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Pancreatic Cancer: Novel Therapy, Research Tools, and Educational Outreach

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Pancreatic Cancer: Novel Therapy, Research Tools, and Educational Outreach

By

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Pancreatic Cancer: Novel Therapy, Research Tools, and Educational Outreach

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Supervisor: Michael A. Hollingsworth, Ph.D.

Abstract: Since the 1980's, legislatures have invested immense effort into strategies to capitalize on the United States' global lead in basic and translational research. This is largely motivated by the discrepancy the US faces in converting its world leading research discoveries and innovations into commercial products, relative to other countries. This truncated productivity leaves the US out of substantial GDP growth and reduces public access to new innovations. Proponents of government and university owned patents reason that enhanced public private partnerships narrow the gap between the technology transfer of basic research discoveries and accessible commercial products.

With these concepts of technology transfer in mind, we set out to develop translationally relevant technologies to improve PDAC patient outcomes. First, we then partnered with pharmaceutical scientists and medicinal chemists to evaluate novel small molecule inhibitors and combined therapeutic approaches to treating PDAC. Second, we engineered a research tool, our Abdominal Imaging Window (AIW), which facilitates in vivo longitudinal deep tissue microscopy of pancreatic tumors and microenvironment throughout disease progression. Then, we collaborated with experts in the field of fluorescent imaging to develop tumor specific contrast agents that could be used in real time intraoperatively to improve surgical resection outcomes. Lastly, we investigated tools for strategic Science, Technology, Engineering, and Mathematics (STEM) outreach programing for not only recruiting future scientists, but for building a community of trust and understanding of scientific practices in the next generation.

In our investigation of novel combination therapeutics, we found that inhibition of CDK5 with small molecule inhibitor CP had the potential to reduce orthotopically implanted murine pancreatic adenocarcinomas of clinically relevant size to radiographically undetectable when used in combination with gemcitabine. These previously tumor bearing mice remained in remission and showed durable treatment free response of up to 40 days. Given this profound response, we pursued a multifaceted investigation into the effects of CDK5 inhibition on the tumor microenvironment, including the effects on perineural invasion and tumor associated neural migration.

To do so, we had to first engineer an Abdominal Imaging Window which would allow us to perform longitudinal intravital microscopy of tumor progression and neural invasion via multiphoton microscopy. These well tolerated AIWs provided macro and microscopic detail of PDAC progression longitudinally, and brought to the market a novel mechanism for performing upright microscopy in vivo.

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TABLE OF CONTENTS

List of Figures

Abbreviations

AKT/PKB Protein kinase B

BMI Body mass index

BRCA2 Breast cancer type 2 susceptibility protein

BSA Bovine serum albumin

Cdk5 Cyclin-dependent kinase 5

CLARITY Hydrogel-tissue fusion and electrophoretic-clearing technology

cm Centimeters

CRISPR Clustered regularly-interspersed short palindromic repeats

CT Computed tomography

ddH2O Double-distilled water

DFS Disease-free survival

DNA Deoxyribonucleic acid

DPC4 Deleted in pancreatic cancer locus 4; (SMAD4)

DRG Dorsal root ganglion

ECM Extracellular matrix

EGF Epidermal growth factor

ELISA Enzyme-linked immunosorbent assay

EUS Endoscopic ultrasound

FBG Fasting blood glucose

FBXO32 F-box protein 32

FDG-PET Fluorodeoxyglucose-positron emission tomography

FGF2 Fibroblast growth factor 2

FGFR Fibroblast growth factor receptor

GFP Green fluorescent protein

GFRα-1, -3 GDNF family receptor alpha 1, 3

GLUT1, 4 Glucose transporter 1, 4

GPCR G-protein coupled receptor

IB Immunoblot

ICAM-1 Intercellular adhesion molecule 1

IGF-1, -2 Insulin-like growth factor 1, 2

IL-1α, β Interleukin 1 alpha, beta

KPC LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre transgenic mouse model of spontaneous PDAC

KPCT KPC mice crossed with ROSA-LSL-tdTomato reporter strain B6.CgGt(ROSA)26Sortm9(CAG-tdTomato)Hze/J

LN Lymph node

LVI Lymphatic vessel invasion

LYVE-1 Lymphatic vessel endothelial hyaluronan receptor 1

MAFbx Muscle atrophy F-box; (Same as Atrogin-1 and FBXO32)

