Specific Aims

The vast majority of patients with pancreatic cancer do not have disease that is curable with resection alone. Most patients have unresectable disease at the time of presentation, and most patients who undergo resection recur. Standard therapies—chemotherapy for distant metastatic disease and chemotherapy with or without irradiation for local/regional disease—are associated with low objective response rates and modest improvements in survival. *There is therefore a dire need for more effective therapies for pancreatic cancer.*

Aptamers are a class of therapeutic nucleic acid (RNA or DNA) molecules, which specifically bind to existing target proteins. Aptamers are generated by an iterative screening process of large combinatorial nucleic acid libraries that can be modified for nuclease resistance. Most aptamers have been generated using *in vitro* selection against purified protein targets that were rationally chosen. However, selection strategies utilizing complex targets such as whole cancer cells or tumor tissue *in vivo* allow the aptamers to choose their own—potentially better—targets. Aptamers can have direct therapeutic effects mediated by binding and interfering with the function of their protein targets. In addition, aptamers that bind to cell surface receptors can be internalized by cells expressing those receptors and be utilized to deliver other therapeutic cargo. The objectives of this project are therefore both to identify better pancreatic cancer targets and to develop novel RNA therapeutics. *Our global hypothesis is that aptamers that bind targets over-expressed on pancreatic cancer cells relative to normal cells can be used to selectively deliver other inhibitory molecules, such as small interfering RNAs (siRNAs) or chemotherapeutic agents, to pancreatic cancer cells.* We have 2 specific aims:

Specific Aim #1: To utilize selection strategies against complex targets to identify new pancreatic cancer targets. The ideal target for aptamer-mediated delivery is one that is highly expressed on the surface of all pancreatic cancers, efficiently internalized, and not expressed on the surface of normal cells. Although a perfect target probably does not exist, there is room for improvement over existing aptamer targets such as epidermal growth factor receptor (EGFR), prostate stem cell antigen (PSCA), and nucleolin. This aim includes *in vitro* selection against whole pancreatic cancer cells as well as *in vivo* selection against pancreatic cancer xenografts and genetically engineered mouse (GEM) models of pancreatic cancer. We will characterize selected aptamers by identifying their specific protein and cellular targets. This aim therefore has the potential to simultaneously identify novel targets and the agents to mediate delivery to them. Aptamers that are shown to be internalized by pancreatic cancer cells will be further evaluated in Aim #2.

Specific Aim #2: To utilize aptamers that are internalized by pancreatic cancer cells for specific delivery of siRNAs and other therapeutic cargo. We have preliminary data demonstrating that an RNA aptamer that binds EGFR and a DNA aptamer that binds nucleolin are internalized by pancreatic cancer cells. In parallel with the identification of new targets and aptamers in Aim #1, we will utilize these extant aptamers to learn how to effectively deliver K-*ras* siRNAs and gemcitabine polymers into cells (i.e., how best to prepare different cargoes and attach them to aptamers). Then, we will use aptamers discovered in Aim #1 with the cargo technology gained in the first part of Aim #2 to formulate more specific and effective constructs for the delivery of K-*ras* siRNAs and gemcitabine polymers into pancreatic cancer cells *in vitro* and *in vivo*.

Research Plan Timeline

		2010-2011	2011-2012	2012-2013	2013-2014
Specific Aim #1	*In vitro* selection against pancreatic cancer cells				
	In vivo selection against xenografts				
	In vivo selection against GEM tumors				
Specific Aim #2	Optimization of constructs for siRNA delivery *in vitro*				
	Optimization of constructs for gemcitabine delivery *in vitro*				
	Formulation and testing of constructs with aptamers from Aim #1 *in vitro* and *in vivo*				

Significance

Pancreatic cancer

Pancreatic cancer has a mortality rate that exceeds nearly all other cancers. In 2009, approximately 42,000 people were diagnosed with pancreatic cancer, and 38,000 died from it[2]. Greater than 90% of patients present with disease that is considered unresectable, due either to local invasion of adjacent structures or distant metastasis. Approximately10% of pancreatic cancer patients have tumors that are amenable to surgical resection, and the vast majority of these patients will develop recurrent disease within 5 years [3]. For patients with locally advanced disease, the combination of chemotherapy and radiation is utilized to help provide local control [4] and—in rare cases—convert unresectable tumors into resectable ones [5]. For patients with distant metastatic disease, systemic chemotherapy is the only treatment option. Gemcitabine has become the "standard of care" for patients with advanced disease despite a radiographic response rate of only 5% and a survival benefit of only a few weeks [6]. Pancreatic cancer is notoriously chemoresistant with response rates ranging from 0 to 20% in most clinical trials, even for combinations of the "best" agents [7, 8]. Therefore, there is a dire need for more effective therapies for pancreatic cancer.

RNA interference

RNA interference (RNAi) is an invaluable tool to study the many complex and deregulated cellular pathways associated with cancer, but *in vivo* study and clinical application of RNAi has been limited by the inability to efficiently deliver small interfering RNAs (siRNAs) or their single-stranded precursors, short hairpin RNAs (shRNAs), to target cells. SiRNAs potently and specifically inhibit target gene expression by either degradation of mRNA or interference with translation. Early approaches to enhance intracellular delivery of siRNAs in animals have included the use of high-pressure injection, cationic lipids, viral vectors, and fusigenic peptides. These approaches have been associated with toxicity and have the disadvantage of delivering siRNAs to cells non-specifically, without regard to cell type. More recent efforts have utilized conjugation to ligands such as transferrin and the sigma receptor ligand anisamide to deliver siRNAs via internalization of their respective receptors [9, 10]. Using RNA ligands (aptamers) for cell type-specific delivery of siRNAs is an approach that was first described by the Mentor's RNA therapeutics program [11]. **For pancreatic cancer, this approach would allow the inhibition of important but elusive targets such as K-*ras* that have not proven amenable to inhibition by other approaches, such as small molecules or antibodies. K-*ras* is an attractive target, as activating mutations are present in almost all pancreatic cancers [12], and K-ras appears to play a critical role in carcinogenesis, growth, and invasion [13, 14].** Several studies using RNAi have demonstrated that K-ras is a valid target in pancreatic cancer [15-17], but these approaches have not come to fruition due to the fundamental difficulty of delivering enough of the therapy to the specific cells that need it without toxicity to the cells that do not.

