating the effect of small molecules on the microRNAome of cancer cells provides an informative perspective on the mechanism of action of several drugs and natural products. Studies have shown that the natural compound curcumin affects the mircroRNAome of pancreatic cancer cells [81], and further that inhibition of oncogenic miRNAs (oncomirs) such as miR-21 by curcumin may overcome resistance to gemcitabine in PDAC cells [22]. MicroRNAs may be manipulated to alter chemosensitivity to and cytoprotection from a drug [28]. Studies have examined cancer cell miRNA expression profiles to understand mechanisms of resistance of non-small

cell lung carcinoma cells to cisplatin [82] and the resistance of melanoma cells to BCL2 inhibitors such as ABT-263 [83]. Although studying the biological role of miRNAs dysregulated in PDAC has proven informative, we and others seek to evaluate the miRNA changes in PDAC following chemotherapy treatment to better understand key miRNAs which regulate cell proliferation.

Rationale and purpose of study

No previous reports have examined the effect of triptolide on the PDAC miRNAome nor evaluated miRNA-mediated regulation of HSP70 in PDAC cells. Consequently, the aims of this study are as follows: (a) examine the effect of triptolide on the miRNAome of PDAC cells *in vitro* and verify this *in vivo*, (b) evaluate whether miRNAs upregulated by triptolide play a tumor suppressive role in PDAC, and (c) validate that predicted miRNAs regulate HSP70 independent of the HSF1 pathway.

Hypothesis

We hypothesize that triptolide will upregulate tumor-suppressive miRNAs which directly regulate HSP70. This is the first evaluation of an miRNA-mediated mechanism of HSP70 regulation in cancer.

Figures

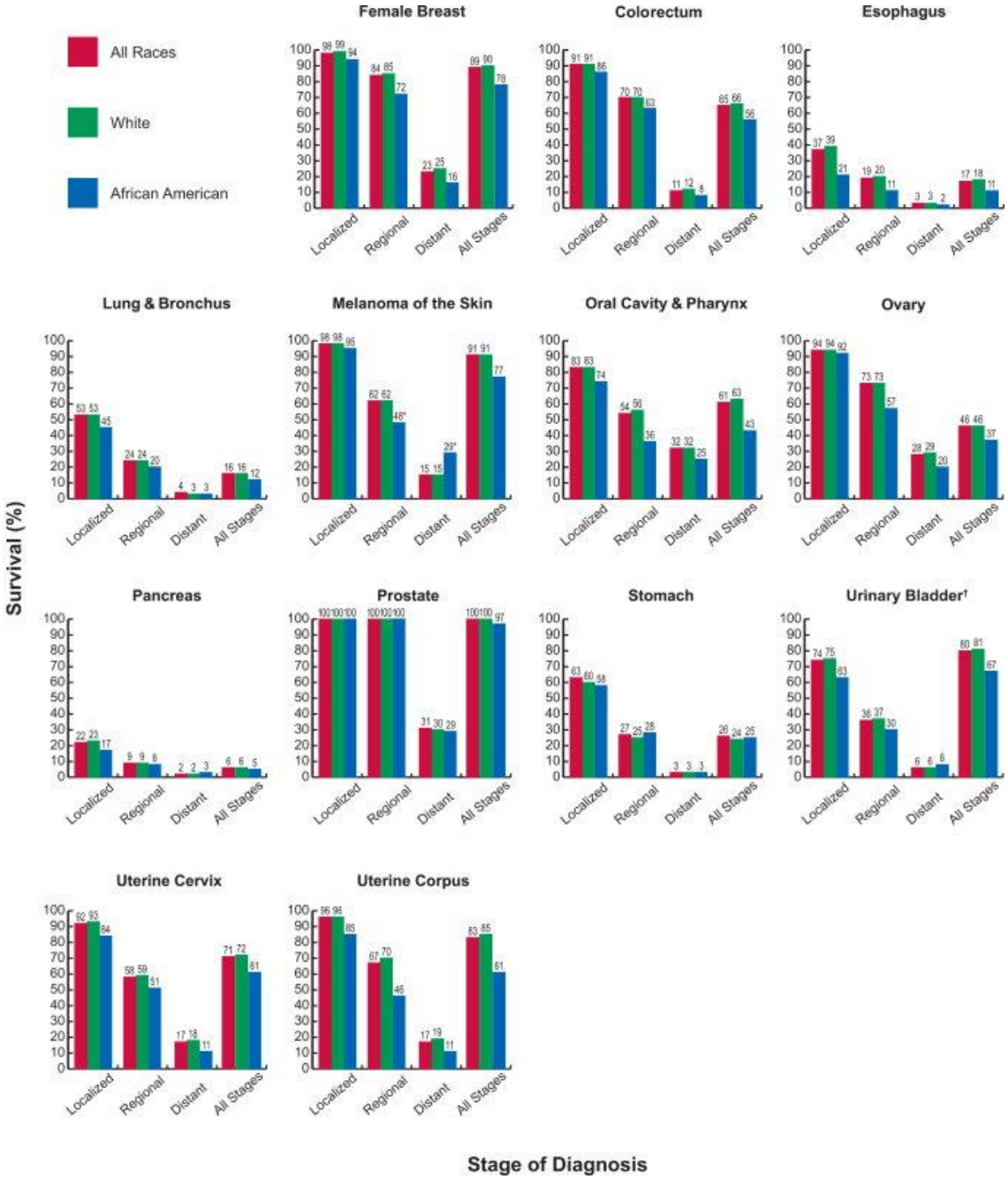

Figure 1-1: Five-Year Relative Survival Rates Among Patients Diagnosed with Selected Cancers by Race and Stage at Diagnosis, United States, 1999 to 2005.
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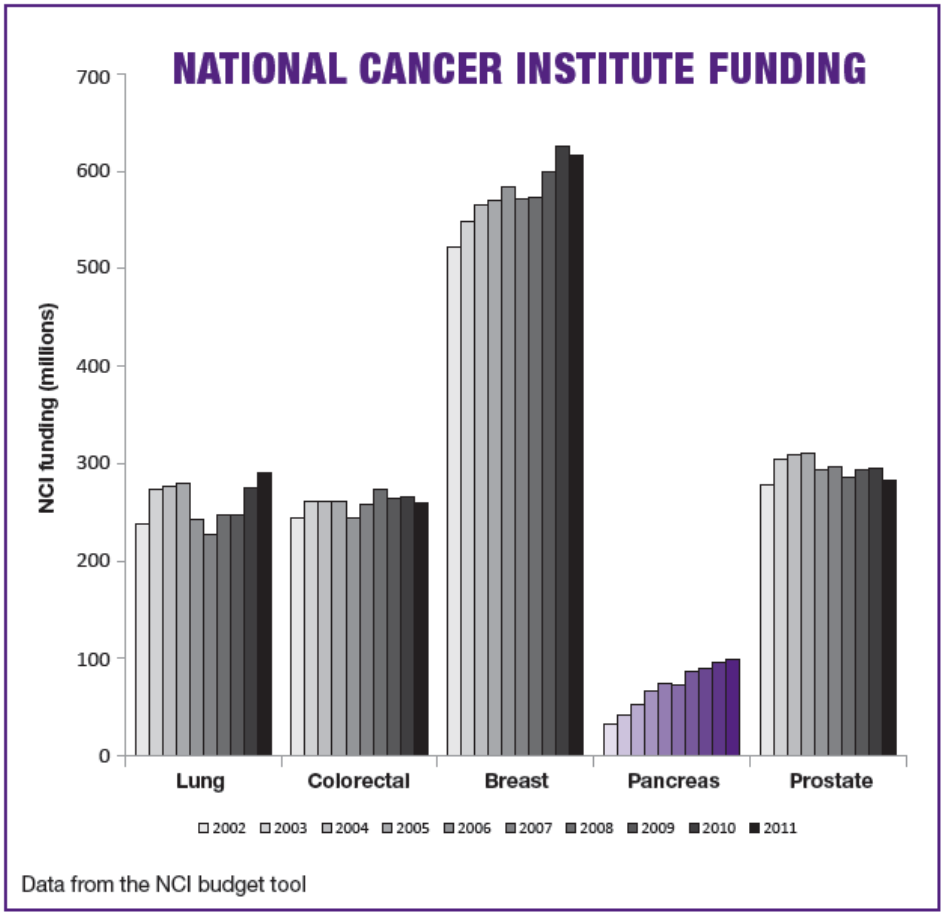

Figure 1-2: Funding for pancreatic cancer research lags significantly behind the other current top five cancer killers.
The largest source of cancer research funding in the U.S. is the National Cancer Institute (NCI). NCI research investment towards pancreatic cancer is just two percent of the NCI's total budget, representing only one-third to one-sixth the amount dedicated to the other top cancer killers. Reprinted with kind permission from the Pancreatic Cancer Action Network.

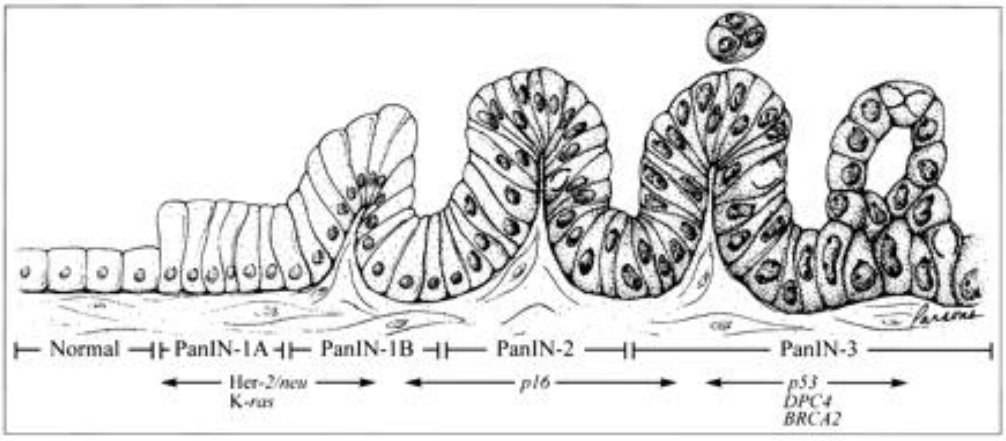

Figure 1-3: Model of accumulation of genetic mutations in PanIN evolution.
Somatic K-*ras* mutations occur very early in the PanIN progression from least to most invasive PDAC. Other mutations, such as in *p53* or *BRCA2*, occur later in the transformation. Reprinted with kind permission from the American Association for Cancer Research.

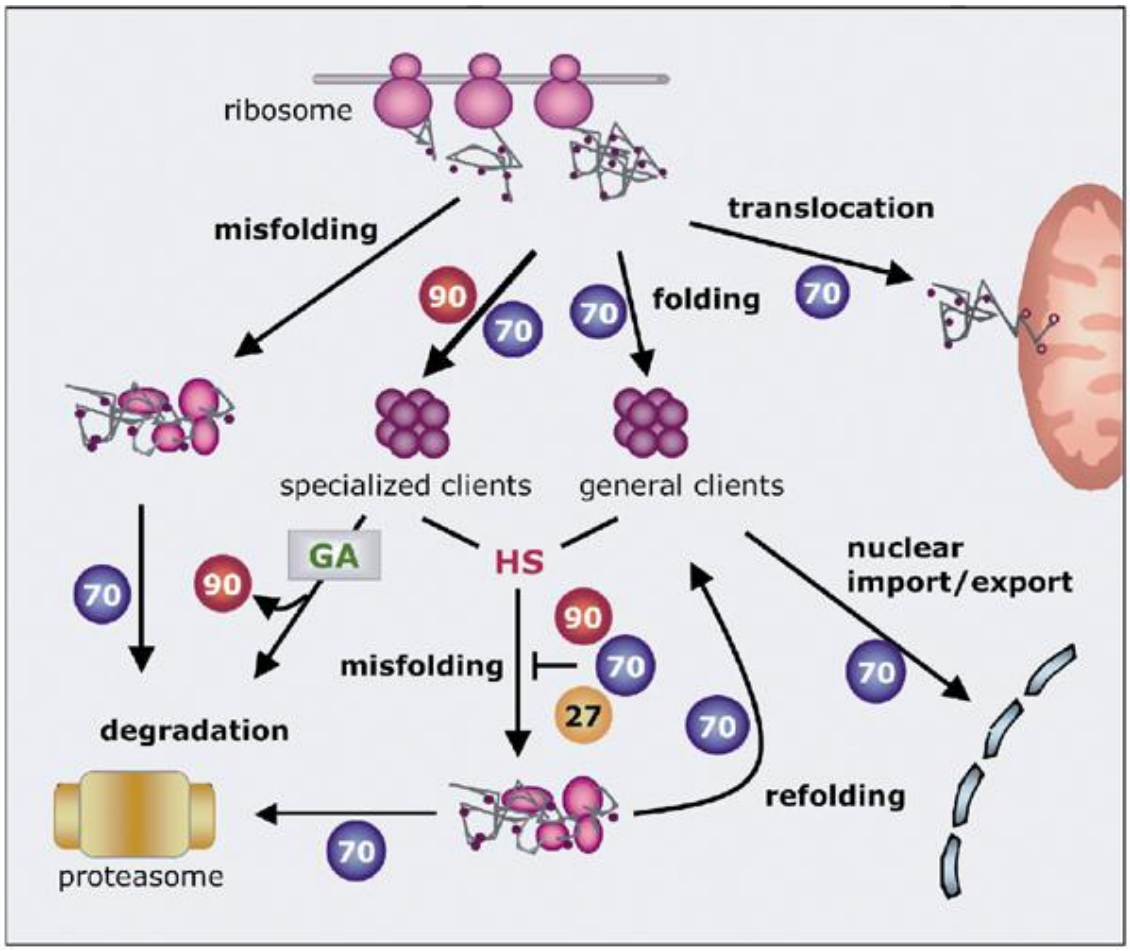

Figure 1-4: Chaperone folding roles of Heat Shock Proteins 70 and 90.
HSP70 is involved in facilitating protein folding and transportation of many client proteins in eukaryotic cells. Reprinted with kind permission from Nature Publishing Group.