MAPK Mitogen-activated protein kinase

mg Milligrams

MHC Major histocompatibility complex

mL Milliliters

mm Millimeters

MMP-2, -9, -10 Matrix metalloproteinase 2, 9, 10

mMs Monoclonal mouse antibody

MRI Magnetic resonance imaging

mRNA Messenger RNA

NFDM Non-fat dehydrated milk

NFκB Nuclear factor kappa B

ng Nanograms

NGF Nerve growth factor

Notch1 Neurogenic locus notch homolog protein 1

p Phosphorylated

p16 (CDKN2A) Cyclin-dependent kinase inhibitor 2A

p53 (TP53) Cellular tumor protein p53

PanIN Pancreatic intraepithelial neoplasia

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

PD Pancreat(ic)oduodenectomy

PDAC Pancreatic ductal adenocarcinoma

PFA Paraformaldehyde

pg Picograms

PNI Perineural invasion

qRT-PCR Quantitative real-time reverse transcription polymerase chain reaction

RAP Rapid autopsy program for pancreatic cancer

RIPA Radioimmunoprecipitation assay buffer

RNA Ribonucleic acid

S2013

SDS Sodium dodecyl sulfate

TBP TATA box binding protein

TBS Tris-buffered saline

TGF-β Transforming growth factor beta

TNF-α Tumor necrosis factor alpha

TR Texas red

VEGF-A, -C, -D Vascular endothelial growth factor A, C, D

VEGFR-1, -2, -3 Vascular endothelial growth factor receptor 1, 2, 3

µg Micrograms

µL Microliters

µm Micrometers

Introduction

Pancreatic Ductal Adenocarcinoma

Progression statistics

Pancreatic Ductal Adenocarcinoma (PDAC) is currently the fourth leading cause of cancer associated death and is predicted to become the second most fatal cancer diagnosis by 2030 (Polireddy, 2016). While advancements in diagnostics and treatment have significantly improved patient outcomes and survival time for many cancer types, decades of research into PDAC treatment options have advanced the 5 year survival time to a dismal 8% (Rahib, 2014, Siegel 2018). Despite making up only 3% of new cases, PDAC patient deaths represent 8% of all cancer associated deaths for both men and women (Siegel, 2021).

One of the greatest struggles of treating PDAC is late stage of detection. However, patient outcomes remain poor even for the estimated 15% of cases that present while still resectable (Winter, 2012). And while perioperative mortality has decreased as a result of improved surgical treatments and earlier detection, survival rates remain poor (Seufferlein 2019).

PDAC associated pain

Severe abdominal and/or lower thoracic pain is a critical presenting symptom in 60% of PDAC patient diagnoses, and a nearly inevitable disease phenotype in almost all patients (Lakatos, 2016). Other symptoms may be more highly conserved across patients and include weight loss, fatigue, and nonspecific gastrointestinal problems which can be vague and perceived as non-emergent (Hawes. 2000). Effective pain control is often unachievable and lacks standardization despite pain's well-established predictive indicator of survival outcomes. A 2018 review of PDAC-associated pain indicates that the multifaceted causes of PDAC pain require equally multitargeted therapies not limited to pharmaceuticals including opioids, interventions such as endoscopy, endosonography, surgery, neurolysis, neuromodulation, radiotherapy, psychotherapy and holistic nursing and supportive care practices (Drewers, 2018).

These multifaceted causes of PDAC associated pain result from both nociceptive and neuropathic processes, causing patients to often experience both visceral pain and, when the brain is unable to differentiate the source of the pain signaling, referred somatic pain (Bogduk, 1987). Typical tumor physiology creates a highly stimulatory microenvironment for sensory nerves via chronic inflammation, tissue necrosis, aberrations in pH and secretory molecules. All of these factors contribute, in part, to nociceptive signaling. Neuropathic sensitization occurs simultaneously in response to external pressure from the burdensome accumulation of desmoplastic stroma and immune cell infiltration, and internal pressure from tumor cell invasion known as perineural invasion. Additionally, tumor hijacking of developmental neuro-signaling cascades and inflammatory signaling drive changes in innervation density and fiber structure, further exacerbating nociceptive signaling.

Studies investigating the correlative density of sympathetic and cholinergic nerves, which are depleted in PDAC and chronic pancreatitis (which also has a prevalent and sever associated pain phenotype) correlate with higher reported incidences of patient reported pain (Ceyhan, 2009). These changes patients' neural density and hypertrophy mirror in vitro finding when myenteric plexus and dorsal root ganglia cells are co-cultured with PDAC or chronic pancreatitis cell line conditioned supernatants which resulted in increased neural density and hypertrophy (Demir, 2010). When compared to normal pancreas pathology, neuroendocrine tumors, papillary mucinous neoplasia, and cystadenomas, PDAC and chronic pancreatitis are the only known pancreatic conditions with increased nerve density (Ceyhan, 2009).

Despite the overwhelming number of physiological factors that need to be mitigated to effectively manage patient pain, special consideration must also be given to secondary causes of PDAC patient pain independent of tumor associated pain. Causes of treatment associated pain include trauma from surgery, endoscopy, pharmaceutical and oncological therapies including radiation (Hidalgo, 2015).

Even opioids can cause pain phenotypes which are not easily discriminated from PDAC pain via opioid induced hyperalgesia.

Post-operative complications can include infection, anastomotic leaks, intra-abdominal collections, acute pancreatitis, perforation and adhesions (Kapoor, 2016). Additionally, partial pancreatic resection can cause exocrine pancreatic insufficiency, and bile acid malabsorption, all of which can cause confounding abdominal pain (Drewers, 2017). In metastatic and unresectable patients obstruction of the bile duct, duodenum, and gastric outlet can exacerbate abdominal pain (Laquente, 2017).