The proposed project is significant because we will use a novel approach to target siRNAs and other cytotoxic cargo selectively to pancreatic cancer cells with the goal of curing more patients with resectable disease and improving survival in patients with unresectable disease.

Innovation

Aptamers

Aptamers are a class of therapeutic oligonucleotides (RNA or DNA), which specifically bind to existing target proteins. Aptamers are generated by an iterative screening process of complex nucleic acid libraries (>10^{14} shapes per library) employing a process termed Systemic Evolution of Ligands by Exponential Enrichment (SELEX) [18, 19]. The Mentor's group has employed this technology to generate potent RNA antagonists of over 25 proteins including coagulation factors, growth factors, and cell surface receptors [20-24]. **Nuclease-resistance is critical for aptamer stability in biological fluids and is generally conferred to RNA by the use of 2'-modified nucleotides, most commonly 2'-fluoro or 2'-O-methyl pyrimidines, with associated half-lives in plasma of several hours [25]. Although aptamers of comparable affinity and specificity can be generated from RNA and DNA, the Mentor's laboratory has generally used 2'-modified RNA, which has greater plasma stability than unmodified DNA. Stimulation of the immune system via Toll-like Receptors by double-stranded regions within RNA aptamers is a valid concern [26], but modified ("artificial") nucleotides do not appear to be potent stimulators of this innate immune response [27]. For large scale chemical synthesis, it is usually necessary to "truncate" or "minimize" aptamers from**

their full length at the end of selection to fewer than 60 nucleotides. Due to the relatively small size of truncated aptamers (8 kDa to 15 kDa), the circulating half-lives of most aptamers are limited not by plasma stability but by renal clearance, which can be improved by conjugation to high molecular weight groups such as polyethylene glycol (PEG) [28]. Although not a new concept, aptamers have only become realistic clinical agents as methods for their efficient synthesis have improved, similar to monoclonal antibodies 30 years ago. Intraocular delivery of a nuclease-resistant RNA aptamer against vascular endothelial growth factor (VEGF) modified with PEG (pegaptinib or Macugen®, Eyetech Pharmaceuticals) is now in clinical use for the wet form of age-related macular degeneration [29]. **The first aptamer to enter clinical trials for cancer therapy is an aptamer that binds nucleolin, a protein that is expressed in the nuclei of all cells but is over-expressed in the cytoplasm and on the plasma membrane of cancer cells relative to normal cells [30]. AS1411 is a 26-mer G-rich DNA aptamer against nucleolin that has direct anti-proliferative effects at relatively high (micromolar) doses but is internalized efficiently even at low (nanomolar) doses [31]. Phase II trials of AS1411 are currently underway in acute myeloid leukemia (NCT00512083) and renal cell carcinoma (NCT00740441). One of the key issues that currently limits the applicability of aptamers as cancer therapeutic agents is drug cost for repeated systemic delivery. This is one reason why we have focused this resubmission on the exploitation of aptamers for delivery of cytotoxic cargo, an approach that has the potential to deliver "more bang for the buck".**

Aptamers for cell type-specific delivery

Recent work by the Mentor's group and others has demonstrated that aptamers may have functions other than just blocking receptor-ligand interactions. For instance, an RNA aptamer against prostate specific membrane antigen (PSMA), a surface protein specifically expressed in prostate cancer, is known to be internalized and has been used to selectively deliver cytotoxins [32] and chemotherapeutic agents [33][[34] into PSMA-expressing cells but not into cells that do not express PSMA. Using a similar strategy, [redacted] et al. from the Mentor's group linked siRNAs against a critical gene for cell survival, *polo like kinase-1* (*Plk1*), to the 3' end of a PSMA aptamer in order to target the siRNAs to PSMA-expressing cells. *In vitro* experiments demonstrated specific uptake of the chimera constructs into PSMA-expressing cells with silencing of *plk1* mRNA and resultant induction of apoptosis. Intratumoral injections of the PSMA aptamer–plk1 siRNA chimera into prostate cancer xenografts resulted in tumor regression, establishing proof of concept for this strategy in preclinical models. **An optimized version of this chimera was modified with PEG to enhance plasma stability and induced tumor regression in prostate cancer xenografts when delivered systemically [35].** This novel approach for cell type-specific delivery has potentially broad applications. PSMA is internalized via endocytosis, suggesting that this approach may be adapted to many other proteins that are internalized, including surface tyrosine kinase receptors such as Epidermal Growth Factor Receptor (EGFR) [36] and nuclear proteins that shuttle to and from the surface such as nucleolin [30]. **We will extend this work by comparing the effectiveness of aptamers that are internalized by a variety of mechanisms, since successful RNA interference requires not only internalization but also proper localization and processing of the construct by Dicer.** Furthermore, the use of RNA aptamers for cell type-specific delivery is an approach that may be more globally applied to different types of therapeutic cargo, such as quantum dots [37], radioisotopes [38], and photosensitizing agents [39]. In Aim #2, we propose the delivery of a gemcitabine polymer that is both novel and very relevant to pancreatic cancer.