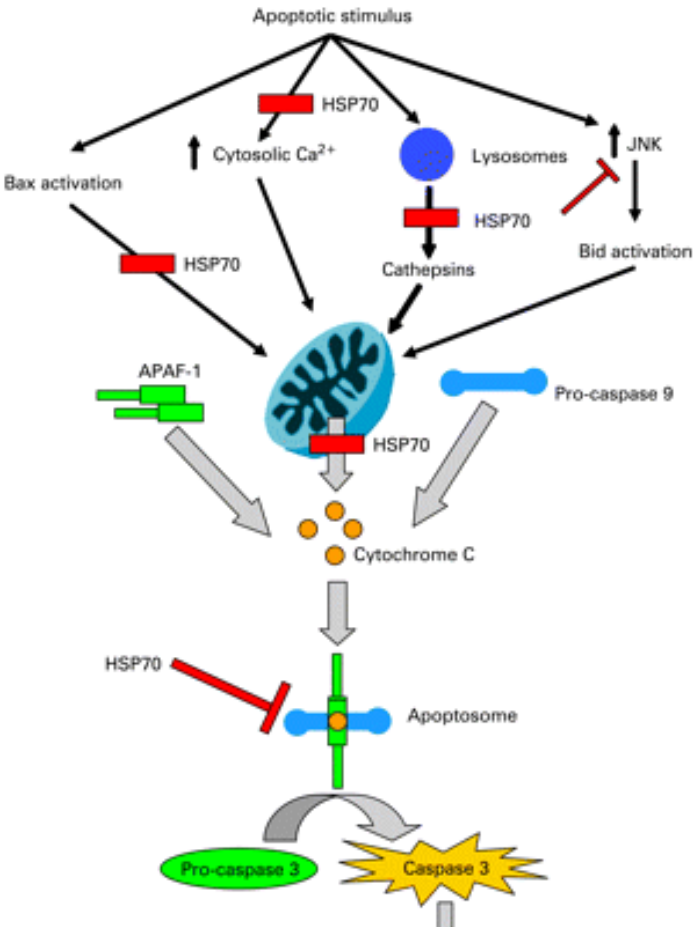

Figure 1-5: Hypothesized sites where Heat Shock Protein 70 blocks intrinsic apoptosis.
HSP70 is hypothesized to prevent such upstream events as surges in intracellular calcium concentration to blocking the formation of the apoptosome following Cytochrome C release. Reprinted with kind permission from BMJ Publishing Group Ltd.

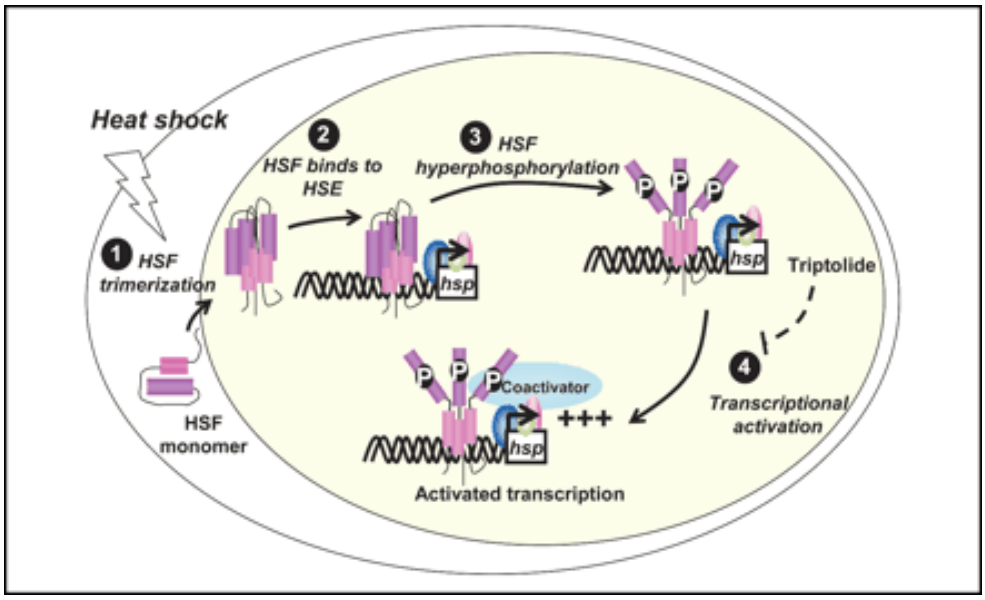

Figure 1-6: Schematic showing that triptolide blocks the transactivation of HSF1, but not its DNA binding capacity.
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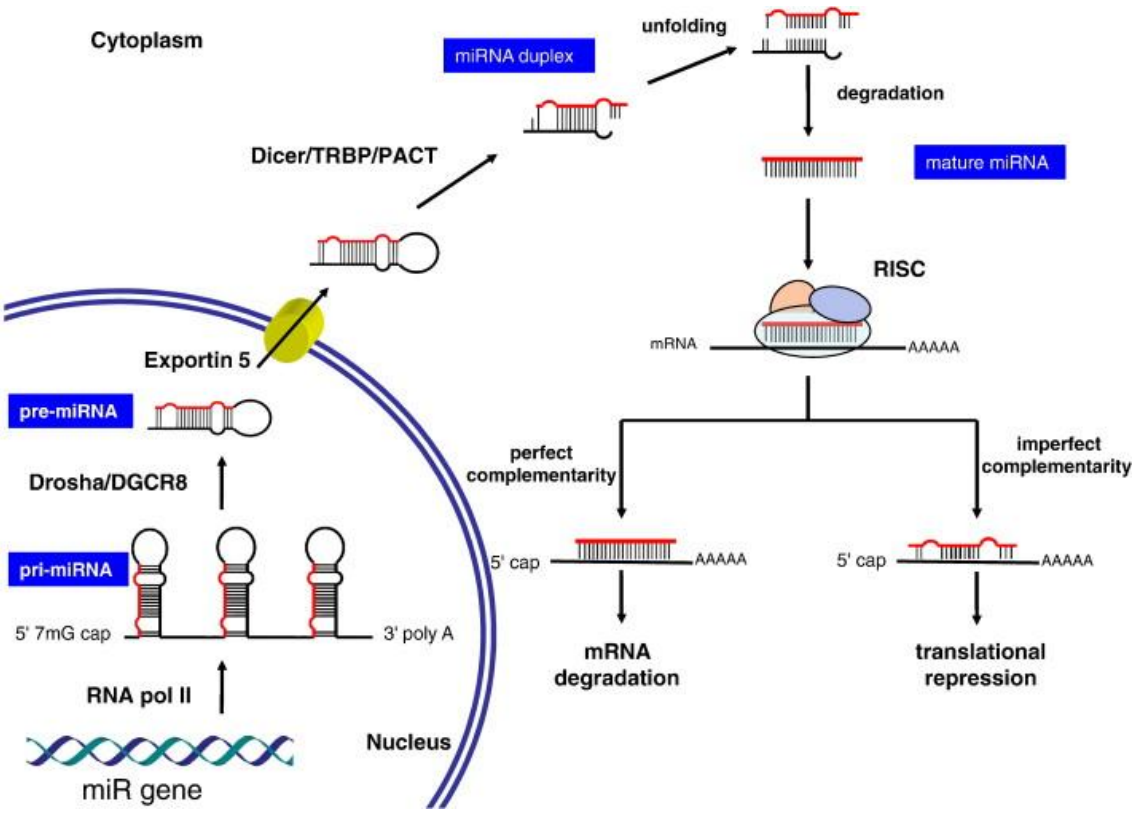

Figure 1-7: Biogenesis of miRNA.
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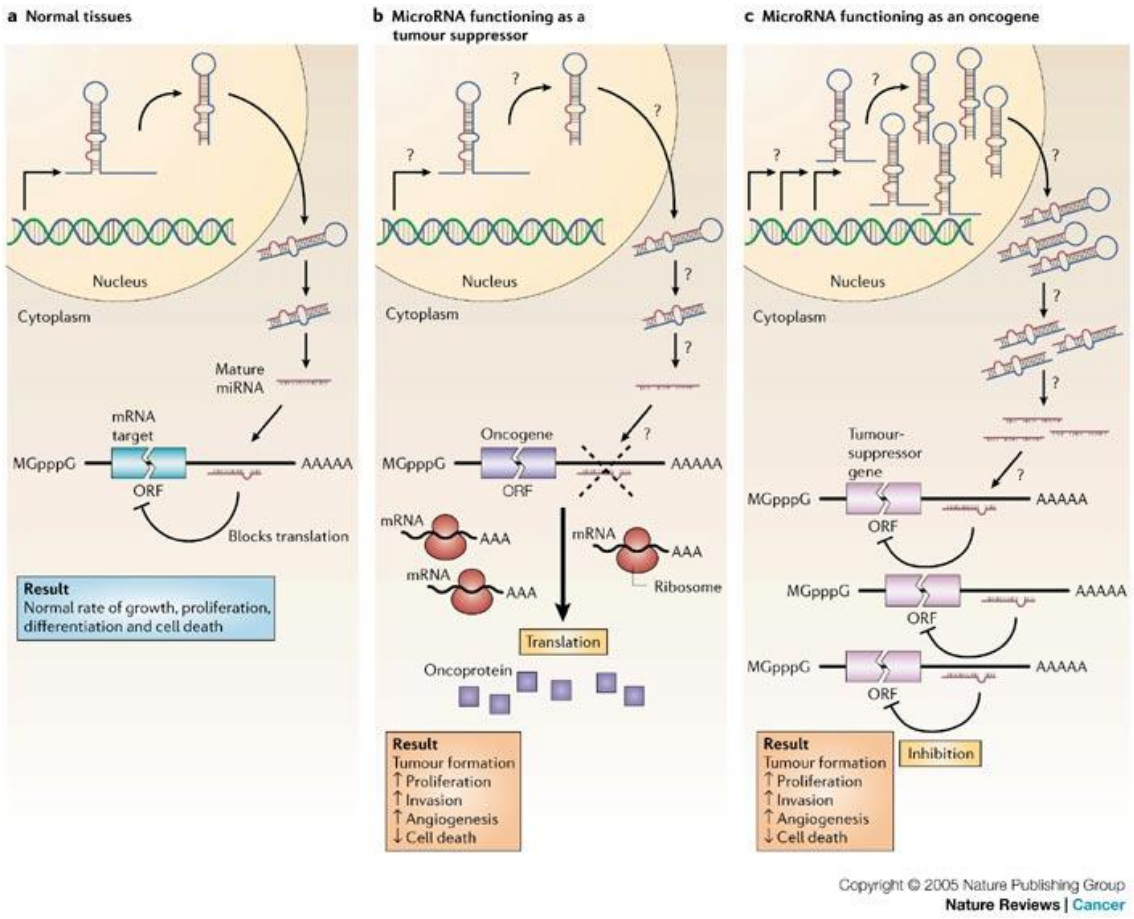

Figure 1-8: MicroRNAs may act as oncogenes or tumor suppressors.

a) MicroRNAs in normal abundance and binding to wild type mRNA targets.
b) MicroRNAs unable to repress target mRNA, leading to upregulation of oncoprotein.
c) MicroRNAs in abundance, abnormally downregulating target tumor suppressor mRNA. Reprinted with kind permission from Nature Publishing Group.

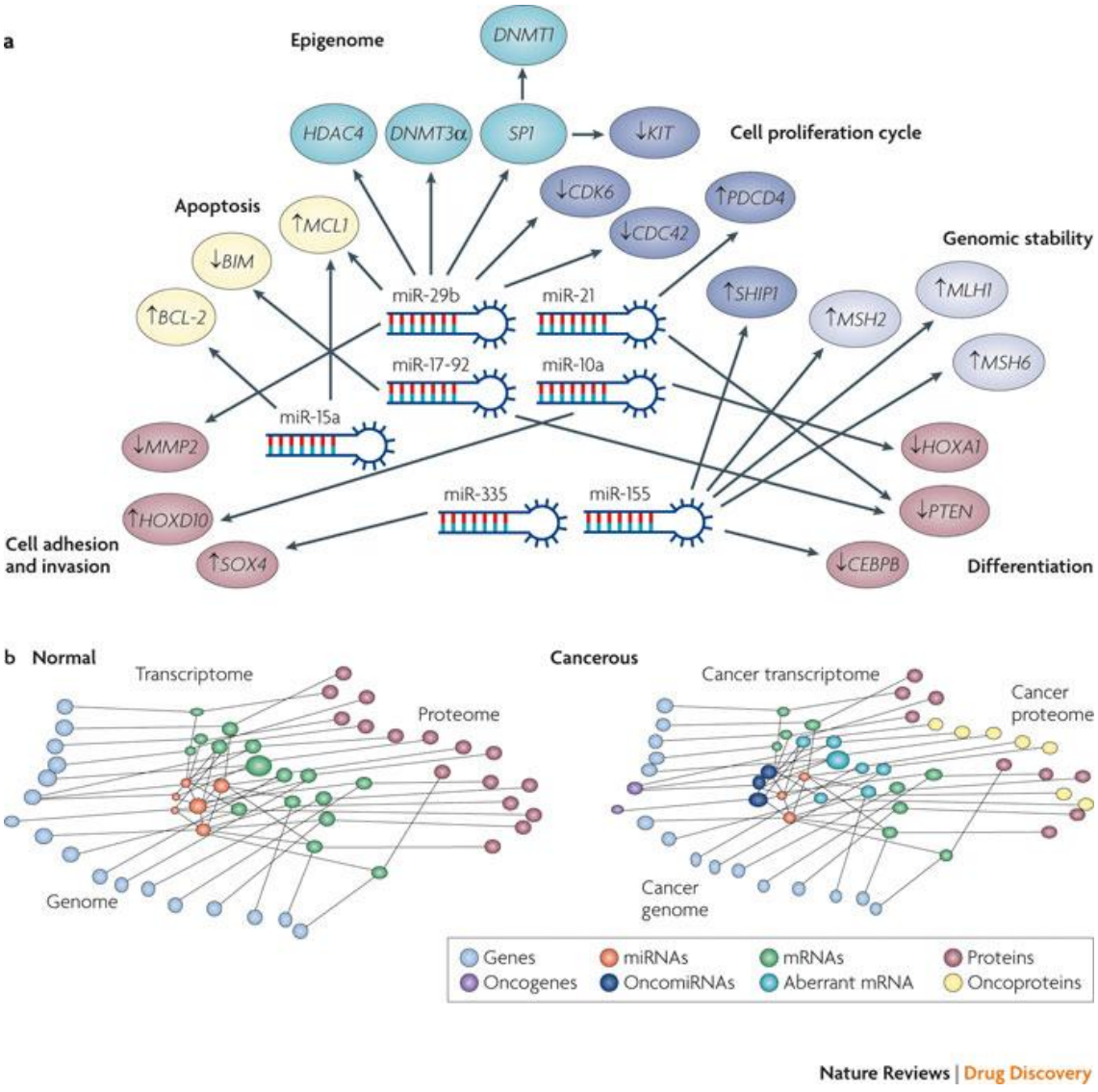

Figure 1-9: Relationship between oncogenic transcriptome networks and miRNAome.

a) Target mRNAs of each pathway represented by circles and miRNAs represented as hairpin structures; arrows connecting miRNAs and mRNAs are validated mRNA-miRNA interactions

b) MicroRNAs as a powergrid keeping gene and protein networks connected - graphical representation of gene-protein network of normal tissues and cancer.

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Chapter 2 Profiling changes in mRNA and miRNA expression following triptolide treatment in pancreatic cancer cells

Tiffany N. MacKenzie, Nameeta Mujumdar, Sulagna Banerjee, Veena Sangwan, Aaron L. Sarver, Selwyn M. Vickers, Subbaya Subramanian, and Ashok K. Saluja
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Tables 2 and 3 and Figures 2-5 through 2-8 are included in the manuscript:
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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in the United States [1]. The 5-year survival rate for patients with localized disease after surgical resection is 20% and for those with metastatic disease, the survival rate is 5-6% [1]. Over the past several decades these survival rates have not improved due in large part to aggressive growth, metastasis, and drug resistance of PDAC cells [1]. Efforts are ongoing to understand the pathobiology of PDAC and to develop innovative and effective therapies. An important part of this process is to understand the mechanism of action of potential chemotherapeutic agents.