Perineural Invasion

Perineural invasion provides an alternative tumor cell dissemination route to vascular and lymphatic escape. Tumor cells have been identified by multiple studies to invade into all three spaces of the nerve sheath (epineural, perineural, and endoneurial) and is not limited to the perineural space (Demir, 2010 Liebig, 2009). Although it has not been explicitly detailed what drives tumor cells to invade nerves, it is clear that nervous tissues provide a highly hospitable environment (Bockman, 1994). It has also been shown that both the nervous and tumor cells are highly responsive to signaling molecules in the microenvironment including neurotrophines, chemokines, cell-surface ligands and receptors, and molecule signals generated during pain signaling (Bapat, 2011).

Regardless of what drives the invasion, there is glaring evidence that perineural invasive events worsen the disease state and complicate treatment. Neuronal growth factor, which has been positively associated with PNI, is also associated with metastasis to the lymphatics, and positive surgical resection margins. Additionally, the degree of nerve infiltration correlates with worsened prognostic factors (Ma, 2008). One 2012 study found PNI to be a consistent predictor of abdominal recurrence after neoadjuvant gemcitabine therapy plus resection, and PNI was associated with reduced disease-free survival (Takahashi, 2012). And while tumor stage is not correlated with perineural invasion, the

presence and extent of perineural invasion is a correlative predictor of disease progression and recurrence (Shimada, 2011).

PDAC pain management

Chemotherapy and radiation

Chemotherapy has been reasoned to reduce pain in unresectable and metastatic patients by reducing overall tumor burden and subsequent pressure and inflammation factors stimulating nociception. Patient data meta-analysis, which include indicators of pain in unresected patients, show a majority of patients self-report pain relief following chemotherapy treatment (Kristensen, 2016). Of note, gemcitabine treatment has been shown to reduce pain in PDAC patients with greater efficacy than 5FU, however is unchanged in gemcitabine combination therapies (Conroy, 2011). Alleviation of pain was also not found to be significantly different between gemcitabine therapy and FOLFIRINOX, however FOLFIRINOX did require more time for symptom relief to manifest compared to gemcitabine (Von Hoff, 2013). In the case of patients with metastatic disease affecting the liver or bone, radiotherapy has been shown to offer analgesic effects (Lanquente, 2017).

Pancreatic enzyme therapy

Although only studied and proven effective in patients suffering from exocrine pancreatic insufficiency, pancreatic enzyme therapy has been argued to have analgesic effects by normalizing digestion via cholecystokinin suppression (Lieb II, 2009).

Ablation

Given the neuroanatomy of the pancreas, the practice of celiac plexus neurolysis (CPN – the direct or bilateral injection of alcohol into the ganglia) has been clinically utilized for decades as a method of ablating the afferent pathways which facilitate pancreas to brain signaling (Arcidiacono,

2011). While CPN is effective at reducing pain with significantly lower doses of opioid analgesics, the highly variable duration of effect and risk of adverse surgical complication generally makes CPN a last resort for end stage patients only. CPN also only targets the visceral pain of the upper abdomen and is ineffective in cases of peritoneum or diaphragm metastatic involvement where somatic innervation has occurred. Additional routes of ablation have been clinically tried, including splanchnicectomy, sympathectomy, and rhizotomy, but are rarely used by clinicians.

Adjuvant analgesics

Adjuvants including antidepressants, anticonvulsants, and anxiolytics have been shown across different disease types of clinically diagnosed PNS or CNS lesions, including cancer, to improve analgesic effectiveness (Attal, 2008). Antidepressants, like those with serotonin-norepinephrine reuptake mechanisms, demonstrate promising analgesic effects through neuromodulation (Mika, 2013). Current clinical evidence suggests combination therapies commonly reduce side effect risks and can be used effectively at lower doses (Holbech, 2015).

Non opioid pharmaceutical analgesics

Before beginning even low dose opioid therapy, non-opioid analgesics are attempted as a first line option. This often includes first line analgesic therapy with NSAIDs to reduce systemic inflammation. Although non-steroidal anti-inflammatory drugs pose risk for upper-gastrointestinal and renal function, they can be used in combination with proton pump inhibitors in certain circumstances (Konijnenbelt-Peters, 2017).

Opioid analgesics

While highly effective at managing the most severe pain phenotypes, opioid analgesics come with an array of moderate to severe side effects and have many contraindications relevant to PDAC

treatments, including those for codeine, tramadol and morphine which can exacerbate renal and hepatic insufficiency in advanced stage patients (Dowell, 2016). Opioid induced hyperalgesia, bowel dysfunction, and narcotic bowel syndrome are amongst the most common side effects (Drewers, 2016). Often additional therapeutics are necessary to mitigate the side effects of opioid use, including laxatives or anti-cholinergics, somatostatin analogues, setrons for nausea, and GABA-agonists for neuropathic pain (Drewers, 2018).

Models and methodologies for animal cancer pain study

Much like the difficulties in generating animal models that recapitulate the diverse and complex genetic features of cancer itself, generating animal models that express the molecular, biochemical, and neurobiologic mechanisms of cancer pain, plus the methodologies to quantify this pain, are considerable. Any feature of the tumor or metastatic microenvironment that causes aberrant physical or chemical stimulus near a neuron contributes uniquely to pain signaling. For extra complexity, surgical, and therapeutic efforts contribute additional extra tumoral pain signaling.