Aptamers as tools for target identification

The versatility of these agents and their selection process have led to the development of techniques for selection against more complex targets, such as whole cancer cells and tumors rather than purified proteins in solution. One goal of such strategies is to generate oligonucleotides that specifically bind the target cell/tissue over normal tissues and which therefore may be useful as diagnostic or therapeutic agents. The most exciting aspect of selection against complex targets is that the aptamer chooses the targets. With this strategy, aptamer targets are not limited to specific proteins and might include specific post-translational modifications, combinations of proteins, or even non-protein targets that are unique to cancer cells. Another goal of complex selections is therefore the potential identification of novel or unexpected targets that may improve our understanding of cancer biology and treatment. DNA aptamers have been generated against several leukemia and solid tumor cell lines using *in vitro* selection against whole cells in culture [40-43]. In at least one case, the aptamer binds a cell surface protein (protein tyrosine kinase-7) that mediates its internalization and allows delivery of doxorubicin specifically to target leukemia cells [44, 45]. In Aim #1, we propose a modification to the cell-based selection strategy that will bias the selection towards aptamers that are internalized. The

Mentor's group has taken this approach further and used an *in vivo* selection technique to identify an RNA aptamer that targets colon cancer [46]. Tumors were established in the livers of mice by injection of the murine colon carcinoma cell line CT26. The nuclease-resistant RNA library was injected systemically into tumor-bearing mice prior to harvesting the tumor. Bound RNA molecules were extracted and amplified by reverse transcription-polymerase chain reaction (RT-PCR). An enriched RNA pool was then *in vitro* transcribed, and the process was repeated, similar to the *in vitro* SELEX technique. After 14 rounds of selection, an aptamer was identified, the target of which was determined by affinity purification to be p68, an RNA helicase that is over-expressed in colon cancer and which is believed to shuttle between the cell surface and nucleus [47, 48]. When fluorescently-labelled p68 aptamer is injected systemically into tumor-bearing mice and the tumor is harvested, the aptamer can be visualized by fluorescence microscopy in the nucleus and cytoplasm of tumor cells but not surrounding normal tissue. *These studies support the feasibility of whole cell and in vivo selections and the potential for these techniques to identify aptamers against unexpected but relevant cancer targets.* In addition to a similar strategy using pancreatic cancer xenografts, we propose an *in vivo* selection strategy in a genetically engineered mouse (GEM) model of pancreatic cancer that will provide an opportunity to generate aptamers that target the unique pancreatic cancer microenviroment.

This project is innovative in that it builds on a promising but immature body of work on aptamer-mediated delivery. We propose creative strategies to identify new aptamers that are specifically bound and internalized by pancreatic cancer cells as well as novel cargoes that are very relevant to pancreatic cancer.

Approach

The ideal target for aptamer-mediated delivery is one that is highly expressed on the surface of all pancreatic cancers, efficiently internalized, and not expressed on the surface of normal cells. Some of the aptamers described in the previous submission may be useful for other applications but are not included in this resubmission because they target soluble ligands (angiopoietin-2) or are not efficiently internalized (prostate stem cell antigen) and thus are not good targets for aptamer-mediated delivery. One aptamer described in the previous submission (EGFR) as well as a DNA aptamer that binds the nuclear transport protein nucleolin are included in this resubmission as tools to optimize to constructs for delivery in Aim #2 while we identify "more ideal" targets in Aim #1.

Specific Aim #1: To utilize selection strategies against complex targets to identify pancreatic cancer targets. This aim includes *in vitro* selection against whole pancreatic cancer cells as well as *in vivo* selection against pancreatic cancer xenografts and genetically engineered mouse models of pancreatic cancer. We will characterize selected aptamers by identifying their specific protein and cellular targets. This aim therefore has the potential to simultaneously identify novel targets and the agents to deliver to them.

Hypothesis #1: "Positive/negative" selection strategies utilizing whole, live pancreatic cancer cells and non cancer cell lines will yield aptamers that specifically bind pancreatic cancer cells.
Whole cell selection strategies have been utilized to target specific surface receptor targets using "positive/negative" selection strategies in which RNAs that bind cells that express the target receptor are recovered and amplified, and RNAs that bind cells that do not express the target receptor are discarded [49]. Selections by the Tan laboratory against whole, live cancer cells (including leukemia, hepatocellular, and small cell lung carcinoma) have identified DNA aptamers that specifically bind the target cancer cells [50-52]. In at least one case, the aptamer binds a cell surface protein (protein tyrosine kinase-7) that mediates its internalization and allows delivery of doxorubicin specifically to target leukemia cells [44, 45]. We propose a similar strategy of complex selection against pancreatic cancer cells that will allow the aptamer to identify pancreatic cancer-specific target(s). Briefly, a 2'-fluoro-modified RNA library with a 40 nucleotide random region ($>10^{14}$ potential sequences) will be generated by *in vitro* transcription as previously described [53] We will use a modified SELEX protocol. Since there are many more non-cancer-specific proteins on the surface of a cancer cell than there are cancer-specific proteins, negative selection against a non-cancerous cell line is important. **We will incorporate a "negative" selection against the non-cancerous, immortalized pancreatic ductal cell line HPDE (generous gift of Ming Tsao, Toronto [54]) to subtract RNAs that bind cell surface proteins that are not cancer-specific. The RNA library will be incubated with cultured HPDE cells. RNAs that bind HPDE cells will be discarded. RNAs that do not bind HPDE cells will then be incubated with one of several pancreatic cancer cell lines that we have established from resected**

primary tumors. These "low passage" cell lines theoretically are more similar to pancreatic cancer cells *in vivo* than commercially available "high passage" cell lines that can undergo genotypic and phenotypic change over time. The cells (and any bound or internalized RNAs) are harvested, and the RNAs are isolated, reverse transcribed, and amplified to generate a pool of RNAs enriched for sequences that bind the cells. The process will be repeated until there is an appreciable increase in binding affinity of the RNA pool for the cells, as measured by a quantitative double-filter binding assay [55] modified for cells. The RNA pool is then reverse transcribed, cloned, and sequenced. In order to identify potentially novel or unexpected protein targets, we will drive the *in vitro* selection to a point at which there is still significant heterogeneity (i.e., several families of sequences) in order to hopefully identify not just one but a few high affinity "winning" aptamers. The binding affinity and specificity of individual RNAs (potential "aptamers") for cancer versus non-cancer cell lines will then be further evaluated by flow cytometry. Selection against complex targets typically requires more rounds (10 – 20 rounds) to reach completion than does *in vitro* selection against purified targets (often fewer than 10 rounds). We will consider the selection successful if at least one aptamer is identified that binds cancer cells at least 100-fold better than HPDE cells by flow cytometry.