Triptolide, a diterpene triepoxide extract from the Chinese herb *Tripterygium wilfordii*, has been shown to inhibit PDAC cell viability *in vitro* [54, 60] and to block growth and metastatic spread *in vivo* [61]. To date, *in vivo* studies have shown that triptolide inhibits the growth of cholangiocarcinoma cells in hamsters [62] and xenografts of human melanoma, breast cancer, bladder cancer, and gastric carcinoma in nude mice [63]. Previous data from our laboratory have shown that triptolide inhibits the growth of neuroblastoma cells *in vitro* and prevents tumor growth *in vivo* [64].

Because triptolide was identified in a small molecule screen to inhibit tumorigenic molecular chaperone heat shock protein gene transcription [53], our laboratory has continued to demonstrate that triptolide likewise inhibits cancer cell proliferation while concurrently inhibiting heat shock protein 70 (HSP70) expression in PDAC cells [52, 54, 60, 61] or neuroblastoma cells [55].

Materials and Methods

Cell culture and drug treatment

Cells from the MIA PaCa-2, Capan-1 and HEK-293 lines were obtained from ATCC (Manassus, VA) and cultured in DMEM (Life Technologies, Carlsbad, CA) containing 10% FBS (Life Technologies, Carlsbad, CA). S2-013 cells were kindly provided by Dr. Buchsbaum (University of Alabama at Birmingham) and cultured in RPMI medium (Life Technologies, Carlsbad, CA) containing 10% FBS. Multiple aliquots of cells were cryopreserved when initially grown. All the cell lines were used within 6 months of resuscitation. No authentication was done by the authors, but ATCC authenticates using Short Tandem Repeat Profiling. For all drug treatments, cells were seeded in 6-well plates (2.5×10^5 cells/well) and incubated for the periods indicated in the figure legends prior to RNA or protein analysis. Triptolide treatment (Calbiochem, San Diego, CA) was performed as previously described [54, 60]. Quercetin (Sigma-Aldrich, St. Louis, MO) or gemcitabine (Eli Lilly Corporation, Indianapolis, IN) treatments were performed as previously reported [51, 84]. Cells were maintained as previously described [54, 60].

MicroRNA expression profiling

MicroRNA was isolated using the *mir*Vana RNA isolation kit (Ambion, Carlsbad, CA), and quantified using a Nanodrop spectrophotometer (Thermo Fisher, Rockford, IL). RNA quality (RNA index number of $\geq$5) was verified by an Agilent 6000 nanochip (Agilent, Santa Clara, CA), prior to miRNA analysis using miRNA BeadArrays

(Illumina, San Diego, CA). Arrays were imaged using an Illumina BeadArray Reader, and the fluorescent intensity of miRNA probes were analyzed using BeadStudio version 3.3.3 (Illumina San Diego, CA). Quality control and statistical analyses of miRNA profiling were carried out as previously described [85].

Quantitative real-time PCR

Total RNA was reverse transcribed using the miScript II RT Kit (Qiagen, Valencia, CA). Real-time PCR was done using the QuantiTect or miScript SYBR green PCR kit (Qiagen, Valencia, CA) on an Applied Biosystems 7300 real-time PCR system. *18S* was used as a control for *HSP70, HSP27 or HSF1*, and *U6* was used as a control for miR-142-3p (miScript Primer Assay; Qiagen). To verify *HSPA1B (HSP70)* overexpression, HSP70 primers targeting the *HSP70* ORF region [54] were used. Quantification was done using the ΔΔCt method.

Statistical Analysis

Values are expressed as the mean ± SEM. All experiments with cells were repeated at least thrice. The significance of the difference between the control and each experimental test condition was analyzed by unpaired Student's t-test and a value of $p<0.05$ was considered statistically significant.

Results

Triptolide alters the mRNAome of PDAC cells

These studies show that triptolide significantly affects the transcriptome of pancreatic cancer cells. Triptolide upregulated 37 transcripts and downregulated 73 transcripts in MIA PaCa-2 and S2-013 cells after treatment with triptolide for 6 – 48 hours (Figure 2-1). The changes in the expression of 5 downregulated transcripts were validated: *HSF2, HSPA8, ABCF1, BCAR3,* and *RGS4.* The decreases in these mRNAs (Table 1) were validated by real-time PCR. Triptolide decreased the expression of transcripts in the heat shock pathway (*HSF2* and *HSPA8*) as measured by real-time PCR (Figure 2-2) and the expression of other transcripts thought to play protumorigenic roles in cancer: *ABCF1*, *BCAR3,* and *RGS4* (Figure 2-3).

Triptolide alters the microRNAome of PDAC cells, in particular inducing miR-142-3p expression

Previous data from our laboratory have shown that triptolide induces PDAC cell death *in vitro* and *in vivo,* while concurrently downregulating HSP70 expression [52, 54, 60, 61]. Triptolide was identified as an HSP70 inhibitor by virtue of its ability to inhibit its main transcription factor, HSF1 [53]. Given that miRNAs are becoming increasingly recognized as major negative regulators of gene expression [75], triptolide-induced upregulation of HSP70-targeting miRNAs was studied. We treated MIA PaCa-2 and S2-013 cells with triptolide and screened the collected miRNAs by microarray. These

cell lines were chosen because triptolide induces different mechanisms of cell death in these primary and secondary (metastatic) tumor types, respectively [60]. A 100 nM concentration of triptolide was chosen, for all studies in this manuscript, because this concentration has been shown to inhibit cell viability of both MIA PaCa-2 and S2-013 by 40% or 70% after 24 h or 48 h of treatment, respectively [60]. Time points as early as 6 h following triptolide treatment were chosen so as to observe early microRNAome changes linked to proliferative pathways. Later time points were evaluated to verify that changes were sustained. In MIA PaCa-2 cells, 15 miRNAs significantly changed (10 upregulated, 5 downregulated) ($p<0.05$; Figure 2-). In S2-013 cells, 14 miRNAs significantly changed (10 upregulated, 4 downregulated) ($p<0.05$; Figure 2-5). Principal component analysis of the resulting dataset shows that miRNA transcript levels changed linearly in both cell lines (Figure 2-7). Over the time-course of triptolide treatment, upregulation of miR-142-3p was one of the most significant changes in both cell lines (8-9 fold at 24 hours, Table 2). This induction of miR-142-3p was validated in a third cell line, Capan-1 (Fig. 1B). Furthermore, independent validation of miRNA microarray was done by real-time PCR (Figure 2-). In addition, the levels of those miRNAs known to play a role in PDAC, such as miR-155, miR-21 and miR-221 [23, 33, 86], were not observed to change in response to triptolide (Figure 2- and Table 3). These results show that triptolide alters the microRNAome of PDAC cells, notably by increasing miR-142-3p.

Discussion

These results show that triptolide alters the transcriptome of PDAC cells, most notably by decreasing about twice as many transcripts as it is upregulating. Because of this observation, we focused on validating the downregulation of several transcripts: *HSF2, HSPA8, ABCF1, BCAR3,* and *RGS4*. *HSF2* and *HSPA8* were chosen because of their role in the heat shock pathway. Our results show that triptolide inhibits *HSF1* levels (Figure 3-9), but our laboratory has not previously evaluated whether triptolide inhibits other members of the heat shock factor family, such as *HSF2*. To date, there are 32 publications discussing HSF2 and cancer. Although less is known about the role of HSF2 as compared to HSF1, studies have shown that HSF2 itself cannot efficiently mediate transcription of HSP70, as HSF1 is required for optimal transcription; however, evidence suggests that it does play a role in *HSP70* transcription by forming heterocomplexes with HSF1 [73, 74]. Data support that HSF2 enhances HSP70 transcription as the most HSP70 transcription is observed with HSF1/HSF2 heterocomplexes as compared with HSF1 or HSF2 homotrimers in leukemia and cervical cancer cells [73, 74]. Further, studies using HSF2 null cells have shown increased stability in the tumor suppressor protein p53, suggesting that HSF2 may play a p53-stabilizing role [87]. Triptolide also markedly decreased HSPA8 levels. To date, there are 93 studies of the role of HSPA8, one of the isoforms of constitutively active HSP70 (heat shock cognate 70 or HSC70) [88], in cancer. One seminal study showed that inhibiting either HSP70, referred to as HSP72 in the manuscript, or HSC70 alone

does not affect proliferation nor heat shock protein 90 expression in colon cancer cells[89]. Interestingly, when both HSP70 (HAPA1A) and HSC70 (HSPA8) are inhibited, degradation of HSP90 client proteins, G1 cell-cycle arrest, and tumors-specific apoptosis are observed [89]. These data support that triptolide inhibits many arms of the heat shock pathway including the following molecules: *HSP70, HSF1, HSF2 and HSC70 (HSPA8).*

These studies show that triptolide alters the miRNAome of PDAC cells, most notably by inducing the expression of miR-142-3p. To date, there have only been three studies evaluating the effect of triptolide on miRNAs. One study found that triptolide inhibited proliferation of lymphocytic leukemic cells via downregulating nuclear factor κB (NF-κB) and miR-16-1* [90]. Another study reported that triptolide enhanced the sensitivity of multiple myeloma cells to dexamethasone via the downregulation of miR-142-5p and miR-181a [91]. Most recently, it has been shown that triptolide may sensitize leukemic cells to adriamycin via inhibiting miR-21 [92]. This is in contrast to what we observe in PDAC cells, given that a reduction in miR-21 levels was not observed in this study (Table 3 and Figure 2-). None of the studies addressed whether triptolide affected miR-142-3p expression.

Among more than 200 reports evaluating the role of miRNAs in PDAC, only two evaluate the role of the mir-142 precursor, and none study miR-142-3p. In one study, SUIT-2, the cell line from which S2-013 was derived [93], and Capan-1 were studied to find miRNAs which were altered upon developing gemcitabine resistance. This report showed that gemcitabine-treated patients with high miR-142-5p and miR-204

expression had longer survival times than those with low expression [94]. Interestingly, triptolide induces miR-204 expression, though not to the degree that it induces the expression of miR-142-3p (Figure 2-). Another real-time PCR profiling study found that 100 miRNA precursors were aberrantly expressed in PDAC, including miRNAs previously reported as upregulated in other human cancers such as miR-155, miR-21 and miR-221 [23, 33, 86]. The mir-142 precursor was found to be 15-fold downregulated in PDAC compared with normal pancreas [95]. These findings support our hypothesis that miR-142-3p plays a tumor suppressive role in PDAC cells. Although studying the biological role of miRNAs dysregulated in PDAC has proven informative, our findings support the importance of evaluating miRNA changes in PDAC following chemotherapy treatment to better understand key miRNAs which regulate proliferation.

Figures and Tables

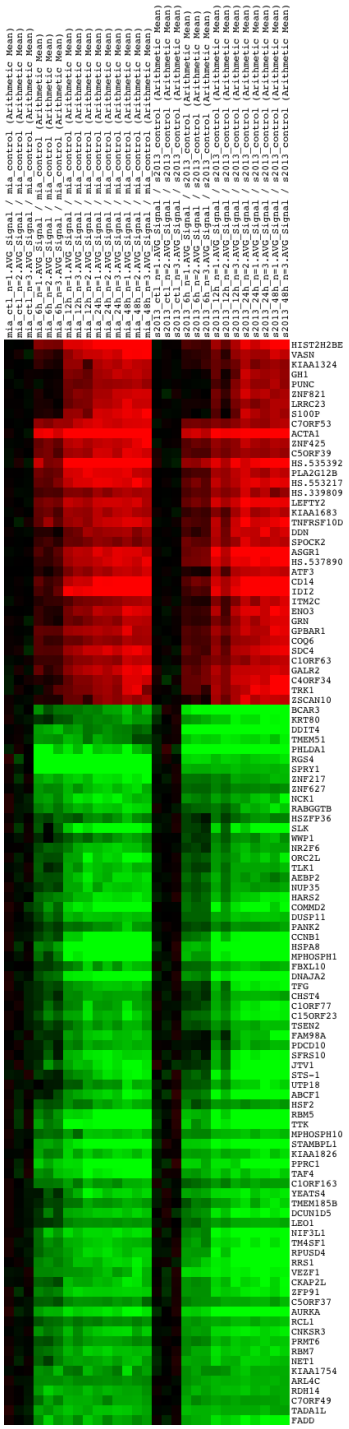

Figure 2-1: Triptolide-regulated mRNAs in PDAC cells.
Heatmap representation of differentially expressed human mRNAs after triptolide (100 nM) treatment (0, 6, 12, 24 or 48 h) in MIA PaCa-2 and S2-013 cells as measured by microarray. Only mRNAs with strong differential expression (t-test between 0 and 24 hour replicate time points, $p<0.05$, average fold change>5) in at least one of the two time-courses are shown. Log transformed expression values are shown compared with the untreated control for each cell line independently.

Table 1: Raw data of differences in select mRNAs downregulated by microarray.
Shown are the mean effect sizes, from each biological replicate, for select mRNAs from the heatmap in highlighting the 0 x 24 hour comparison for both the MiaPaCa-2 and S2-013 cell lines.