Even in the case of cancer associated pain studies, hypersensitivity testing in the form of heat paw-withdrawal test and von Frey test remain standard in the absence of consensus on measures for chronic pain conditions in small animals (Jourdan, 2001; Lariviere, 2002). Numerous nocifensive behaviors, those correlated with increased nociceptive signaling, which more closely correspond to chronic pain conditions like cancer, include self-administration of analgesic, gait disturbances, aberrant grooming, guarding or haunching, hypolocomotion, weight loss, and ultrasonic vocalization among others (Blackburn-Munro, 2004). However, the literature is inconsistent, and each behavior is only represented with few publications of unrelated materials (Mogil, 2004).

Bone and nonbone cancer animal models

Given the debilitating pain associated with bone cancers and their moderately more less variable microenvrionemnts, several of the earliest animal models for pain were based on engineered femur and humerus models of sarcoma, namely osteosarcoma and fibrosarcoma (45, 57, 82, 97, 130, 148/21, 79, 176). Since then, murine pancreatic cancer, and rat squamous cell carcinoma and neuroma have been engineered and nociceptively profiled to have consistently reported features (Lindsay, 2005; Sevcik, 2006; Nagamine, 2006; Dorsi, 2008; Tyner, 2007).

Methodologies for assessing animal pain

Using a spontaneous murine model of pancreatic cancer wherein the first 127 amino acids of simian virus 40 large T antigen were under the promoter control of rat elastase-1 (ET mice), Sevcik et al were able to profile an inducible and reversible profile of conserved nociceptive behaviors throughout spontaneous tumor progression. Their methodology included mouse posturing and vocalization.

Briefly, animals were placed individually into an open arena and allowed to explore. Over the course of three minutes, animal posture was scored on a scale of zero to five based on the degree of haunchedness as well as other nocifensive behaviors such as ambulation, exploratory behavior, and others. The animals were scored for every second they spent in each criteria in order to generate a composite score for each animal. Recorded high frequency vocalizations and other physiological criteria were also monitored throughout disease progression. These methods are consistent across the literature and easily recapitulated in any vivarium (Sevcik, 2006).

CDK5 role in pain

Normal pain physiology

Neuropathic pain pathways stimulate immediate nociception in response to tissue injury and inflammation. After prolonged stimulation, secondary signaling systems are thought to enhance chronic

pain as an indicator of nerve injury. This secondary signaling system utilizes several activating kinases, including mitogen-activated protein kinase (MAPK), protein kinase C (PKC), phosphatidylinositol 3-kinase (PI3K), and cyclin-dependent kinase 5 (CDK5) (Ji, 2004; Ma, 2005; Zimmerman, 2001; Xu, 2007; Aley, 1999;). It is unsurprising that such an inflammatory disease like cancer and PDAC would conserve several of these signaling pathways pain signaling pathways.

Cyclin dependent kinase 5

Cyclin dependent kinase 5 (CDK5) is a member of the cyclin-dependent kinase (CDK) family which phosphorylates serine and threonine when activated by its regulatory partner p35, or p39 (Tang, 1995; Humber, 2000). CDK5 is a proline-directed kinase with substrate specificity mirroring that of CDK1 and CDK2 with highly conserved homology to cell cycle regulator CDK2 making selective inhibition of CDK5 pharmacologically challenging (Meyerson, 1992; Moreno, 1990). CDK5 has no known role in cell cycle regulation, and while CDK5 is expressed in all tissues, CDK5 activity is predominantly observed in nervous tissues (Tsai, 1993).

CDK5 deficiency is embryonically lethal in murine models and yields animals with substantial brain structure abnormalities throughout the cerebral cortex, hippocampus, cerebellum, and olfactory bulb (Gilmore, 1998). In addition to several other cellular processes, CDK5 activation has been reported to impact neuronal migration, axon guidance, cell adhesion, microtubule stability, and actin dynamics (Dhavan, 2001). Active CDK5 persists in many cancer types including over 95% of PDACs, and has been identified to be contribute to tumor proliferation, migration, angiogenesis, and chemotherapy resistance (Eggers, 2011; Pozo, 2016)

CDK5 in pain signaling

The role of activated CDK5 on pain is not fully understood. What has been shown is that tumor necrosis factor alpha (TNFa) increases activator p35 via transcriptional activation (Utreras, 2009). CDK5 is able to

phosphorylate transient receptor potential vanilloid 1 (TRPV1) (Pareek, 2007). CDK5 and p35 have also been shown to increase in response to inflammation at both transcript and protein levels. Inflammatory states also appear to drive hydrolysis of p35 to the much more active p25 through the activation of calpain (Pareek, 2006).

Effects of inhibition of CDK5 on pain signaling

CDK5 inhibitor Roscovitine has not only been shown to attenuate nocifensive behaviors in rats during tail-flick test when administered intrathecally, but when used in combination with morphine, significantly left shifted morphine dose-response curves in morphine desensitized mice (wang 2004).