Although this approach has the potential to identify aptamers that are internalized by cells [56], we will attempt to modify our cell-based selection protocol to increase the likelihood of isolating RNAs that are internalized by cells. The selection approach described above is not able to distinguish between RNA molecules bound to the outside of tumor cells and those that have been internalized into tumor cells. The Ellington laboratory has described an assay in which cells are treated with a cocktail of harsh RNases (Riboshredder®, Epicentre) to degrade the RNA molecules that are bound to the outside of cells, while RNAs that have been internalized are spared [57]. We have reproduced this assay using a nuclease-resistant EGFR aptamer (E07) generated by the [redacted] on the high EGFR-expressing pancreatic cancer cell line Panc-1 (Figure 1). The EGFR aptamer was *in vitro* transcribed with a 24-nucleotide extension at the 3' end and labeled by annealing a complementary "wing" oligonucleotide conjugated to a fluorophore. At 4°, internalization is inhibited, and all RNAs (even 2'-fluoro-modified) are degraded in the presence of such high concentrations of RNases. At 37°, approximately 15% of the EGFR aptamer is not degraded in the presence of RNases, suggesting internalization ((pink curve – blue curve)/pink curve). A scrambled mutant control aptamer is almost completely degraded, suggesting a very small amount of nonspecific internalization. We will modify our pancreatic cancer cell selection protocol by treating cells with a similar cocktail of RNases prior to isolating RNA from the cells. The selection will otherwise be monitored, as described above, and individual RNAs will be evaluated for binding and internalization by flow cytometry, as described above. We do not know how much aptamer needs to be internalized for effective delivery, and this likely depends on both the abundance of the target and the cargo. Since the EGFR aptamer has been used for successful delivery of gold nanoparticles [57], we will consider 15% internalization to be a *minimum* for evaluation of an aptamer as a delivery agent.

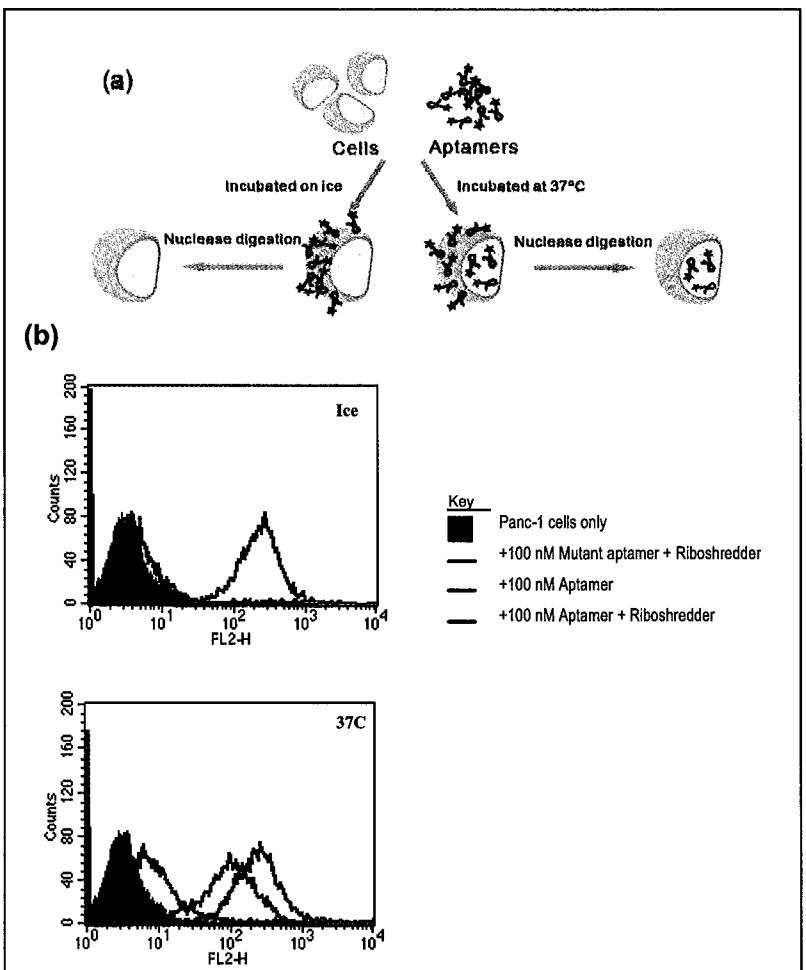

Figure 1. (a) Schematic of internalization assay [1]. (b) Flow cytometry demonstrating (top) aptamer degradation at 4° when internalization is inhibited and aptamer internalization at 37° in EGFR-expressing pancreatic cancer cells.

Aptamers that demonstrate specific binding (with or without internalization) for pancreatic cancer cells over non-cancer cells will be further evaluated by electrophoretic mobility shift ("gel shift") analyses. Radiolabelled RNAs are incubated with cell lysate, and specific binding of the RNA to a protein target is suggested by a single upward band shift. Specificity is confirmed by the ability of the same unlabelled RNA to compete for binding. Cross-competition between individual RNAs indicates binding to common protein targets. RNAs that demonstrate specific binding to unique protein targets will be further characterized. In order to isolate and identify the protein(s) that bind the aptamer(s) that we generate, we will employ affinity purification techniques followed by mass spectrometric analyses, as have been successfully used by the Mentor's group [46]. Briefly, biotinylated aptamers(s) will be immobilized on streptavidin magnetic beads and incubated with cell lysate. Following incubation, the beads will be washed and the bound proteins will be eluted and resolved using SDS/polyacrylamide gel electrophoresis. The Coomassie stained polypeptide band(s) will be excised and will be analyzed by mass spectrometry to identify the binding protein(s).

Aptamers that demonstrate specific internalization into pancreatic cancer cells will be further evaluated in Aim #2 for their ability to deliver therapeutic cargo. Aptamers that demonstrate specific binding but not internalization will be evaluated in future applications for either direct therapeutic effect on pancreatic cancer cell growth or as imaging/diagnostic agents.

Hypothesis #2: In vivo selection utilizing established pancreatic tumors in mouse models will yield aptamers that specifically bind pancreatic tumor tissue.