Row	mia_24h_n=1.AVG_Signal / mia_control (Arithmetic Mean)	mia_24h_n=2.AVG_Signal / mia_control (Arithmetic Mean)	mia_24h_n=3.AVG_Signal / mia_control (Arithmetic Mean)	s2013_24h_n=2.AVG_Signal / s2013_control (Arithmetic Mean)	s2013_24h_n=1.AVG_Signal / s2013_control (Arithmetic Mean)	s2013_24h_n=3.AVG_Signal / s2013_control (Arithmetic Mean)
BCAR3	0.35010394	0.26206794	0.256342	0.0652702	0.077031456	0.05879243
RGS4	0.026692554	0.025275081	0.027604604	0.21769299	0.1913515	0.18088351
HSF2	0.249785	0.27574927	0.22023267	0.3296176	0.28104874	0.2797904
HSPA8	0.050015952	0.04084095	0.046934683	0.03873686	0.03072275	0.031204354
ABCF1	0.18993859	0.16861483	0.20254804	0.09084443	0.083247386	0.07282318

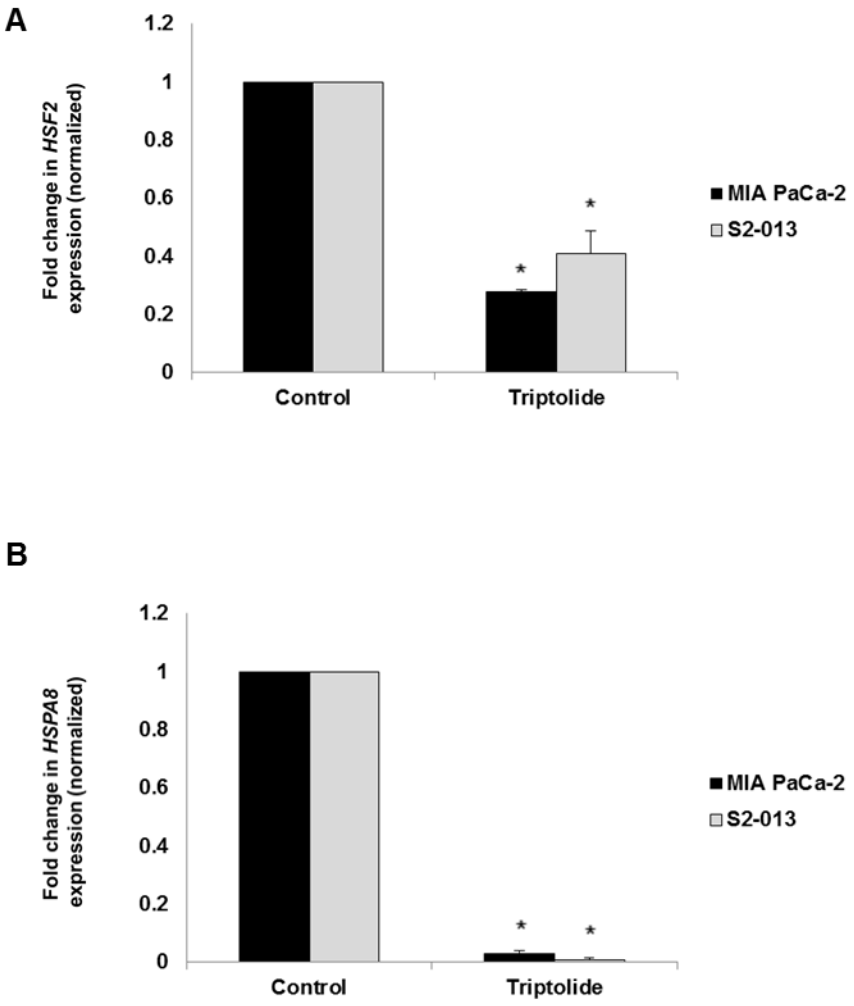

Figure 2-2: Effect of triptolide on heat shock mRNAs downregulated in microarray

Triptolide (100 nM) decreases both *HSF2 and HSPA8* (as assessed by real-time PCR) in MIA PaCa-2 and S2-013 cells. Expression of mRNA was normalized against *18S*. The bars represent mean $\pm$ SEM, n=3, *p<0.002 (t test).

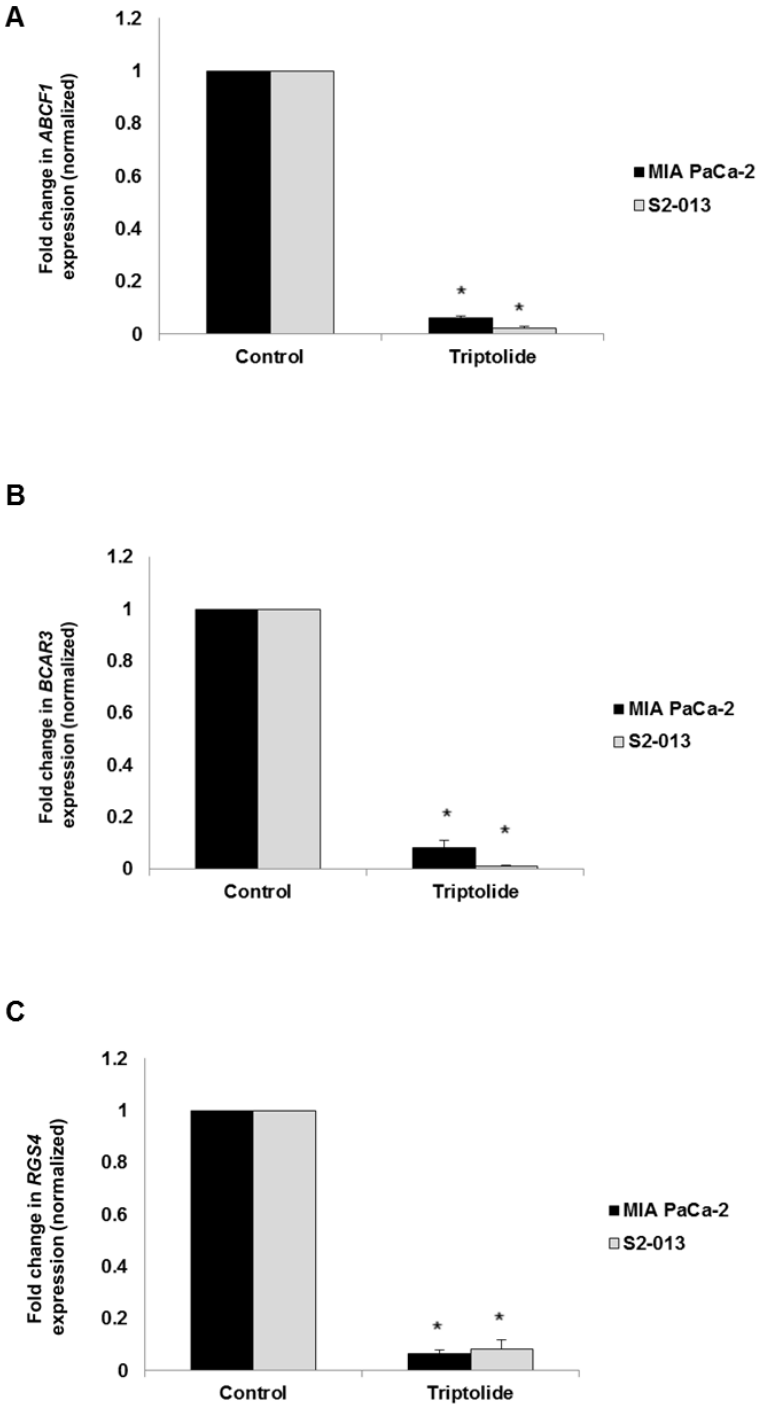

Figure 2-3: Effect of triptolide on select mRNAs downregulated in microarray.
Triptolide (100 nM) decreases *ABCF1*, *BCAR3* and *RGS4* (as assessed by real-time PCR) in MIA PaCa-2 and S2-013 cells. Expression of mRNA was normalized against *18S*. The bars represent mean $\pm$ SEM, n=3, *p<0.002 (t test).

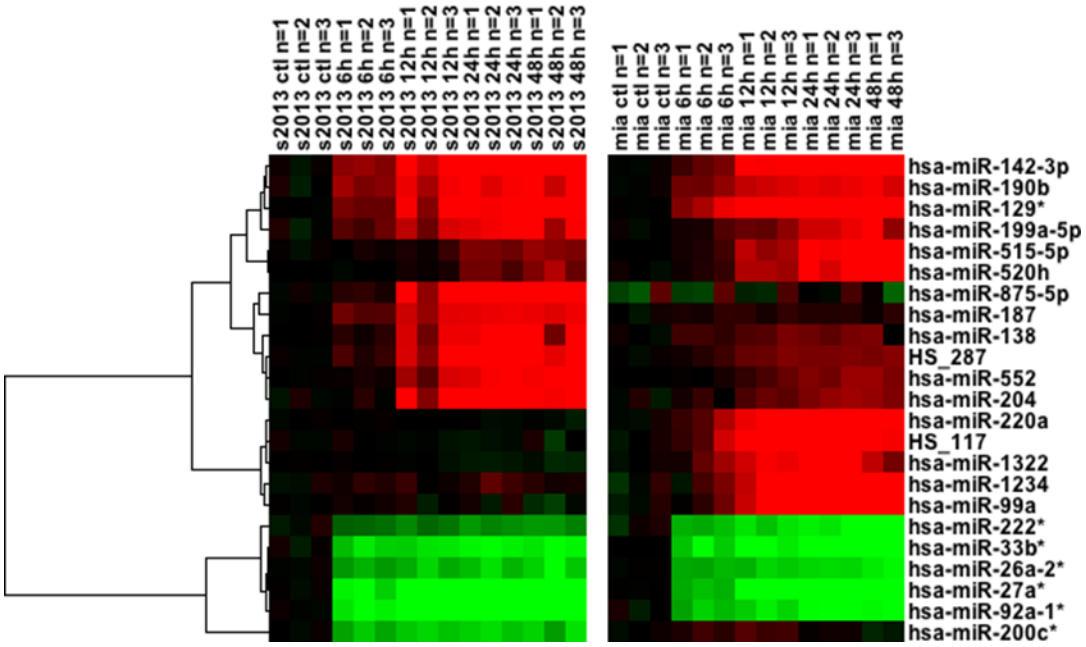

Figure 2-4: Triptolide-regulated miRNAs in PDAC cells.
Heatmap representation of differentially expressed human miRNAs after triptolide (100 nM) treatment (0, 6, 12, 24 or 48 h) in MIA PaCa-2 and S2-013 cells as measured by microarray. Only miRNAs with strong differential expression (t-test between 0 and 24 hour replicate time points, $p<0.05$, average fold change>5) in at least one of the two time-courses are shown. Log transformed expression values are shown compared with the untreated control for each cell line independently.

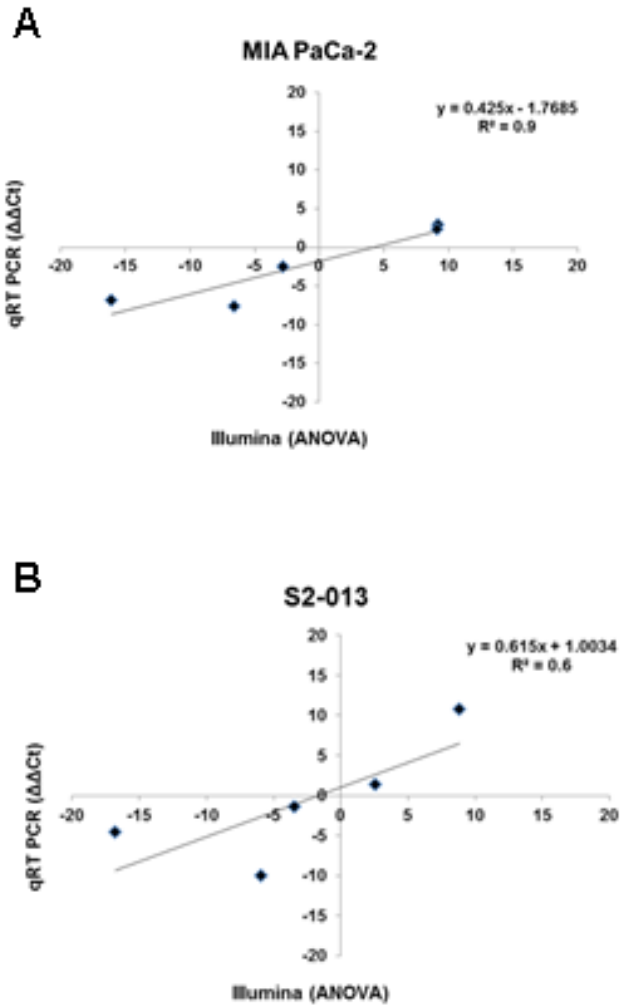

Figure 2-5: Validation of miRNA microarray by real-time PCR. Correlation of fold change in control versus triptolide-treated PDAC cell lines as determined by microarray and real-time PCR. (A) Fold change correlation for increasing human miRNA (miR-129*, miR-142-3p, miR-515-5p) and decreasing miRNA (miR-1244, miR-125a-3p) in MiaPaCa-2. (B) Fold change correlation for increasing human miRNA (miR-142-3p, miR-190b, miR-515-5p) and decreasing miRNA (miR-1244, miR-125a-3p) in S2-013. Linear regression analysis and the corresponding R^2 value are presented in each plot.

Table 2: Statistics for all miRNAs from microarray.
Shown are the p-values and the mean effect sizes for each miRNA regarding the 0 x 24 hour comparison for both the MiaPaCa-2 and S2-013 cell lines for those miRNA listed in **Error! Reference source not found.**.

Row	s2013-0x24: T-Test — p-value	s2013-0x24: Effect Size — Effect (Group Means)	mia-0x24: T-Test — p-value	mia-0x24: Effect Size — Effect (Group Means)
hsa-miR-142-3p	1.7433E-05	8.726648331	5.28732E-06	9.207952499
hsa-miR-190b	0.000332436	5.77222681	1.10057E-05	5.133922577
hsa-miR-129*	2.86542E-05	6.705578327	1.30827E-06	15.18275166
hsa-miR-199a-5p	0.000400818	5.72492075	7.55243E-05	4.552599907
hsa-miR-515-5p	0.000330781	2.29041934	3.32274E-05	6.705559254
hsa-miR-520h	0.002029971	1.990178466	0.000370154	6.066987514
hsa-miR-875-5p	1.01893E-05	10.65709305	0.506596148	1.392009735
hsa-miR-187	1.36152E-06	5.210954666	0.052495822	1.279753327
hsa-miR-138	2.89802E-06	5.773931026	0.001038195	2.110512972
HS_287	4.80362E-06	8.742925644	0.000113936	2.358536482
hsa-miR-552	3.16362E-05	5.567242622	0.000342159	2.489617109
hsa-miR-204	5.69249E-05	8.033229828	0.002903677	2.754431963
hsa-miR-220a	0.347338736	0.941894829	3.38929E-05	13.36408997
HS_117	0.166315049	0.87775749	1.82683E-05	15.1333847
hsa-miR-1322	0.001015333	0.820091426	3.95357E-05	6.738529205
hsa-miR-1234	0.051600713	1.559337497	0.000638866	8.129764557
hsa-miR-99a	0.673706889	1.069320321	5.04835E-05	12.94771481
hsa-miR-222*	0.00151316	0.328711271	0.000625794	0.150241226
hsa-miR-33b*	7.59828E-05	0.143694758	9.29939E-05	0.102604099
hsa-miR-26a-2*	0.000169325	0.206653729	9.31357E-07	0.180258021
hsa-miR-27a*	6.08382E-05	0.065840103	1.01391E-05	0.062193315
hsa-miR-92a-1*	4.10449E-06	0.083203852	8.84133E-05	0.132626757
hsa-miR-200c*	2.87745E-05	0.192971796	0.203511104	1.101228118

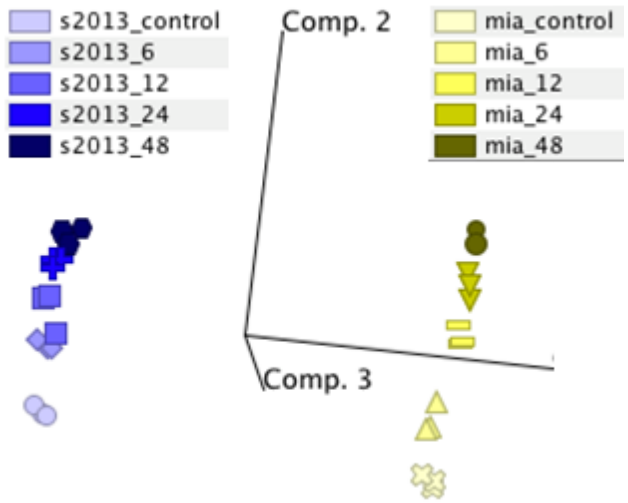

Figure 2-6: Principle components analysis.
Principle component analysis (PCA) to visualize all trends in miRNA transcript level changes over time-course of triptolide treatment (0, 6, 12, and 24 h) in MIA PaCa-2 cells and S2-013 cells. The first PCA component is driven by the differences in miRNA transcript levels between the two cell lines; while the second component is driven by the length of time the cells have been exposed to drug.