Taken together, the biology of CDK5 (potential to inhibit peri-neural invasion, tumor growth, and pain signaling) led to the studies presented in Chapter 1 of this dissertation.

Add paragraphs on why or how you came to undertake the studies in Chapters 2-4.

CHAPTER ONE:

Pharmacological inhibition of CDK5 kinase activity decreases pancreatic tumor growth, invasion, and metastasis

Abstract

Prior research indicates CDK5, a developmental director of neural organization and reported exacerbator of neoplasm proliferation, migration and invasion, is overexpressed in more than 90% of pancreatic ductal adenocarcinomas. Given the early invasive and metastatic properties that contribute to the sever lethality of PDAC, we explored the effects of pharmacological inhibition of CDK5 using inhibitor CP668863 in vitro and in multiple preclinical murine models of PDAC. CDK5 inhibition nearly eliminated soft agar growth and colony formation of S2-013, FG, and HPAF PDAC cells at physiological concentrations. Combination CP668863 and nucleoside analog gemcitabine therapy was tested in human derived orthotopic models using athymic animals, as well as in syngeneic models using immune competent Bl6/C57 animals challenged with KPC derived cell lines. In both models we found combination therapy significantly reduced or eliminated tumor burden in animals previously radiographically confirmed to have tumors. Additionally, combination therapy significantly reduced invasion and metastasis, and increased survival time. Animals radiographically cleared of tumor burden experienced durable tumor-free progression out to nine weeks without either treatment. Post survival analyses show significantly reduced blood vessel formation and neural formation at the invasion front of treated animals. The results suggest that CP668863 has therapeutic potential when used in combination with gemcitabine for the treatment of pancreatic cancer.

Introduction

Pancreatic cancer is a highly proliferative, invasive, and lethal disease which has retained dismally low five-year survival rates over the last fifty years (American Cancer Society, 2008). With fewer than one in five pancreatic ductal adenocarcinomas (PDACs) diagnosed at a clinically resectable localized state, there is a clear need for molecular therapies which target proteins involved in PDAC pathogenesis (Pannala, 2009). Previously we have shown that cyclin dependent kinase 5 (CDK5) is aberrantly over expressed in more than 90% of PDACs (Eggers, 2011). Here we sought to investigate the in vitro and in vivo effects of PDAC progression when disrupted by pharmacological inhibition of CDK5.

Prior work with CDK5 inhibitor roscovitine demonstrated decreased migration and invasion of five PDAC cell lines by more than 37% in vitro (Deramaudt, 2005). Furthermore, dominant negative constructs of CDK5 decreased tumorigenesis in vitro and in vivo in multiple PDAC cell lines, likely via effects on RalA and RalB signaling (Feldmann, 2010). For these studies we investigated the potential for roscovitine analog CP668863 which has been previously characterized in vitro (Robb, 2017).

Perineural invasion (PNI), a hallmark of pancreatic ductal adenocarcinomas (PDAC), occurs when opportunistic tumor cells metastasize to distal tissues by penetrating the neural sheath of surrounding neurons, and leverage the neuronal network against the body. Neural infiltration promotes the severity of disease and contributes to acute and chronic pain signaling via stimulation of nociceptive (pain) receptors inside the nerves (Feldmann, 2006). Evidence strongly indicates that upregulation of neurotrophic signaling molecules in the tumor microenvironment closely correlated to the onset of cancer associated pain and encourages this invasion process (Saikkonen, 2008).

Amongst the molecules upregulated during perineural invasion is Cyclin Dependent Kinase 5 (CDK5), a kinase with highly tissue specific cofactors, which regulate developmental neural migration. In adults, CDK5 also modulates nociceptive response via phosphorylation of pain receptors (Cruz, 2003).

Though CDK5 is not expressed in the normal exocrine pancreas, CDK5 produced by pancreatic tumors may promote the tumor directed growth of neurons and commensurate perineural invasion (Huang, 2009). Through pharmacological inhibition of CDK5, we interrogated CDK5's role in disease progression and survival, invasion and metastasis, and formation of new vasculature and neuronal filaments, while evaluating compound efficacy in multiple murine preclinical drug trial models of pancreatic cancer.

Given the overlay between aberrant pathways in PDACs and the pathways regulated by CDK5, we sought to investigate the role of pharmacological inhibition of CDK5 in multiple preclinical models of pancreatic cancer. This investigation began in 2011 with Dr. John Eggers, MD, PhD's publication on the hyperactivity of CDK5 in human PDAC samples (Eggers, 2011). Dr. Eggers had also begun preliminary studies on the in vivo impacts of CDK5 inhibitor CP 668863 (Eggers, 2011b). The extreme hydrophobicity of CP 668863 made stable suspension and dosing difficult, but the preliminary in vivo data he was able to generate yielded promising trends in orthotopic animal models. In 2015, we resumed these pre-clinical trials with a newly formulated vehicle that reduced compound precipitation.