Although *in vitro* selection is much easier than *in vivo* selection, cell culture does not recapitulate the complex tumor microenvironment that exists *in vivo*. We will therefore perform *in vivo* selection in two different but complementary models. Subcutaneous pancreatic cancer xenografts are easy to establish and monitor but are limited in that the tumors that arise in immunocompromised mice may not completely reflect the complex interactions that exist between tumor and host *in vivo*. Genetically engineered mouse (GEM) models of pancreatic cancer are more difficult to establish and monitor but more fully recapitulate features of human pancreatic cancer [58]. By pursuing these three different approaches in parallel (cultured cells *in vitro*, xenografts *in vivo*, and GEM tumors *in vivo*), we increase our chances of identifying aptamers with different properties and against different targets and creating a "portfolio" of useful aptamers.

In vivo selection against pancreatic cancer xenografts

In order to optimize the *in vivo* selection technique, we have embarked on an *in vivo* selection using the commercially available human pancreatic cancer cell line BxPC3. Subcutaneous tumors are established in the flanks of athymic nude mice by direct injection of tumor cells in 50% Matrigel (BD). We generated a 2'-fluoro-modified starting RNA library, as described above for cell selection. When tumors measure at least 1 cm in diameter, 10 nanomoles of RNA in 200 µl of phosphate-buffered saline (PBS) are injected via tail vein. In contrast to Mi et al., we wait one hour (rather than 20 minutes) prior to harvesting the tumor in order to decrease the recovery of RNAs that bind contaminating blood within the tumor and increase the enrichment of RNAs that durably bind tumor cells and associated stroma. With this approach, the "negative" selection and "positive" selection occur simultaneously, as RNAs that bind non-tumor-specific targets are bound up by non-tumor tissues as the RNAs circulate. The tumor is snap frozen in liquid nitrogen, and total RNA is isolated using Trizol reagent (Invitrogen). Due to the large excess of natural tumor RNA, the isolated total RNA is treated with RNase to reduce natural RNA prior to reverse transcription of the injected 2'-fluoro-modified RNA using a primer complementary to the 3' flanking region. A new RNA library, enriched for sequences that target the tumor, is then regenerated by PCR amplification and *in vitro* transcription. The new library is then injected into a new tumor-bearing mouse, and the process is repeated.

After 4 rounds of selection, the binding affinity of the RNA pools to tumor lysate was compared to the binding affinity of the starting library using the standard "double-filter" method [55]. A modest increase in binding affinity was seen from the starting library to round 4 (data not shown). RNA pools should demonstrate increased binding affinity for proteins extracted from tumor with successive rounds of selection. However, since lysate preparation may disrupt secondary and tertiary protein structures that are also potential aptamer targets, this technique may underestimate the progress of the selection. Therefore, in parallel, we measured the amount of injected RNA recovered from harvested tumor by quantitative real-time PCR (qRT-PCR). The total amount of RNA recovered increased from 0.1 picomole in the first round (0.001% of 10 nanomoles injected) to 5 picomoles in the latest round (0.1% of 5 nanomoles injected). These data suggest that—even

after only 4 rounds of selection—there has been enrichment for RNA sequences that target tumor tissue. We will continue to monitor the progress of selection by both methods (binding affinity and qRT-PCR). As described above for cell selection, when enrichment reaches a plateau, the RNA pool will be reverse transcribed, cloned, and sequenced.

The most direct way to confirm that selected RNAs are specifically targeting pancreatic cancer tissue is to fluorescently label the RNA then inject it systemically into tumor-bearing mice. Fluorescent labeling can be accomplished in a variety of ways; we routinely use 5' biotinylation and complexation to streptavidin-fluorophore conjugates. Tumors will be harvested, and RNA localization to tumor tissue and—ideally—absence of localization to adjacent normal tissue will be assessed by fluorescence microscopy. RNAs that demonstrate specific localization to tumors *in vivo* will be further evaluated as described for *in vitro* cell selection above. More quantitative assessment of internalization will be obtained by flow cytometry using isolated pancreatic cancer cells in the presence of RNases, as described above. We believe that extending the length of time between injection and tumor harvest will promote the identification of aptamers that are internalized by cells. However, if we do not identify RNAs that are internalized by cells, we can use a similar strategy to the one described above for *in vitro* cell-based selection to bias the selection toward RNAs that are internalized. Specifically, tumors will be harvested, dissociated, and treated with harsh cocktails of RNases to remove RNAs that are bound to but not internalized by cells prior to isolating RNA from the cells.

In vivo selection in genetically engineered mouse models of pancreatic cancer
Activating mutations in the K-*ras* proto-oncogene are present in greater than 90% of human pancreatic cancers, and K-*ras* mutation is thought to be an initiating event in pancreatic cancer development. Mice that are engineered to endogenously express mutant K-*ras* in the presence of Cre recombinase (Lox-STOP-Lox(LSL)Kras^{G12D}) in combination with a pancreas-specific Cre recombinase transgene (PdxCre) develop a full range of pancreatic cancer precursor lesions (pancreatic intraepithelial neoplasia or PanIN) before eventually developing invasive pancreatic ductal adenocarcinoma [59]. Because of the late onset and incomplete penetrance of invasive pancreatic cancer in this model, subsequent models have incorporated additional mutations. Mice engineered to endogenously express the mouse ortholog of the most common p53 mutation in humans (LSL-TP53^{R172H}) in addition to mutant K-ras in the presence of pancreas-specific Cre recombinase develop invasive pancreatic cancer with 100% penetrance [60]. The cancers that develop have clinical, histopathological, and genomic features similar to human pancreatic cancers, including the dense desmoplastic stroma that is believed to contribute to contribute pancreatic cancer chemoresistance. The tumors demonstrate local invasion (biliary and small bowel obstruction) and metastases to lymph nodes, liver, and peritoneum. The predominant histology is ductal adenocarcinoma, and there is evidence of loss of heterozygosity at the wild-type P53 allele and a high degree of chromosomal instability. Recently, it has been shown that the tumors associated with this model demonstrate resistance to gemcitabine and are poorly vascularized and perfused, similar to pancreatic cancers in human patients [61].