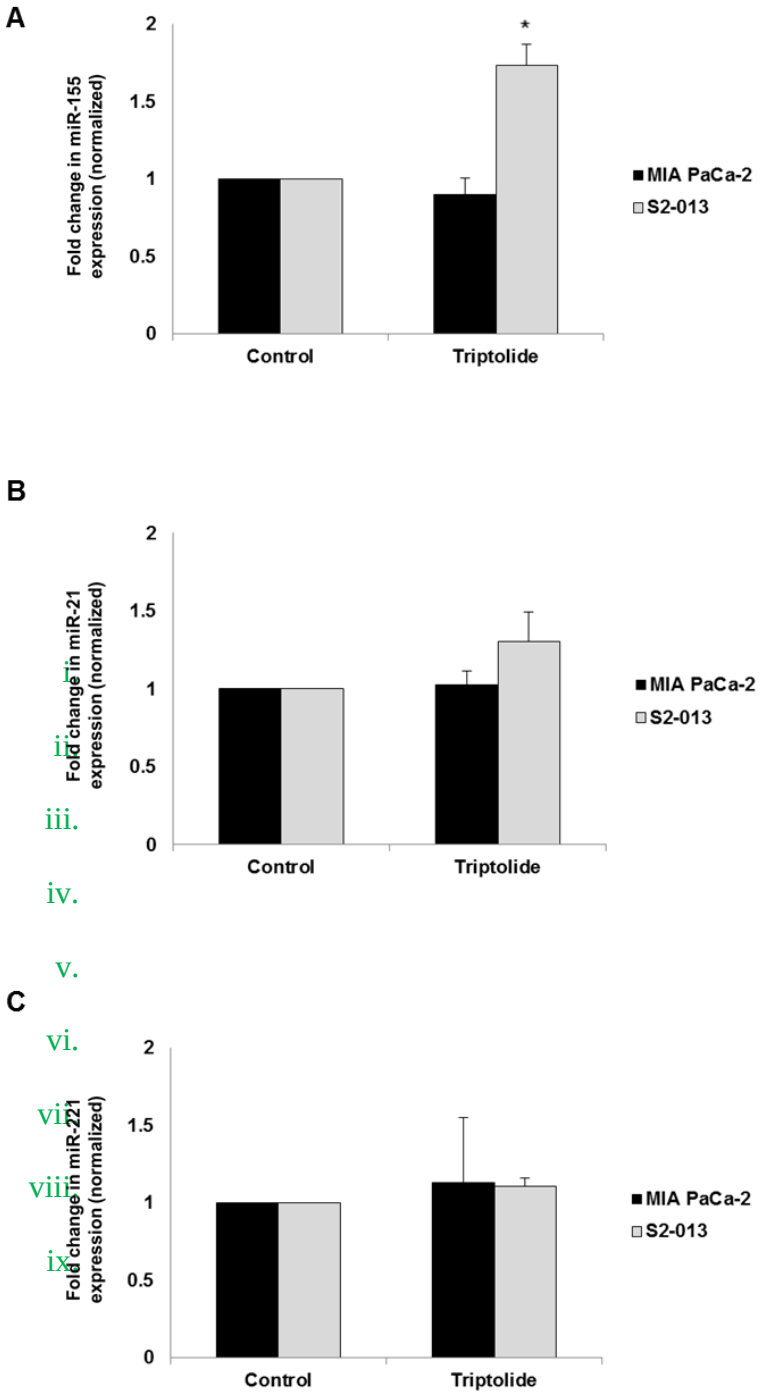

Figure 2-7: Effect of triptolide on miRNAs known to play a role in PDAC. Triptolide (100 nM) has no observed effect on miR-155, miR-21 nor miR-221 (as assessed by real-time PCR) in MIA PaCa-2. Triptolide modestly increases miR-155 in S2-013 (but this change is not validated in the microarray data tabulated in Supplemental Table S2), but has no effect on miR-21 nor miR-221 in this cell line. Expression of miRNA was normalized against *U6*. The bars represent mean ± SEM, n=3, *p<0.05 (t test).

Table 3: Statistics miRNAs from microarray which are known to play a role in PDAC.
Shown are the p-values and the mean effect sizes for each miRNA regarding the 0 x 24 hour comparison for both the MIA PaCa-2 and S2-013 cell lines.

Row	s2013-0x24: T-Test — p-value	s2013-0x24: Effect Size — Effect (Group Means)	mia-0x24: T-Test — p-value	mia-0x24: Effect Size — Effect (Group Means)
hsa-miR-155	0.675511181	1.024030685	0.244509667	0.850677907
hsa-miR-21	0.833157063	1.007005334	0.724045694	0.986084461
hsa-miR-221	0.663820207	1.02396524	0.845810592	1.008038282

Chapter 3 Triptolide induces the expression of miR-142-3p: a negative regulator of heat shock protein 70 and pancreatic cancer cell proliferation

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Triptolide induces the expression of miR-142-3p: a negative regulator of heat shock protein 70 and pancreatic cancer cell proliferation.

All figures in this chapter (with the exception of Table 4) are included in the manuscript: ***Molecular Cancer Therapeutics*** Accepted for publication on April 22, 2013.

Introduction

Some studies suggest that triptolide inhibits the transcription factor heat shock factor 1 (HSF1) and in this way suppresses HSP70 transcription [53], but as microRNAs (miRNAs) become increasingly recognized as major negative regulators of gene expression, we asked whether triptolide may regulate HSP70 expression via miRNAs. In cancer cells, the dysregulation of miRNAs expression serves as an efficient means to rewire the cell gene expression map and generate a cancer phenotype. In particular, the loss of tumor-suppressive miRNAs upregulates oncogenic targets [75]. No previous reports have examined the effect of triptolide on the PDAC miRNAome nor evaluated miRNA-mediated regulation of HSP70 in PDAC cells. Consequently,
the aims of this study are as follows: evaluate whether miRNAs upregulated by triptolide play a tumor suppressive role in PDAC, and validate that predicted miRNAs regulate HSP70 independent of the HSF1 pathway. We hypothesize that triptolide will upregulate tumor-suppressive miRNAs which directly regulate HSP70. This is the first evaluation of an miRNA-mediated mechanism of HSP70 regulation in cancer.

Materials and Methods

Cell culture and drug treatment

Cells from the MIA PaCa-2, Capan-1 and HEK-293 lines were obtained from ATCC (Manassus, VA) and cultured in DMEM (Life Technologies, Carlsbad, CA) containing 10% FBS (Life Technologies, Carlsbad, CA). S2-013 cells were kindly provided by Dr.

Buchsbaum (University of Alabama at Birmingham) and cultured in RPMI medium (Life Technologies, Carlsbad, CA) containing 10% FBS. Multiple aliquots of cells were cryopreserved when initially grown. All the cell lines were used within 6 months of resuscitation. No authentication was done by the authors, but ATCC authenticates using Short Tandem Repeat Profiling. For all drug treatments, cells were seeded in 6-well plates (2.5×10^5 cells/well) and incubated for the periods indicated in the figure legends prior to RNA or protein analysis. Triptolide treatment (Calbiochem, San Diego, CA) was performed as previously described [54, 60]. Quercetin (Sigma-Aldrich, St. Louis, MO) or gemcitabine (Eli Lilly Corporation, Indianapolis, IN) treatments were performed as previously reported [51, 84]. Cells were maintained as previously described [54, 60].

Quantitative real-time PCR

Total RNA was reverse transcribed using the miScript II RT Kit (Qiagen, Valencia, CA). Real-time PCR was done using the QuantiTect or miScript SYBR green PCR kit (Qiagen, Valencia, CA) on an Applied Biosystems 7300 real-time PCR system. *18S* was used as a control for *HSP70, HSP27 or HSF1*, and *U6* was used as a control for miR-142-3p (miScript Primer Assay; Qiagen). To verify *HSPA1B (HSP70)* overexpression, HSP70 primers targeting the *HSP70* ORF region [54] were used. Quantification was done using the ΔΔCt method.

Transfection of miR-142-3p mimic or inhibitor

Cells were seeded in 6-well (8 x 10^4 cells/well) or 24-well plates (1.5 x 10^4 cells/well) and incubated overnight prior to transfection. Transfection mastermixes were diluted in Opti-MEM (Life Technologies, Carlsbad, CA) containing HiPerFect (Qiagen, Valencia, CA), miR-142-3p mimic, miR-142-3p inhibitor or negative control (NC) miRIDIAN reagents (Thermo Scientific Dharmacon, Rockford, IL). Transfected cells were analyzed for Western blotting or cell viability as previously reported [54].

Cell proliferation using Electric Cell-substrate Impedance Sensing (ECIS)

Using the ECIS method, cells are grown on the surface of planar gold-film electrodes and the AC impedance of the cell-covered electrode is measured continuously at a frequency of 4000 Hz. Due to the insulating properties of cell membranes, the impedance increases with increasing coverage of the electrode. MIA PaCa-2 or Capan-1 cells (6 x 10^4 cells/well) and S2-013 (1 x 10^5 cells/well) were plated in 8-well, gold-film electrode coated 10E+ arrays (Applied Biophysics, Troy, NY). Proliferation rates were normalized to 6 h following transfection.

Dual-Luciferase reporter assay and 3'UTR binding site mutagenesis

HEK-293 cells were seeded in seeded in 24-well plates (6 x 10^4 cells/well). Mastermixes diluted in serum-free media (Life Technologies, Carlsbad, CA) containing Attractene (Qiagen, Valencia, CA), pGL4.73 control vector expressing firefly luciferase (Promega, Madison, WI), GoClone (HSPA1B) containing the wild-type HSPA1B 3'UTR expressing renilla luciferase (SwitchGear Genomics, Menlo Park,

CA), miR-142-3p mimic or NC (Thermo Scientific, Rockford, IL). Mutagenesis was done using the QuickChange Site-Directed Mutagenesis Kit (Agilent Stratagene, Santa Clara, CA). The Dual-Luciferase Reporter Assay System (Promega, Madison, WI) was used on a Synergy2 luminometer (BioTek, Winooski, VT).

Transfection of HSPA1B (HSP70) or HSF1 ORF vector

Cells were seeded in 24-well plates (1.5×10^4 cells/well) and were incubated overnight prior to transfection. Transfection mastermixes diluted in Opti-MEM (Life Technologies, Carlsbad, CA) containing Attractene (Qiagen, Valencia, CA), HSP70 (HSPA1B isoform) ORF, HSF1 ORF, or negative control (GeneCopoeia, Rockville, MD), along with miRIDIAN reagents (Thermo Scientific Dharmacon, Rockford, IL). 400 ng of either plasmid was added to each well along with transfection of miRIDIAN reagents.

Transfection of HSF1 siRNA

Cells were seeded in seeded in 6-well plates (8.0×10^4 cells/well) and were incubated overnight prior to transfection. Transfection mastermixes diluted in Opti-MEM (Life Technologies, Carlsbad, CA) containing HiPerFect (Qiagen, Valencia, CA), HSF siRNA or non-silencing siRNA (Qiagen, Valencia, CA) at a final concentration of 25 nM.

Measurement of miR-142-3p and HSPA1B (HSP70) levels from human xenograft PDAC tumor model

Three de-identified human pancreatic tumors were implanted subcutaneously into female severe combined immunodeficient (SCID) mice (The Jackson Laboratory, Bar Harbor, ME). When tumor volumes reached 500mm^3, tumors were dissected, cut into 10 mm^3 pieces, and propagated in additional SCID mice (one animal per tumor, n=3 animals). Strict animal care procedures from the University of Minnesota Institutional Animal Care and Use Committee were followed. Animals were randomized and tagged before daily intraperitoneal (i.p) injections of Minnelide (0.42 mg/kg) or saline for 7 days. Mice were sacrificed, and tumors were stored in RNA*later* (Qiagen, Valencia, CA). Samples were homogenized in one ml of Trizol (Life Technologies, Carlsbad, CA). Gene levels were analyzed by real-time PCR.

Statistical Analysis

Values are expressed as the mean ± SEM. All experiments with cells were repeated at least thrice. The significance of the difference between the control and each experimental test condition was analyzed by unpaired Student's t-test and a value of $p<0.05$ was considered statistically significant.

Results

Quercetin, but not gemcitabine, also induces miR-142-3p expression

To evaluate whether the induction of miR-142-3p was unique to HSP70 inhibiting compounds such as triptolide, we tested whether other chemotherapeutic agents upregulated miR-142-3p. As our laboratory has previously shown that quercetin (100 μM) inhibited HSP70 protein levels and decreased MIA PaCa-2 cell viability by 50% after 24 h [51], we tested whether quercetin likewise induced miR-142-3p. To address this, we treated PDAC cells with quercetin and assayed for miR-142-3p expression via real-time PCR. Expression of miR-142-3p was upregulated 3-fold by quercetin in MIA PaCa-2 and S2-013 cells and upregulated 8-fold in Capan-1 cells (Figure 3-1). To test whether the induction of miR-142-3p may be common among other chemotherapeutic agents, we measured miR-142-3p levels following gemcitabine treatment. We chose 1 μM gemcitabine treatment because it has been shown to inhibit MIA PaCa-2 [84, 96] viability by at least 50% after 72 h. This concentration allows us to evaluate early changes in the microRNAome directly linked proliferative pathways. Gemcitabine treatment did not alter miR-142-3p levels (Figure 3-1). These results show that miR-142-3p induction is common among HSP70 inhibitors triptolide and quercetin but is not present with the nucleoside analog gemcitabine.

Triptolide and ectopic expression of miR-142-3p inhibit PDAC cell proliferation

Because triptolide induced the expression of miR-142-3p, (Figure 2-) and inhibited cell viability in MIA PaCa-2, S2-013 and Capan-1 cells [60], we verified whether triptolide

inhibits proliferation in these cell lines using Electric Cell-substrate Impedance Sensing (ECIS) assay, an established method to test cancer cell proliferation in real-time [97]. Due to the insulating properties of cell membranes, the measured impedance increases with accumulating coverage of the electrode. We found that triptolide treatment inhibited proliferation of MIA PaCa-2, Capan-1, and S2-013 as early as 15 hours following treatment (Figure 3-2). To test whether ectopic expression of miR-142-3p was playing a tumor suppressive role in PDAC cells, we measured PDAC cell proliferation rates following miR-142-3p overexpression. Likewise, we found that overexpression of miR-142-3p inhibited proliferation of MIA PaCa-2, Capan-1, and S2-013 as early as 15 h following transfection (Figure 3-3). These data show that miR-142-3p and triptolide both suppress PDAC cell proliferation.