Materials and Methods

Human Pancreatic Cancer Cell Lines

S2013, MiaPaca-2, Capan-1, and T3M4 human pancreatic cancer cell lines were maintained in RPMI media with 7% Fetal Bovine Serum supplement and 100 units of penicillin and or 100 ug/ml of streptomycin. Cells were maintained at a constant 37o C humidifier with 5% CO2.

Mouse Pancreatic Cancer Cell Lines

KPC8060 and KPC8069 mouse pancreatic tumor cells were derived from the LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre (also known as KPC) murine tumors from the Hollingsworth laboratory. These spontaneously occurring PDAC tumors replicate the human PanIN lesion occurrence. To isolate tumor cells from KPC primary tumors, the primary tumor was resected from terminal stage animals, homogenized in a 2mg/ml collagenase A (Roche, Basel Switzerland) with iterative agitating 37^{o} C incubations. Cell lines were maintained under that same conditions as described previously for human pancreatic cancer cell lines. All orthotopic injections throughout these studies utilized the same KPC cell passage.

S2-013 Orthotopic Tumor Challenge

Female athymic mice between the ages of 4-6 weeks were weighed, anesthetized and monitored in accordance with UNMC IACUC approved protocol. Once unresponsive with a sterile surgical field, 1x106 S2-013 tumor cells were injected into the body of the pancreas and sutured closed. Once recovered, all animals were randomly assigned new housing and monitored twice daily for infection, surgical complication, and aberrant behavior. Starting seven days post orthotopic tumor challenge, all animals underwent weekly abdominal ultrasound screening for the presence of tumor-indicative masses. Once a detectable pancreatic mass greater than 3mm but no larger than 5mm

developed, animals were randomly enrolled into one of five treatment groups (saline control, vehicle control, standard of care/gemcitabine control, inhibitor monotherapy, and combination standard of care with inhibitor dual therapy). Because gemcitabine's vehicle is saline, the saline control serves as both the no treatment and standard of care vehicle control arm.

KPC 8069 Orthotopic Tumor Challenge

Immune competent C57BL/6 were also evaluated in our pre-clinical trial to evaluate any unexpected immunogenicity responses to our combination therapy. Female C57BL/6 mice between 4-6 weeks of age were weighed, anesthetized and monitored in accordance with IACUC approved protocol. Once unresponsive with a sterile surgical field, 50,000 KPC8069 tumor cells were injected into the body of the pancreas and sutured closed. Once recovered, all animals were randomly assigned new housing and monitored twice daily for infection, surgical complication, and aberrant behavior. Because of the numerous resource constraints of longitudinally ultrasound imaging fur bearing animals, all animals were randomized and enrolled into one of the same five treatment groups described previously 5 days post tumor challenge.

Dosing strategies

The CP668863 CDK5 inhibitor was dosed every other day for the duration of the trial at 8mg/kg via subcutaneously administration. Gemcitabine was administered every 4 days at 100mg/kg via intraperitoneal injection. Dual therapy recipients were placed on a schedule such that their gemcitabine and CP668863 injections would always occur at least 24 hours apart. For animals enrolled in longitudinal ultrasound imaging, dosages scheduled on the day of imaging were administered 12 hours post imaging to prevent confounding effects of the animal fasting pre-imaging and effects of isoflurane.

Progression Evaluation

C57/Bl6 animal tumor progression was monitored by palpation. Athymic animal tumor progression was monitored via abdominal ultrasound weekly or every other week. Animal weight, grooming, posture/haunching, and behavior were also monitored weekly.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 5 software suite. For evaluation of tumor volume, weight, and metastasis student t tests were used. Survival studies were determined to be significant or not via log-rank tests. Significance level was defined as a $p<0.05$ and error bars are representative of standard of deviation.

Results

Two 3, 5-disubstituted pyrazoles inhibit CDK5 kinase activity

Two 3, 5-disubstituted pyrazoles, CP668863 (Fig. 1), initially developed by Pfizer with the intent to inhibit CDK5 kinase activity for treatment of Alzheimer's Disease, showed significant inhibition of CDK5 kinase activity in vitro and significantly inhibited CDK5 kinase activity in cells (Table 1). CP668863 is selective towards CDK5 compared to other kinases, receptors, and transport channels; although modest inhibitory activity against CDK2 is detected at higher concentrations (Table 1).

CDK5 Inhibitor CP668863 decreases colony formation in FG, HPAF, S2-013 PDAC cell lines in a dose dependent manner

We evaluated the effect of CP668863 on soft agar colony formation for FG, HPAF, and S2-013 PDAC cell lines. The results indicated that CP668863 reduced colony formation in a dose dependent manner in all cell lines. Thus, 1.0 M CP668863 blocked nearly all CDK5 kinase activity and ablated colony formation.

Subcutaneous Injection of CP668863 achieves physiologically relevant serum concentrations and has greatest efficacy with lowest toxicity when administered Q 48 hour at 8mg/kg.