LSL-Kras$^{G12D/+}$, *LSL-Trp53*$^{R172H/+}$, and *Pdx-1-Cre* mice have been obtained through the NCI's Mouse Models of Human Cancer Consortium. *LSL-Kras*$^{G12D/+}$;*LSL-Trp53*$^{R172H/+}$;*Pdx-1-Cre* ("triple mutant") mice will be bred as previously described, and genotyping confirmed by specific PCR amplification of tail DNA [14]. Triple mutant mice have a median survival of 5 months and 100% mortality by 12 months [60]. Disease burden becomes apparent in animals by 10 weeks of age. Primary tumors are palpable prior to development of widespread metastatic disease [62]. When primary tumors are palpable, we will inject 10 nanomoles of RNA library in 200 µl PBS via tail vein, similar to the xenograft selection described above. If tumors are not consistently palpable, we will use ultrasound to confirm the presence of primary tumor within the pancreas. We acknowledge the likelihood that metastatic disease will be present by the time the primary tumor is palpable. Since the molar amount of RNA that we are injecting is in vast excess of the amount of even an abundant protein target within the primary tumor, the presence of limited, concurrent metastatic disease should not significantly interfere with RNAs binding to the primary tumor. If, however, there is extensive metastatic disease by the time the primary tumor is palpable, we will use ultrasound to identify primary tumors at an earlier stage. One hour after injection, the mouse will be sacrificed and the primary tumor dissected from the surrounding structures. The selection will otherwise be performed and monitored as described for the xenograft selection above with recovered RNAs being amplified and the new enriched library being injected into new tumor-bearing mice.

Pitfalls/Alternatives: 1) It is conceivable that—in early rounds—we will not be able to recover a sufficient amount of RNA from the harvested tumors with the selection protocol as described. Hicke et al. have described the biodistribution of a 2'-fluoro-modified RNA aptamer against the extracellular matrix protein tenascin-C [63]. Accumulation of the aptamer within the tumor peaked at 10 minutes, and clearance of both the tenascin-C aptamer and a non-binding control aptamer from the blood was extremely rapid. Although biodistribution may vary with the aptamer target, these observations are useful estimates. Based on these observations of aptamer biodistribution, we can increase the absolute amount of RNA recovered by decreasing the time between injection and tumor harvest. The stringency of selection can then be increased in later rounds by increasing the time between injection and tumor harvest.
2) If only a single aptamer target is identified or if all selected aptamers bind the same cellular target, we may be able to manipulate the selection to target other components of the tumor using laser capture microdissection (LCM) [64]. LCM allows the procurement of specific cellular (or extracellular) components of the tumor mass from tissue sections up to 15 μm thick. Since the yield of RNA from microdissected tissue will obviously be much less than the yield of RNA from bulk tumor tissue, this process is not likely to be feasible during initial rounds of selection. However, after several rounds of selection against the whole tumor have enriched the pool for sequences that target tumor tissue, there should be sufficient RNA present in specific tumor components that it can be recovered by LCM and amplified for use in subsequent rounds.
3) By using a genetically engineered mouse model of pancreatic cancer, it is possible that an aptamer selected against murine protein target may not cross-react with its human homolog. If we do select an aptamer that is potentially useful but which does not cross-react with human tumors, it may be necessary to "redirect" the selection to the human homolog, using either purified protein or a xenograft model.

Specific Aim #2: To utilize aptamers that are internalized by pancreatic cancer cells for specific delivery of therapeutic cargo.

Aptamers that are internalized by pancreatic cancer cells will be tested for their ability to deliver therapeutic cargo. We will focus our efforts initially on the delivery of K-*ras* siRNAs and on the delivery of gemcitabine, the most effective chemotherapeutic agent for pancreatic cancer. All surface receptors cycle intracellularly to some extent as part of the continuous turnover of the plasma membrane. However, many receptors are actively internalized in response to ligand binding through one of several well-characterized processes. In the classic clathrin-mediated endocytosis, the receptor and ligand are transported via endosomes to lysosomes for receptor degradation and ligand release. In potocytosis, the receptor and ligand are transported by caveolae directly into the cytoplasm. The processes by which some receptors, such as nucleolin, are directed to the nucleus are not as well-characterized.

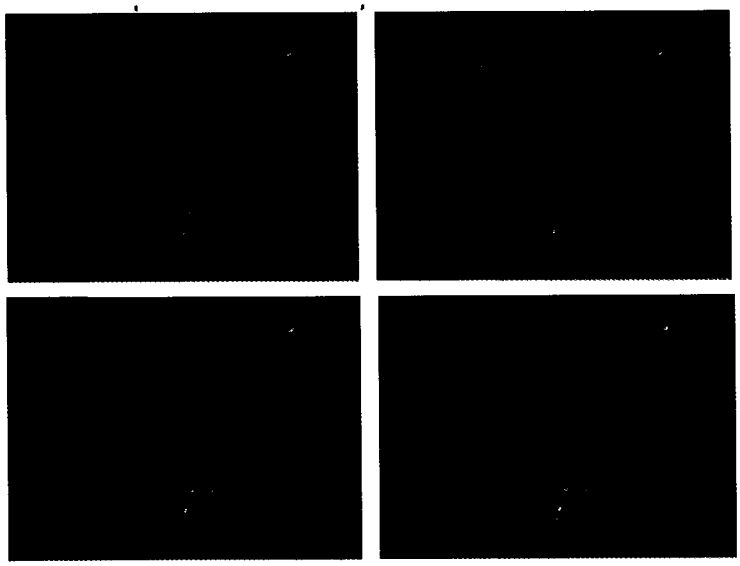

Figure 2. EGFR aptamer (green), nucleolin aptamer (red), nuclei (blue)