Upregulation of miR-142-3p inhibits HSPA1B (HSP70) expression

We tested whether ectopic expression of miR-142-3p inhibited HSPA1B (HSP70) expression for several reasons: first, our lab has previously shown that triptolide inhibits HSP70 expression [52, 54, 60, 61], second, triptolide inhibited *HSPA1B (HSP70)* mRNA expression by 74% or more (Figure 3-4), and third, miR-142-3p is predicted to target the *HSPA1B* isoform of *HSP70* by three independent computational prediction programs (miRDB updated 4.2012, TargetScanHuman Release 6.2, MicroCosm Targets Version 5). Overexpression of the miR-142-3p mimic (5 nM) inhibited at least 31% of *HSPA1B (HSP70)* mRNA expression following 24 h of transfection (Figure 3-4). Similarly, overexpression of miR-142-3p inhibited total HSP70 (both HSPA1B and

HSPA1A) protein expression by at least 50% following transfection for 72 h (Figure 3-5). These results show that ectopic expression of mIR-142-3p decreases HSPA1B (HSP70) mRNA and protein levels. Further, pancreatic cancer cells do not compensate for the loss in HSPA1B expression, induced by miR-142-3p, by upregulating the HSPA1A isoform of HSP70 (Figure 3-4).

MicroRNA-142-3p directly binds to the 3'UTR of HSPA1B (HSP70)

Three independent programs (miRDB updated 4.2012, TargetScanHuman Release 6.2, MicroCosm Targets Version 5) predict that miR-142-3p binding site is located within the *HSPA1B (HSP70)* 3'UTR (Figure 3-6, Table 4, and Table 5). To verify this interaction, a double point mutation (C to G and T to A) was inserted into the predicted binding site of *HSPA1B (HSP70)*, preventing the miR-142-3p seed sequence from interacting with this region (Figure 3-6). Overexpression of miR-142-3p decreased the renilla-to-firefly ratio to 43% of control (Figure 3-7). Upon mutating the miR-142-3p binding site of the renilla-expressing construct, the renilla-to-firefly ratio was rescued to 92% of control (Figure 3-7). These results support the prediction that miR-142-3p regulates *HSPA1B (HSP70)* transcript levels by binding to its 3'UTR.

HSPA1B (HSP70) is a functional target of miR-142-3p in PDAC cells

Because one miRNA may control the expression of many targets within the cell [75], we evaluated whether miR-142-3p was targeting *HSPA1B (HSP70)* as a means to control PDAC cell viability. To test this, we measured whether the loss in cell viability induced by miR-142-3p could be rescued by *HSPA1B (HSP70)* ORF overexpression.

This construct was used because it lacks the miR-142-3p binding site. Following 24 h of transfection, *HSPA1B (HSP70)* was upregulated 13 x 10^3-fold in MIA PaCa-2 cells overexpressing miR-142-3p and upregulated 8 x 10^3-fold upregulation in the same cells but in the control group (Figure 3-8). Following 48 h of transfection, miR-142-3p caused cell viability to decrease to 56% of control, but cotransfection of *HSPA1B (HSP70)* ORF rescues this level to 77% of control. Because a significant, but not complete, rescue was observed, miR-142-3p may be targeting other predicted downstream targets (Table 5). These results show, however, that miR-142-3p is targeting *HSPA1B (HSP70)* in PDAC cells and that this interaction is important in regulating cell viability.

HSF1 and miR-142-3p independently regulate HSPA1B (HSP70)

We tested whether miR-142-3p regulation of *HSPA1B (HSP70)* was independent of HSF1 because our lab has shown that HSF1 inhibition decreases HSP70 expression [98]. This was evaluated by measuring HSF1 levels by real-time PCR in PDAC cells following ectopic expression of miR-142-3p. While triptolide inhibited *HSF1* mRNA expression (Figure 3-9), as well as downstream transcriptional targets *HSPA1B (HSP70)* (Figure 3-4) and *HSP27* (Figure 3-9), ectopic expression of miR-142-3p had no effect on either *HSF1* or *HSP27* levels (Figure 3-9). These data show that miR-142-3p does not regulate *HSF1,* but these results do not address whether HSF1 controls miR-142-3P levels. To test this, we silenced HSF1 and measured miR-142-3p levels. Both HSF1 siRNA sequences independently inhibited *HSF1* mRNA expression (Figure

3-10). Levels of miR-142-3p did not change compared with the control cells following 24 h of transfection (Fig. 5B).

HSF1 and miR-142-3p mediate triptolide-induced suppression of PDAC proliferation

HSF1 and miR-142-3p have each been shown to be inhibited and induced by triptolide, respectively, and to regulate HSP70. For this reason, we tested whether HSF1 and miR-142-3p were important in mediating triptolide-induced suppression of PDAC proliferation via HSP70 modulation. We found that simultaneous overexpression of HSF1 and inhibition of miR-142-3p significantly rescued from triptolide-induced suppression of cell proliferation. The *HSF1* ORF vector yielded sufficient overexpression (Figure 3-11) and this rescued triptolide-induced loss in cell proliferation (Figure 3-11). We also tested whether HSF1 overexpression and miR-142-3p inhibition could rescue from triptolide-induced loss of *HSPA1B (HSP70)* expression. Triptolide suppresses *HSPA1B (HSP70)* levels to 26% of control, but while concurrently overexpressing of HSF1 and inhibiting miR-142-3p, this is increased to 35% of control. Although this was expected, the difference was not statistically significant (Figure 3-12). These data corroborate our results showing that *HSPA1B (HSP70)* is an important, although not the only, target of miR-142-3p. Likewise, *HSPA1B (HSP70)* is one of several transcriptional targets of HSF1 [99]. Both HSF1 and miR-142-3p play an important role in mediating triptolide-induced suppression of cell

proliferation, each independently regulate *HSPA1B (HSP70)* expression, but each also regulate other targets in PDAC cells (Figure 3-13).

Minnelide induces miR-142-3p expression in a mouse model of PDAC

Our laboratory has shown that Minnelide, a prodrug of triptolide, is effective in decreasing tumor burden *in vivo* [61]. To verify that the induction of miR-142-3p observed *in vitro* would also take place *in vivo*, we tested whether Minnelide (0.42 mg/kg for 7 days i.p.) upregulated miR-142-3p levels in 3 independent human PDAC tumor tissues propagated in SCID mice. Minnelide treatment, as compared to saline treatment, led to a 6-fold upregulation of miR-142-3p levels in SCID mice bearing human PDAC tumors (Figure 3-14). In these same samples, Minnelide led to a 55% inhibition of *HSPA1B (HSP70)* mRNA. These results show that Minnelide is concurrently inducing miR-142-3p and inhibiting *HSPA1B (HSP70) in vivo*.

Discussion

While this study is the first to evaluate the role of miR-142-3p in PDAC, over 20 studies have evaluated the tumor suppressive role of miR-142-3p in other cancer types. In miRNA profiling studies of acute myeloid leukemia patients, miR-142-3p was downregulated [100] and shown to regulate tumorigenic targets: CCNT2 and TAB2 [101]. In hepatocellular carcinoma, miR-142-3p was downregulated compared with normal liver and shown to target RAC1, a GTPase involved in cell growth, migration, and the activation of protein kinases [102]. Because *HSPA1B (HSP70)* significantly, though not completely, rescued from miR-142-3p-induced cell death (Fig. 4D), and the miR-142-3p inhibitor somewhat, although not significantly, rescued from triptolide-induced suppression of *HSPA1B (HSP70)* (Figure 3-7), miR-142-3p may bind to additional targets in PDAC cells. There are a nine other targets predicted by multiple algorithms (Table 5). Testing whether mir-142-3p targets RLF rearranged L-myc fusion protein merits further investigation because it has been established as an oncogene in small cell lung carcinoma [24]. CCNT2, TAB2, RAC1 or RLT may be important miR-142-3p targets in PDAC, and this is worthy of future study. Our data support the hypothesis that miR-142-3p plays a tumor suppressive role by regulating *HSPA1B (HSP70)* in PDAC (Fig. 3 & 4).

Although there are several cardiovascular and pulmonary studies evaluating miRNA regulation of HSP70, none have focused on HSPA1B isoform of (HSP70). Hsp70.3 (HSP2A isoform) has been shown to possess general cytoprotective properties in

preventing ischemic damage. One study has shown that the Hsp70.3 (HSP2A) gene product is subject to miRNA regulation via miR-378* and miR-711 [103]. In lung tissue, it has been shown that HSP70 is regulated by miR-146a and miR-146b-5b. These miRNAs were found to increase greatly, and inversely correlate with HSP70 levels, following treatment with gefitinib; this may contribute to pulmonary fibrosis [104]. The results obtained in this study support an miRNA-mediated mechanism of HSP70 regulation independent of HSF1 (Figure 3-10). Though HSF1 regulation of HSP70 has been well-documented [53, 98, 99], miRNA regulation of HSP70 merits further investigation.

Because miR-142-3p negatively regulates protumorigenic genes, it holds promise as a target for future PDAC therapeutic development. As with many miRNAs, miR-142-3p may play an opposite role in different cancer types. For example, miR-142-3p has been reported to be oncogenic and upregulated in human T-cell acute lymphoblastic [105]. Although the majority of studies evaluating miR-142-3p in cancer demonstrate its tumor suppressive role [100-102], those who further develop miR-142-3p as a therapy in PDAC will need to verify its tumor suppressive role in prospective patients. Restoring repressed miRNA levels in patients holds promise because it has been shown to be feasible via systemic delivery of lipid nanoparticles carrying miR-34a, miR-143 and miR-145 in treating orthotopic PDAC tumors *in vivo* [106]. This study was especially important as blood flow to the pancreas is thought to be low [79]. Understanding an miRNA-mediated mechanism of triptolide action, especially the

induction of miR-142-3p, will be useful as Minnelide moves into clinical trials. Moreover, miR-142-3p can be a target for future PDAC therapeutic development.

Figures and Tables

Table 4: Computational prediction of miRNAs which target *HSPA1B (HSP70)*

miRDB	TargetScan	MicroCosm
hsa-miR-142-3p	miR-142-3p	miR-142-3p
hsa-miR-497-5p	miR-15abc/16/16abc/195/322/424/497/1907	miR-497
hsa-miR-424-5p		miR-424
hsa-miR-15b-5p		miR-15b
hsa-miR-15a-5p		miR-15a
hsa-miR-195-5p		miR-195
hsa-miR-16-5p		miR-16-1*
		miR-16
hsa-miR-561-3p		miR-561
		miR-322
hsa-miR-449a	miR-34ac/34bc-5p/449abc/449c-5p	miR-449a
hsa-miR-449b-5p		
hsa-miR-34a-5p		miR-34a
hsa-miR-34c-5p		miR-34c-5p
		miR-449b
	miR-144	miR-144
	miR-132/212/212-3p	miR-132
		miR-212
hsa-miR-495		miR-495
hsa-miR-7-1-3p		miR-7-1*
hsa-miR-518a-5p		miR-518a-5p
hsa-miR-545-3p		miR-545
hsa-miR-524-5p		miR-524-5p
hsa-miR-186-5p		miR-186

Table 5: Computational prediction of miR-142-3p target mRNAs.
Shown are those mRNAs bearing sequence homology to miR-142-3p and thus predicted, by miRDB updated 4.2012, TargetScanHuman Release 6.2 or MicroCosm Targets Version 5, to interact. Those mRNAs predicted by all three algorithms are listed at the top of the table (in no particular order). Those common to two of the three algorithms comprise the remainder of the table (in no particular order). Target mRNAs predicted only by one algorithm are not shown.

miRDB	TargetScan	MicroCosm
CLTA	*CLTA*	*CLTA*
FAM114A1	*FAM114A1*	*FAM114A1*
RLF	*RLF*	*RLF*
NR2C1	*NR2C1*	*NR2C1*
HSPA1B	*HSPA1B*	*HSPA1B*
LRRC3B	*LRRC3B*	*LRRC3B*
PGM1	*PGM1*	*PGM1*
ZNF479	*ZNF479*	*ZNF479*
MON2	*MON2*	*MON2*
WASL	*WASL*	
TSEN34	*TSEN34*	
BOD1	*BOD1*	
DCUN1D4	*DCUN1D4*	
ASH1L	*ASH1L*	
GAB1	*GAB1*	
SLC1A3	*SLC1A3*	
CD1D	*CD1D*	
WHAMM	*WHAMM*	
RAB2A	*RAB2A*	
USP6NL	*USP6NL*	
ATF7IP	*ATF7IP*	
MYLK	*MYLK*	
TMEM200B	*TMEM200B*	
LCOR	*LCOR*	
SMARCA5	*SMARCA5*	
TWF1	*TWF1*	
SLC37A3	*SLC37A3*	
APC	*APC*	
ZNF90	*ZNF90*	
UTRN	*UTRN*	
DSCR6	*DSCR6*	
CLDN12	*CLDN12*	
PDZD9	*PDZD9*	
ZEB2	*ZEB2*	
RHOBTB3	*RHOBTB3*	
C20orf194	*C20orf194*	
ANKRD46	*ANKRD46*	
HEATR5A	*HEATR5A*	
SYN2	*SYN2*	
SLC35F5	*SLC35F5*	
CRIPT	*CRIPT*	
FBXO3	*FBXO3*	
CALCOCO2	*CALCOCO2*	
ZNF217	*ZNF217*	
PRLR	*PRLR*	
PLA2G12A	*PLA2G12A*	
ARNTL	*ARNTL*	
FGF9	*FGF9*	
USP9X	*USP9X*	
CCDC165	*CCDC165*	
WDR5B	*WDR5B*	
ANKRD11	*ANKRD11*	
PCSK1	*PCSK1*	
FLVCR1	*FLVCR1*	
CXADR		*CXADR*
	SMR3A	*SMR3A*
	ZNF676	*ZNF676*

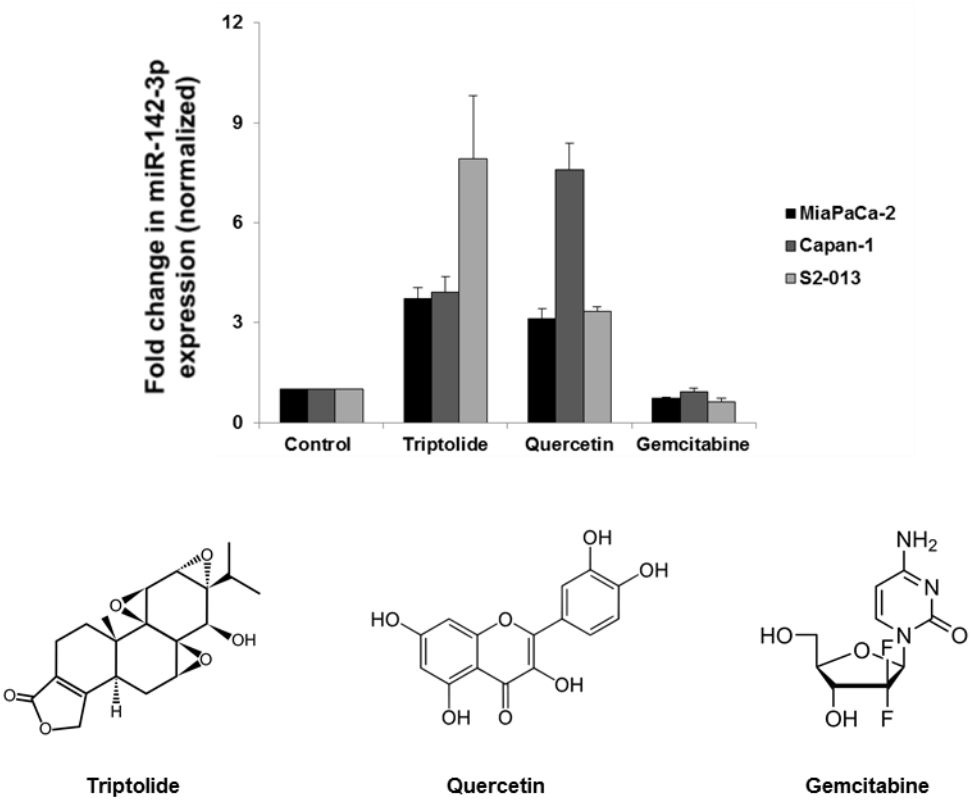

Figure 3-1: Effect of triptolide, quercetin or gemcitabine on miR-142-3p levels in PDAC cells.