Non compartmental pharmacokinetics of single and daily replicate doses of CP668863 were evaluated in the plasma of immune competent Bl6/C57 mice (Envigo, Indianapolis, IN), for subcutaneous injection of 1, 8, and 16mg/kg. Blood samples were collected after single administration at 0, 15, 30, 60, 120, 240, and 480 hours post injection. Animals were reinjected with the same drug concentration daily for five days with blood collected two hours post injection each day. Mean plasma CP668863 levels are reported in Figure 1. Peak circulating CP668863 levels occurred 30 minutes post injection. On a once daily dosing schedule, 1mg/kg doses were not found to accumulate in circulation and 16mg/kg daily

injection was found to be fatally toxic when administered daily. Given the day to day accumulation and clearance of circulating CP668863 at an 8mg/kg dosage, a dosage range of 4mg/kg, 6mg/kg, and 8mg/kg was decided on to move forward into efficacy trials.

The therapeutic window of CP668863, alone and in combination with gemcitabine, was experimentally determined by treating C57/Bl6 mice orthotopically challenged with KPC derived murine PDAC tumor cells (6x10^5) which developed into solid tumors in vivo over three days. After their three day tumor initiating period, animals were randomly enrolled into one of ten treatment cohorts: gemcitabine vehicle, CP668863 vehicle, gemcitabine plus CP668863 vehicle, gemcitabine, 4mg/kg CP668863 with and without gemcitabine, 6mg/kg CP668863 gemcitabine with and without gemcitabine, and 8mg/kg CP668863 with and without gemcitabine. Gemcitabine and its vehicle (saline) were administered IP every 4 days at 100mg/kg as previously described (Olive, 2009), CP668863 and its previously described vehicle were administered subcutaneously every other day at its given dose in on alternative days than gemcitabine. While all CP668863 recipients experienced reduced tumor burden as compared to gemcitabine and control recipients, the greatest efficacy was observed in the 8mg/kg with gemcitabine cohort, as was the greatest survival advantage of any CP668863 recipient. Of note, one 8mg/kg CP668863 and gemcitabine recipient experienced complete reduction of tumor burden.

CDK5 inhibition with CP668863 reduces tumor growth, migration, and invasion while significantly improving survival times in highly aggressive syngeneic KPC cell line in vivo

A highly aggressive and metastatic PDAC cell line passaged from the spontaneous tumors of KPC animals were orthotopically implanted into the pancreas of Bl6/C57 (Envigo Lab, Indianapolis, IN). 5x10^4 cells were implanted into 51 animals. Tumors progressed in vivo for three days at which point a sacrificial animal was necropsied and evaluated for positive tumor burden greater than 2 mm. Remaining animals were randomly designated to one of five treatment groups: no treatment saline

control, inhibitor vehicle no treatment control, inhibitor CP668863 monotherapy, standard of care Gemcitabine (Sigma, St. Louis, MO), and combination CP668863 with Gemcitabine.

CP668863 monotherapy and combination therapy recipients had a significant reduction in tumor burden and invasion and metastasis to the spleen, lymph, intestine, stomach, diaphragm, liver and peritoneum. Of the n=10 combination recipients, only one animal had invasion into the spleen and no other metastasis. Combination therapy recipients also improved survival time significantly over gemcitabine monotherapy recipients. Glucose tolerance and nocifensive behaviors were also monitored between cohorts, but we were unable to determine any significant difference using previously described techniques (Shukla, 2019; Sevcik, 2006; Lindsay, 2005).

CDK5 inhibition with CP668863 reduces tumor growth, migration, and invasion while significantly improving survival times in preclinical murine model using human S2-013 cell line in vivo

We confirmed these findings in a preclinical athymic murine model utilizing human derived S2013 cells orthotopically challenged with 1x10^6 cells into the pancreas of each mouse prior to ultrasound surveillance for tumor formation. Once tumor diameter reached 3mm in any axis, animals were randomly enrolled into one of the same five treatment cohorts (n=10-12/cohort). Tumors greater than 6mm in any diameter were excluded. Combination gemcitabine plus inhibitor CP668863 again significantly improved animal survival over all other treatment modalities. CP668863 as a monotherapy and in combination with gemcitabine significantly reduced tumor burden over treatment course and reduced metastatic lesions to the diaphragm, and inhibited metastases in the spleen, lymphatics, stomach, diaphragm, liver, and peritoneum. Final tumor burden was calculated as a percent change from tumor enrollment volume. Control animal tumors progressed up to 2300% their enrollment size, while 55% of combination recipients experienced 100% tumor regression. Tumor progression quantified

via high resolution ultrasound as a percent change from enrollment volume and graphed over the course of treatment weeks.

Combination CP668863 and gemcitabine recipient animals radiographically cleared of tumors showed durable disease-free response for up to nine weeks

Combination therapy tumors which were twice radiographically cleared via high resolution ultrasound imaging over the course of two successive weeks showed durable response of up to 40 days with no continued treatment. Animals resolved of tumor burden and withdrawn from treatment were ultimately euthanized due to either injection site infection secondary to saline injections used for imaging, malnutrition, gastric/bile duct obstruction, or malaise.