Initially, we will utilize extant aptamers to EGFR and nucleolin as tools to optimize constructs for delivery. EGFR is a tyrosine kinase receptor that is expressed on the cell surface and is internalized by receptor-mediated endocytosis. EGFR is over-expressed in at least 60% [8, 65] of pancreatic cancers but—unlike in brain and lung cancer—is highly conserved in pancreatic cancer [66], making it a good candidate for aptamer-mediated therapy of pancreatic cancer. EGFR, however, is expressed at lower levels in normal tissue, which is why even specific inhibitors of EGFR (such as the monoclonal antibodies cetuximab and panitumumab) may have side effects on normal tissue.
As shown above (Figure 1), we have preliminary data demonstrating that a nuclease-resistant RNA aptamer against EGFR (E07) is internalized by pancreatic cancer cells. Nucleolin is a protein that is expressed in the nuclei of all cells but is over-expressed in the cytoplasm and on the plasma membrane of cancer cells relative to normal cells [30]. AS1411 is a 26-mer G-rich DNA aptamer that binds nucleolin and has direct anti-proliferative effects

at relatively high (micromolar) doses but is internalized efficiently even at low (nanomolar) doses [31]. **Similar to the EGFR aptamer, the nucleolin aptamer was chemically synthesized with a 24-nucleotide extension at the 3' end and labeled by annealing a complementary "wing" oligonucleotide conjugated to the red fluorophore Cy5. The EGFR and nucleolin aptamers were co-incubated with the pancreatic cancer cell line Panc-1 for one hour, washed, then fixed in paraformaldehyde with DAPI to counterstain nuclei. The cells were then imaged using fluorescence microscopy and differential interference contrast (DIC) microscopy to help visualize unstained cells. Figure 2 demonstrates staining of the cell surface and cytoplasm by the EGFR aptamer (green) and staining of cell cytoplasm and nucleoli by the nucleolin aptamer (red). Mutant control aptamers for each did not demonstrate significant staining (not shown). The EGFR and nucleolin aptamers are likely internalized by different mechanisms and thus provide an opportunity to correlate intracellular localization with effectiveness of delivery. *In the parallel Aim #1, we hope to identify aptamers that bind more specific targets than EGFR and nucleolin and that are capable of mediating intracellular delivery by a variety of mechanisms.* Aptamers that bind these targets can then be evaluated for their ability to deliver siRNAs, gemcitabine, and other therapeutic cargo.**

Hypothesis #1: Aptamers internalized by pancreatic cancer cells can be used for specific delivery of siRNAs. The approach used by the Mentor's group has been the con-synthesis of a "long" strand of RNA containing the aptamer and sense siRNA sequences, prepared by in vitro transcription from a DNA template, annealed to a "short" chemically synthesized antisense RNA. One of the advantages of this "all RNA" approach is that it eliminates the need for conjugation and is theoretically less immunogenic [27]. The binding affinity of the aptamer – siRNA construct for its target protein will be compared to the binding affinity of the aptamer alone to ensure that the addition of the siRNA does not affect binding. Adverse effects of attachment of the siRNA to the 3' end may be overcome by attaching the siRNA to the 5' end.

The efficacy of aptamer-mediated delivery requires not only internalization but also release of the cargo into the correct intracellular compartment for activity. Ultimately, the most important measure of aptamer:siRNA constructs will be their effects on target cells. **For functional testing of aptamer:siRNA constructs, we will use validated siRNAs against K-*ras*.** Most studies of RNAi in pancreatic cancer have targeted mutant K-*ras*, although siRNAs to conserved regions of the gene have also proven effective *in vitro* [67]. Point mutations in codon 12 constitute the vast majority of mutations in pancreatic cancer, but actual sequences vary among pancreatic cell lines and human tumors [66]. To effectively target all tumors with activating K-*ras* mutations, one would need to either sequence each tumor's K-ras gene and select the appropriate siRNA or utilize a cocktail of siRNAs encompassing all common mutations. Since, with our approach, tumor specificity is mediated by the aptamer, we will use siRNAs to conserved portions of the K-*ras* gene, which should allow the same siRNA sequence to be effective in all patients, even patients with wild-type K-*ras*. Aptamer:siRNA constructs will be tested for activity by treating pancreatic cancer cell lines with increasing concentrations of the aptamer:siRNA construct without transfection reagent. Aptamer alone, mutant (non-binding) aptamer:siRNA, and aptamer:nonsense control siRNA constructs will be used as negative controls. Duplex siRNA in the presence of lipid-based transfection reagent will be used as a positive control. Total RNA and cell lysates will be collected, and K-*ras* knockdown will be quantified at the mRNA and protein levels by qRT-PCR and Western blotting, respectively. Apoptosis and proliferation will be measured using fluorimetric assays. We will assess for off-target immune-mediated effects by measuring interferon release by ELISA [26]. We will consider this proof-of-concept experiment successful if there is evidence of gene expression knockdown in response to treatment with the aptamer:siRNA construct but not the negative controls. If the K-ras knockdown does not have the expected effects on cell proliferation or apoptosis, we will substitute siRNAs to other validated pancreatic cancer targets such as SRC [68].

Pitfalls/Alternatives: Successful RNA interference requires not only internalization but also proper localization (i.e., release from the endosome) and processing of the construct by Dicer. Although guidelines have been compiled that assist in the design of siRNAs to a given target [69], it is still an empirical process, and the rules that govern efficient siRNA-directed silencing are still being determined. For receptors that target the nucleus, short hairpin RNAs (shRNAs) may be more effective cargo than siRNAs as they may be able to be channeled into the cell's normal microRNA pathways [70, 71]. Dassie et al. have already demonstrated that even small changes to an aptamer:siRNA construct can have significant effects on the efficiency of processing and knockdown [35]. RNA interference is a relatively new area of research for the Applicant and an area of

expertise for the Mentor. A significant amount of optimization may be required to produce constructs that induce efficient knockdown, and the Mentor's assistance will be particularly valuable in this aim.

Hypothesis #2: Aptamers internalized by pancreatic cancer cells can be used for specific delivery of gemcitabine polymers.