Triptolide (100 nM) and quercetin (100 µM) treatments induced, whereas gemcitabine (1 µM) treatment had no effect on, miR-142-3p expression (as assessed by real-time PCR) in human MIA PaCa-2, Capan-1 and S2-013 cell lines following 24 h of treatment. Expression of miR-142-3p was normalized against *U6*. The bars represent mean ± SEM, n=3, **p*<0.02 (t test).

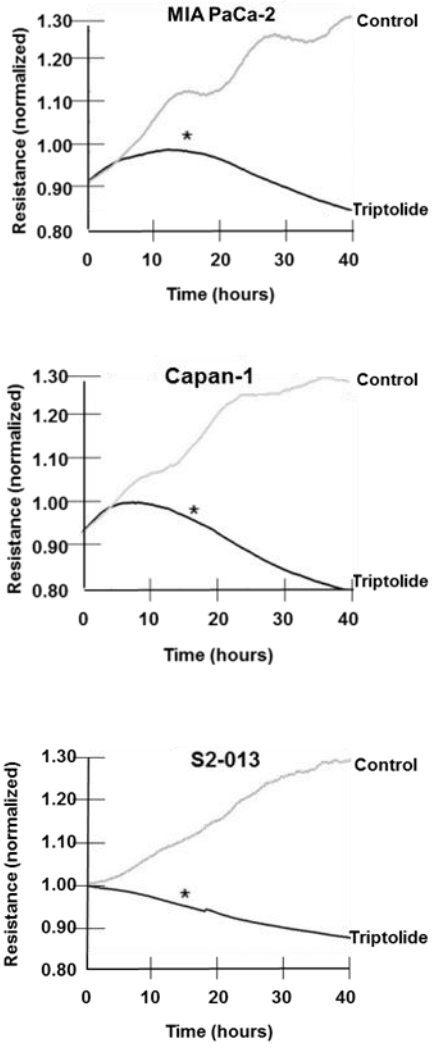

Figure 3-2: Effect of triptolide treatment on PDAC cell proliferation. Triptolide (100 nM) reduced the rate of cell proliferation as measured in real-time on the Electric Cell-substrate Impedance Sensing (ECIS) instrument in MIA PaCa-2, Capan-1 and S2-013 cell lines. Proliferation rates are representative of n>4 experiments and average of 4 replicates. Because proliferation rates are normalized to 6 h following transfection, the 0 h time point represents 6 h following transfection. SEM bars (not shown) overlap from 0-15 h, * represents point after which proliferation rates are different.

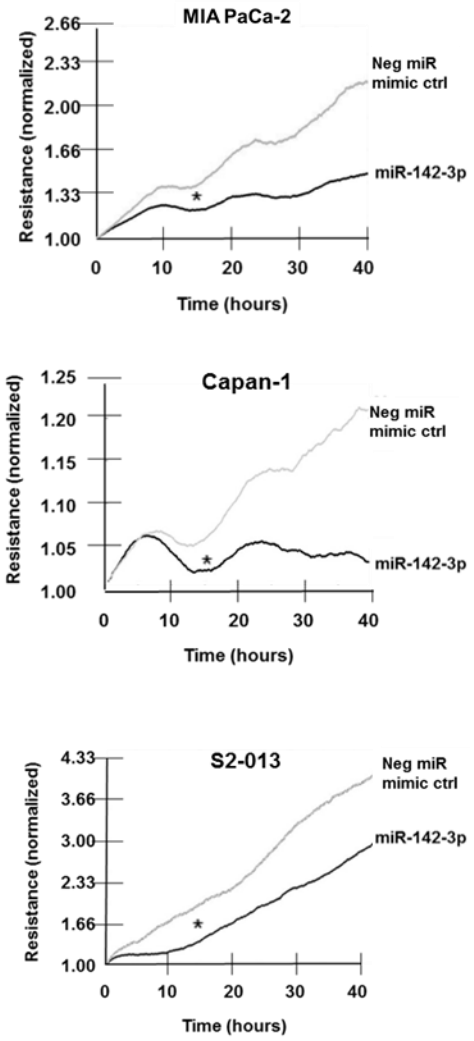

Figure 3-3: Effect of miR-142-3p mimic overexpression PDAC cell proliferation.
MicroRNA-142-3p mimic (5 nM) reduced the rate of cell proliferation as measured in real-time on the ECIS instrument in MIA PaCa-2, Capan-1 and S2-013 cell lines. Proliferation rates are representative of $n \geq 4$ experiments and average of 4 replicates. Because proliferation rates are normalized to 6 h following transfection, the 0 h time point represents 6 h following transfection. SEM bars (not shown) overlap from 0-15 h, * represents point after which proliferation rates are different.

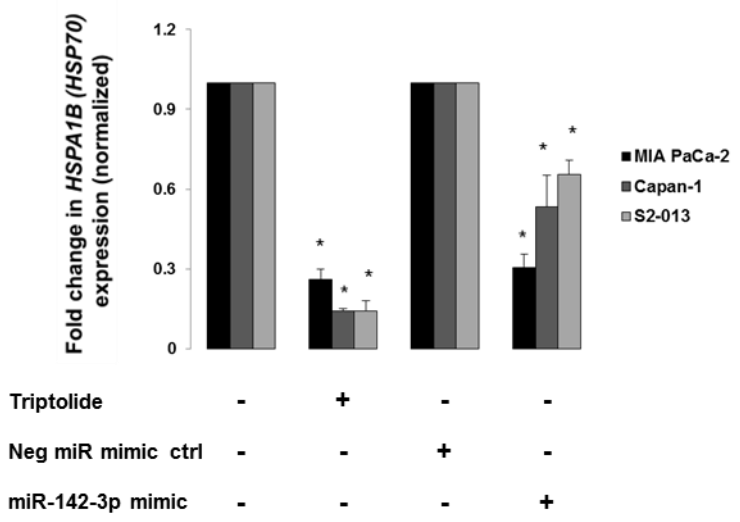

Figure 3-4: Effect of triptolide treatment or microRNA-142-3p mimic overexpression on *HSPA1B (HSP70)* mRNA levels.
Triptolide treatment (100 nM) and miR-142-3p (5 nM) ectopic expression reduced *HSPA1B (HSP70)* mRNA expression (as assessed by real-time PCR) in MIA PaCa-2, Capan-1 and S2-013 cell lines following 24 h of exposure. Expression of *HSPA1B (HSP70)* was normalized against *18S*. The bars represent mean $\pm$ SEM, n=3, *$p<0.02$ (t test).

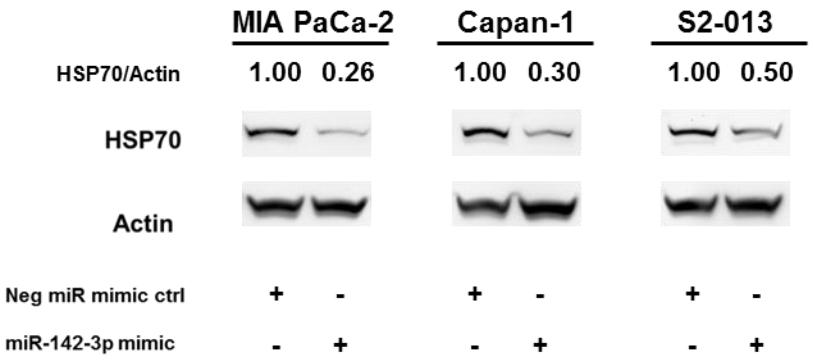

Figure 3-5: Effect of triptolide treatment or microRNA-142-3p mimic overexpression on HSPA1B (HSP70) protein levels.
MicroRNA-142-3p mimic (5 nM for 48 h) reduced HSP70 protein expression in MIA PaCa-2, Capan-1 and S2-013 cell lines. Expression of HSP70 was normalized against actin.

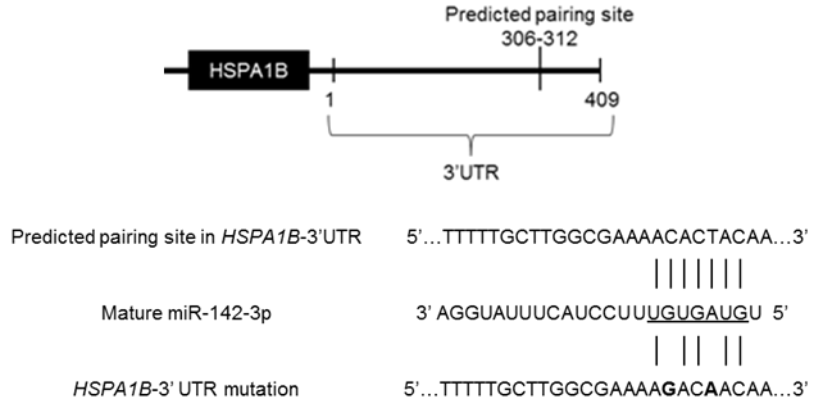

Figure 3-6: Predicted binding site of miR-142-3p in *HSPA1B (HSP70)*. Schematic of *HSPA1B (HSP70)* mRNA showing predicted miR-142-3p interaction site (upper). Seven-nucleotide interaction sequence between wildtype (wt) *HSPA1B (HSP70)*-3'UTR and miR-142-3p and mutant *HSPA1B (HSP70)*-3'UTR construct shown (lower).

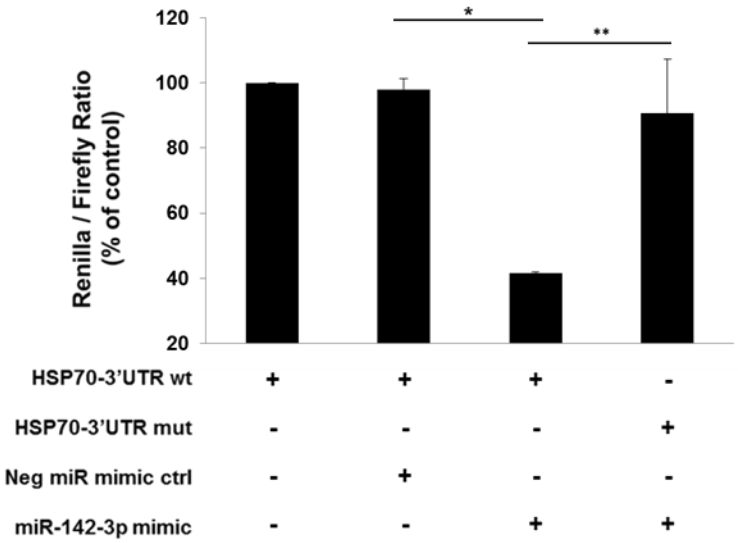

Figure 3-7: MicroRNA-142-3p modulates *HSPA1B (HSP70)* expression by binding to the predicted binding site of its 3'UTR. Luciferase reporter assay using HEK-293 cells to demonstrate the direct interaction of miR-142-3p and the 3'UTR of *HSPA1B (HSP70)*. After 24 h, miR-142-3p mimic (10 nM) reduced the ratio of renilla-to-firefly expression but not when the 3'UTR bears two point mutations in the miR-142-3p binding site. The bars represent mean $\pm$ SEM, n=3, *or **$p<0.05$ (t test).

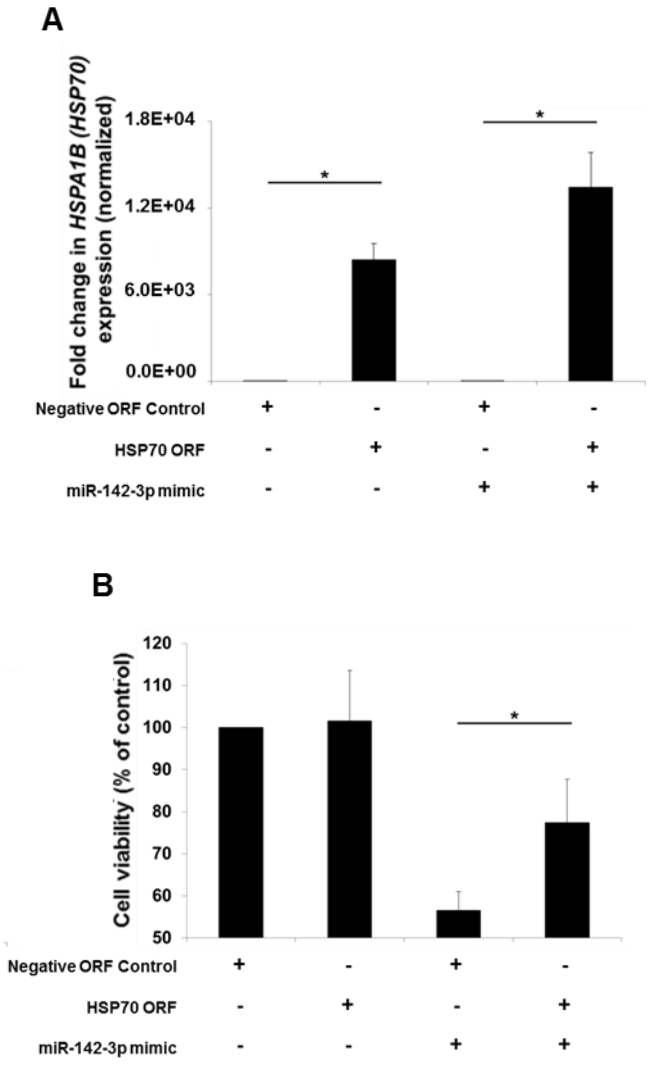

Figure 3-8: *HSPA1B (HSP70)* is a target of miR-142-3p which mediates PDAC cell death.
(A) *HSPA1B (HSP70)* ORF (lacking the 3'UTR containing the miR-142-3p binding site) transfection causes overexpression of *HSPA1B (HSP70)* expression (as assessed by real-time PCR) in MIA PaCa-2. (B) *HSPA1B (HSP70)* ORF overexpression rescued loss in cell viability caused by miR-142-3p (5 nM) overexpression for 48 h in MIA PaCa-2 cells. The bars represent mean $\pm$ SEM, n=3, *or **$p<0.05$ (t test).

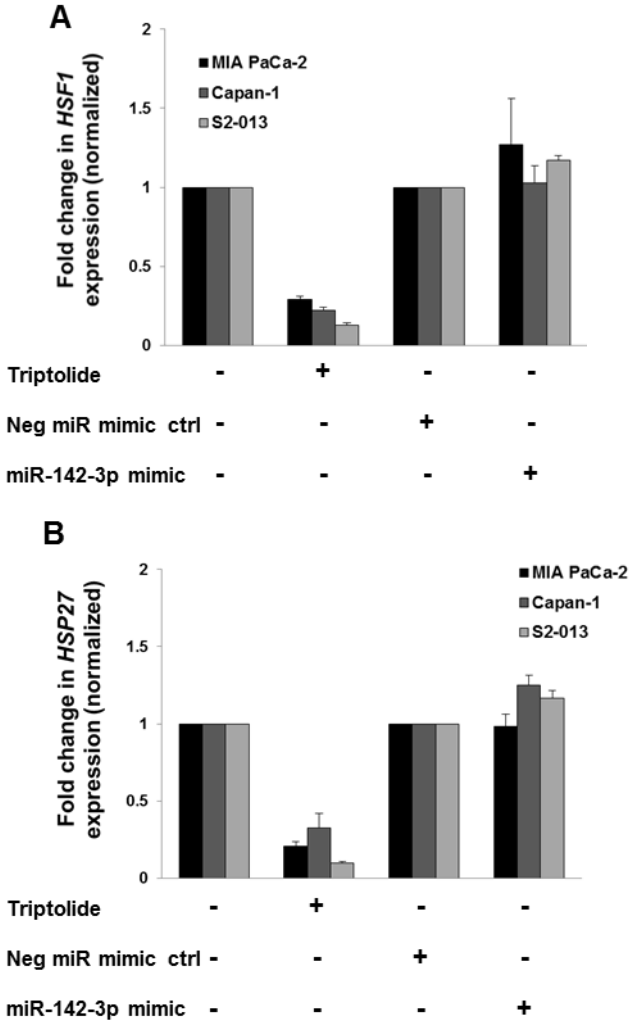

Figure 3-9: Effect of triptolide or miR-142-3p overexpression on *HSF1* and *HSP27* levels in PDAC cells.
(A) Triptolide (100 nM) treatment for 24 h decreases *HSF1* levels, but ectopic expression of miR-142-3p mimic (5 nM for 24 h) has no effect (as assessed by real-time PCR) in MIA PaCa-2, Capan-1 and S2-013 cell lines. (B) Triptolide (100 nM) treatment for 24 h decreases the level of *HSP27* mRNA, but 24 h ectopic expression of miR-142-3p mimic (5 nM) has no effect (as assessed by real-time PCR) in MIA PaCa-2, Capan-1 and S2-013 cell lines. Expression of *HSF1* or *HSP27* was normalized against *18S*. The bars represent mean $\pm$ SEM, n=3, *$p<0.05$ (t test).

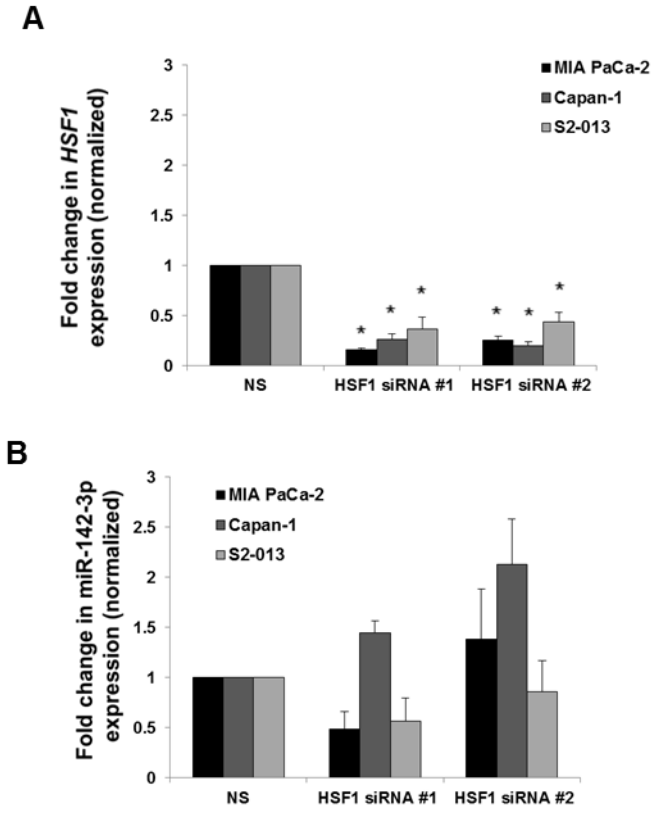

Figure 3-10: Effect of *HSF1* silencing on miR-142-3p levels in PDAC cells. (A) Real-time PCR verification of *HSF1* knockdown by two different siRNA (25 nM) sequences in MIA PaCa-2, Capan-1 or S2-013 cells 24 h-post-transfection. Expression of *HSF1* was normalized against *18S*. (B) Real-time PCR measurement of miR-142-3p following HSF1 silencing (25 nM for 24 h). Expression of miR-142-3p was normalized against *U6*The bars represent mean $\pm$ SEM, n=3, *$p<0.05$ (t test).

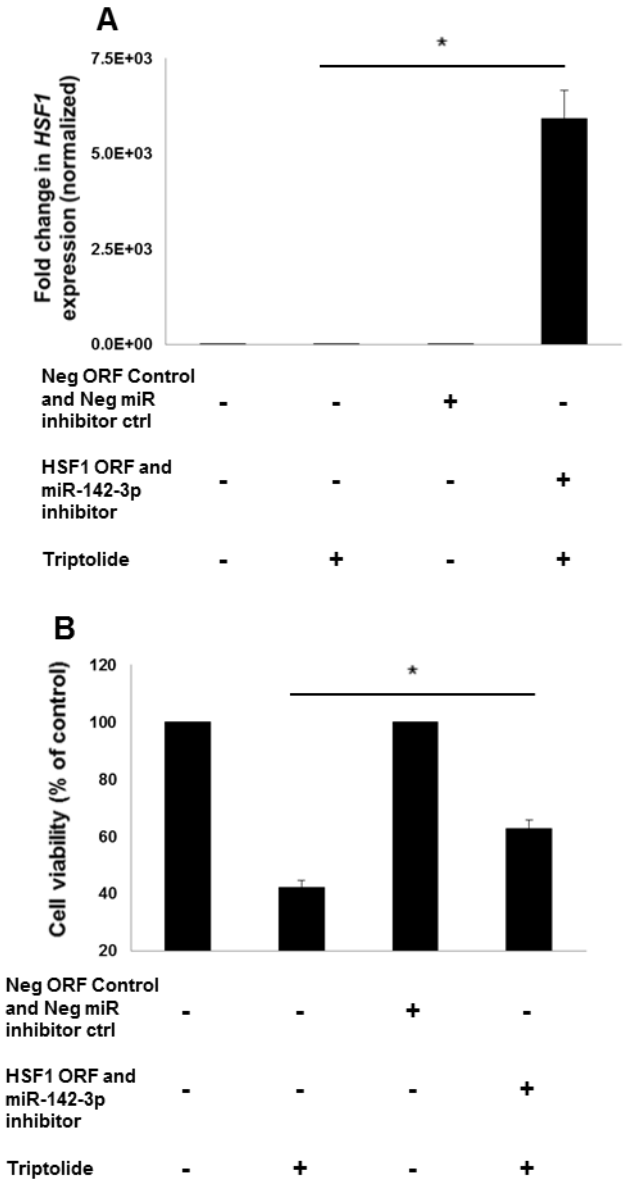

Figure 3-11: *HSF1* and miR-142-3p mediate triptolide-induced suppression of PDAC proliferation.
(A) *HSF1* ORF transfection causes overexpression of *HSF1* expression (as assessed by real-time PCR) in MIA PaCa-2. (B) *HSF1* ORF overexpression rescues loss in cell viability caused by triptolide (100 nM for 48 h) overexpression in MIA PaCa-2 cells. The bars represent mean $\pm$ SEM, n=3, *$p<0.05$ (t test).

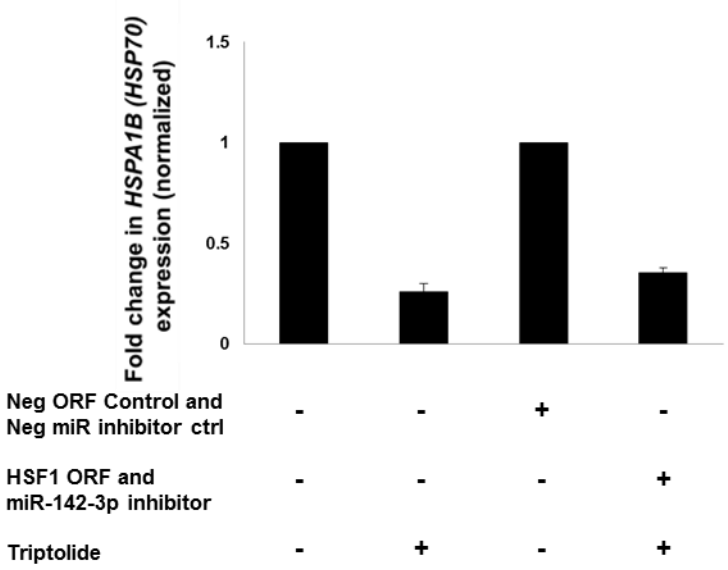

Figure 3-12: Effect of HSF1 overexpression and miR-142-3p inhibition on triptolide-induced suppression of *HSPA1B (HSP70)*.
Triptolide (100 nM) treatment for 24 h decreases the level of *HSPA1B (HSP70)* mRNA expression (as assessed by real-time PCR) in MIA PaCa-2, and this level is somewhat reversed, although not significantly, by overexpression of *HSF1* and inhibition of miR-142-3p. Expression of *HSPA1B (HSP70)* was normalized against *18S*. The bars represent mean $\pm$ SEM, n=3.

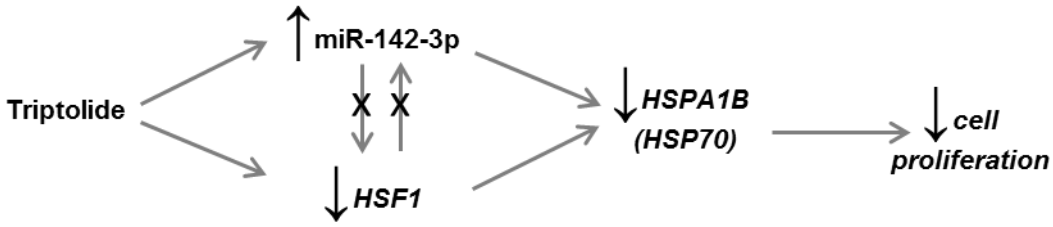

Figure 3-13: Model of triptolide action in PDAC cells.

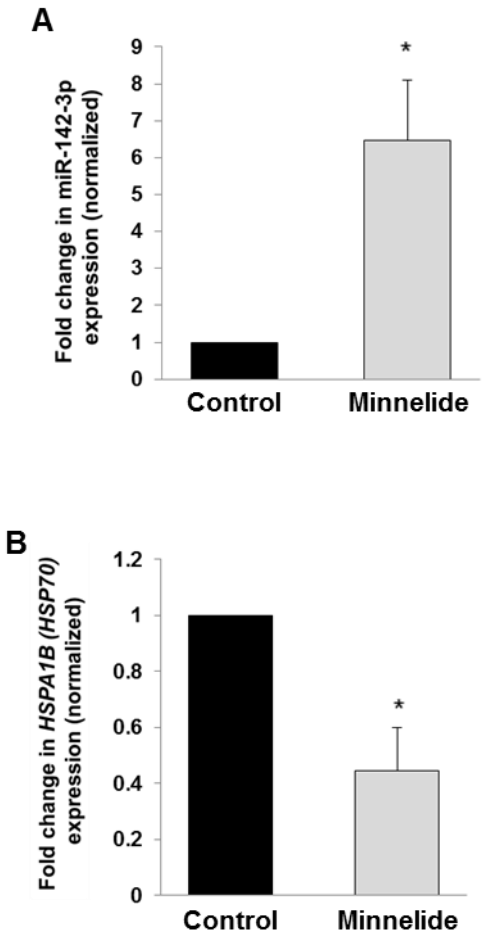

Figure 3-14: Effect of Minnelide on miR-142-3p or *HSPA1B (HSP70)* expression *in vivo*.
(A) Minnelide (0.42 mg/kg daily for seven days i.p.) induces miR-142-3p expression (as assessed by real-time PCR) in three independent human tumor xenografts grown *in vivo*. Expression of miR-142-3p was normalized against *U6*. (B) Minnelide reduces *HSPA1B (HSP70)* mRNA expression (as assessed by real-time PCR) of these same samples *in vivo*. Expression of *HSPA1B (HSP70)* was normalized against *18S*. The bars represent mean $\pm$ SEM, n=3, *$p<0.05$ (t test).

Chapter 4 Concluding remarks

These are the first studies to show the effect of triptolide on the transcriptome and microRNAome of pancreatic cancer cells. We have shown that triptolide globally downregulates about twice as many transcripts as it upregulates. Most notably, triptolide decreases the expression of two molecules in the heat shock pathway which our laboratory has not previously studied: HSF2 and HSPA8 (HSC70). It would be worthwhile to further study the effect of triptolide on these players. More specifically, it would be interesting to test whether the mechanism of triptolide action is on HSF1 versus HSF2. Further, it would be valuable to test whether the mechanism of triptolide action is dependent upon HSPA8 (HSC70) as opposed to HSPA1B (HSP70), as discussed in chapter 2. We can now depict a more clear mechanism of how triptolide affects heat shock pathways in pancreatic cancer cells.

These are also the first studies to examine the effect of triptolide on the miRNAome of pancreatic cancer cells and to evaluate the role of miR-142-3p in pancreatic cancer. In contrast to the transcriptome data set, triptolide upregulates twice as many miRNAs as it downregulates, and one of the most significant changes observed was the upregulation of miR-142-3p. As miR-142-3p has been shown to be downregulated in pancreratic cancer, relative to normal tissue, and was predicted by multiple algorithms to target one of the inducible isoforms of HSP70 (HSPA1B), we hypothesized that miR-142-3p would play a tumor suppressive role in pancreatic cancer via downregulating downregulating HSPA1B (HSP70). We found that overexpression of miR-142-3p inhibited proliferation of pancreatic cancer cells. Importantly, our findings may have an impact clinically because we found miR-142-3p to be induced in a mouse model of

pancreatic cancer as well. We found this miRNA-mediated mechanism of HSPA1B (HSP70) regulation to be independent of the HSF1 pathway. These results were discussed in chapter 3. It would be interesting to test what transcription factors are responsible for the induction of miR-142-3p. In other words, we would like to know what is upstream of triptolide induction of miR-142-3p in pancreatic cancer cells. For instance, it has been shown that the oncogenic transcription factor LMO2 downregulates miR-142 in leukemic cells [107]. It would be interesting to test whether triptolide inhibits LMO2 in pancreatic cancer cells, and further, what may be regulating LMO2. Understanding the mechanism of triptolide action as well as possible will aid in the execution of the upcoming phase I clinical trials of Minnelide.

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