As a prodrug, gemcitabine (2',2'-difluoro, 2' deoxycytidine or dFdC) requires cellular uptake and intracellular phosphorylation. The rate-limiting step is conversion to gemcitabine monophosphate (dFdCMP) by deoxycytidine kinase (dCK). Ultimately, dFdCMP is then converted to the di- and triphosphate (dFdCDP and dFdCTP, respectively), which are the major active metabolites [72] dFdCTP is a DNA chain terminator [73], and dFdCDP inhibits ribonucleotide reductase [74], and these are thought to be the primary mechanisms for cytotoxicity. *In vitro*, all pancreatic cell lines are sensitive to gemcitabine...if you treat them with concentrations of gemcitabine that are not safely achievable in humans. *In vivo* efficacy is limited—at least, in part—by the inability to get enough of the active metabolite into the cells that need it.

Each step can potentially be performed enzymatically or chemically, and the ultimate method of production will be dictated both by efficiency and efficacy, which will be dependent on the intracellular location and rate of hydrolysis to monomers. However, we propose the following methodology. Gemcitabine (dFdC) is a pyrimidine nucleoside analog and is commercially available (Eli Lilly). **Our collaborators in the Shaw laboratory have already generated the precursor dFdCTP by chemical means from dFdC. dFdCTP is known to be incorporated by eukaryotic polymerases into DNA, and it is therefore predicted that dFdCTP will also be incorporated by a variety of other enzymes known to incorporate nucleotides. For example, mutant prokaryotic RNA polymerases (T7) are routinely used for the *in vitro* synthesis of RNA containing non-canonical 2'-modified pyrimidine nucleotides [75]. This strategy will require a DNA template and could therefore be used to produce very specific and potentially long chain lengths. We propose to anneal the gemcitabine polymer to a short 3' "wing" extension of the aptamer by encoding a complementary sequence at the end of the polymer DNA template, similar to the constructs used for labeling aptamers for imaging and flow cytometry described above.** Once the construct is within the cell, the acidic environment of the endosome should promote hydrolysis of the polymer. Importantly, the drug should be released in its monophosphate form, bypassing the rate-limiting step of conversion by dCK.

The aptamer:gemcitabine constructs will first be evaluated using *in vitro* proliferation and apoptosis assays. We will utilize a pancreatic cancer cell line that and is relatively resistant to gemcitabine (e.g., Panc-1) to screen various aptamer:gemcitabine constructs for activity. Cell lines will be treated with aptamer:gemcitabine constructs, mutant (non-binding) aptamer:gemcitabine constructs, gemcitabine alone, or aptamer alone at concentrations ranging from 0 to 50 μM. Since the most common dose-limiting toxicity of gemcitabine in pancreatic cancer patients is hematologic toxicity [6], we will also measure gemcitabine levels in human peripheral blood mononuclear cells (PBMCs) that have been treated with aptamer:gemcitabine constructs using anion exchange LC/MS as previously described [76]. By comparing gemcitabine levels in PBMCs that have been treated with aptamer:gemcitabine constructs to PBMCs that have been treated with gemcitabine alone, we hope to directly confirm that not only is the aptamer able to increase delivery to pancreatic cancer cells but that it reduces uptake in normal cells and thereby decreases toxicity to normal cells.

Pitfall/Alternative: If enzymatic polymerization is unsuccessful, chemical polymerization of nucleotides via standard phosphoramidite technology is always an option and is the likely method we would use for eventual larger scale applications. However, there are several limitations to this approach for initial proof-of-concept experiments. For one, the current limit of efficient oligonucleotide chemical synthesis is on the order of 100 nucleotides, whereas nucleic acid sequences of greater than 1000 nucleotides can be "synthesized" enzymatically *in vitro* using polymerases. We really do not know what the ideal gemcitabine:aptamer ratio will be but anticipate that a higher ratio will be more effective. In addition, although chemical synthesis is more cost effective for large scale synthesis, it is much less cost effective for small scale synthesis, and cost would limit how many different constructs we could synthesize and compare for efficacy in our *in vitro* assays.

Hypothesis #3: Aptamer constructs that inhibit pancreatic cancer growth in vitro will inhibit pancreatic cancer growth in vivo.

Although the EGFR and nucleolin aptamers are good candidates for aptamer-mediated delivery, we hope to identify in Aim #1 additional aptamers that are more specific for pancreatic cancer cells and which are internalized by a variety of mechanisms. New aptamers will be substituted into the aptamer:siRNA and

aptamer:gemcitabine constructs optimized in Hypotheses #1 and #2, respectively. Aptamer constructs that demonstrate inhibitory effects on pancreatic cell growth *in vitro* will be evaluated *in vivo*. If the EGFR or nucleolin aptamer is as effective or more effective than the new aptamers *in vitro*, we will proceed to evaluate the extant aptamer(s) *in vivo*. In general, we will initially use a subcutaneous xenograft model to evaluate for tumor growth inhibition. Briefly, xenografts will be established in the flanks of athymic nude mice by injection of a tumorigenic human pancreatic cell line (e.g., BxPC3). When tumors are easily palpable, groups of 10 mice will be randomized to treatment with the aptamer construct or appropriate controls. For initial proof-of-concept experiments, we will use direct intra-tumoral injection, which requires much less material than systemic delivery. For aptamer:siRNA constructs, appropriate controls include aptamer:control siRNA constructs and mutant aptamer:siRNA constructs. For aptamer:gemcitabine constructs, appropriate controls include mutant aptamer:gemcitabine constructs and aptamer alone. Relative tumor growth inhibition at four weeks will be calculated as the average volume of the treated tumors divided by the average volume of the control tumors. Tumor will be collected at the time of sacrifice for assessment of treatment effect.

Future Plans

Aptamer constructs that inhibit growth in intra-tumoral injection models will be further optimized for *in vivo* applications by truncation and—depending on the size of the construct—PEGylation to enhance circulating half-life. Constructs can then be delivered systemically in animal models either by tail vein injection or continuously via implantable osmotic pumps (Alzet). In addition to tumor effects, daily weights and behavior of the mice will be monitored, and normal tissue and blood will be collected at the time of sacrifice for assessment of toxicity. Aptamer constructs that demonstrate inhibitory effects *in vivo* will be subjected to further IND-enabling studies, such as pharmacokinetic/pharmacodynamic characterization and toxicology. Our medium-term goals include the translation of at least one of these aptamer constructs into a phase I trial for patients with advanced pancreatic cancer.