CDK5 inhibition decreases vascularity at the invasion front

Histological examination of tumors showed decreased neovascularization as compared to saline controls, as evidenced by decreases in the number of blood vessels at the invasion front of tumors (Eggers, 2011b). The invasion front included the interface between the tumor and normal pancreas at the leading edge of the tumor. At least three independent counts were obtained for both treatments in which blood vessels were detected as endothelial cell epithelia with a lumen containing red blood cells and confirmed with immunohistochemical (IHC) staining of the endothelial marker CD31. All treatment groups had a statistically significant reduction in blood vessel number at the invasion front with CP668863.

CP668863 recipient tissues were pathologically normal

Of note, emaciation and dehydration were observed in both athymic and immune competent models in some combination therapy recipients despite no change in food interest. Once pathology confirmed no apparent systemic toxicity via macroscopic necropsy and histology, the study was

repeated under the same conditions with the addition of the dietary supplement Diet Recovery Gel (ClearH2O, vendor site). To mitigate animal association of disease with supplement, animals were provided access to the supplement ad libitum for one week prior to orthotopic challenge, and throughout the duration of the study. Providing supplement significantly improved animal mass retention throughout the duration of study when compared to the previous trial ran under otherwise identical conditions.

Discussion

Previously we have shown that CDK5 and p35 are aberrantly overexpressed in greater than 90% of primary PDAC tumor samples and PDAC cell lines, and that CDK5, p35 or p39 were amplified at the genomic level in more than 67% of pancreatic cancer samples, and that treatment with the CDK5 inhibitor roscovitine inhibited growth of Capan2 cells in vitro and decreased migration and invasion for all five tested PDAC cell lines in vitro (Eggers, 2011). Similarly, Feldmann et al observed decreases in tumorigenesis in vitro and in vivo with CDK5 inhibition using either a dominant negative expression construct or shRNA targeting CDK5 (Feldmann, 2010).

Thus, we found it compelling to evaluate the effects of CDK5 inhibition in a preclinical model of pancreatic cancer via a novel 5-disubstituted pyrazole small molecule inhibitor developed by Pfizer, CP668863. CP668863 significantly inhibited neoplastic cell growth and colony formation in soft agar, which led us to investigate its pharmacological profile in vivo.

When dosed subcutaneously the compound achieved serum concentrations greater than the 1 uM concentration required to inhibit colony formation. Peak serum levels occurred thirty minutes post bolus, and were accumulative when dosed daily. Pharmacokinetic distribution and clearance of CP668863 led us to a dosing regimen of Q 48 hours delivered subcutaneously. Combination therapy recipients received Gemcitabine on an every four day cycle previously described (Olive, 2009).

To evaluate efficacy of the compound on inhibiting tumor and metastatic formation, we first challenged immune competent Bl6/C57 female animals with 5x10^4 syngeneic murine PDAC cells derived from spontaneously occurring KPC animals. Tumors progressed in vivo for three days at which point a sacrificial animal was necropsied and evaluated for positive tumor burden greater than 2 mm. Remaining animals were randomly designated to one of five treatment groups: no treatment saline

control, inhibitor vehicle no treatment control, inhibitor CP668863 monotherapy, standard of care Gemcitabine (Sigma, St. Louis, MO), and combination CP668863 with Gemcitabine.

CP668863 monotherapy and combination therapy recipients had a significant reduction in tumor burden and invasion and metastasis to the spleen, lymph, intestine, stomach, diaphragm, liver and peritoneum. Of the n=10 combination recipients, only one animal had invasion into the spleen and no other metastasis. Combination therapy recipients also improved survival time significantly over gemcitabine monotherapy recipients. Glucose tolerance and nocifensive behaviors were also monitored between cohorts, but we were unable to determine any significant difference using previously described techniques (Shukla, 2019; Lindsay, 2005).

Next we wanted to evaluate the impact of inhibition on models of human disease progression, so we utilized SUIT2 derived human S2-013 PDAC cells orthotopically implanted into the pancreas of athymic female mice (1x10^6 cells per animal). Animals were evaluated daily for palpable abdominal masses. Any suspected mass was radiographically imaged and quantified via high resolution ultrasound. Tumor diameters in any axis greater than 3 mm but less than 6 mm were randomly enrolled into the same five treatment groups as before. Animals with tumor burden greater than 6 mm were disqualified from the study. Animals were imaged weekly for tumor progression.

Combination gemcitabine plus inhibitor CP668863 again significantly improved animal survival over all other treatment modalities. CP668863 as a monotherapy and in combination with gemcitabine significantly reduced tumor burden over treatment course and reduced metastatic lesions to the diaphragm, and inhibited metastases in the spleen, lymphatics, stomach, diaphragm, liver, and peritoneum. Final tumor burden was calculated as a percent change from tumor enrollment volume. Control animal tumors progressed up to 2300% their enrollment size, while 55% of combination recipients experienced 100% tumor regression. Tumor progression quantified via high resolution

ultrasound as a percent change from enrollment volume and graphed over the course of treatment weeks.

Combination therapy tumors that completely regressed (radiographically via high resolution ultrasound imaging) over the course of two successive weeks showed durable response of up to 40 days with no continued treatment. Animals resolved of tumor burden and withdrawn from treatment were ultimately euthanized.

Examination of H & E slides and IHC staining for CD31 of the primary tumors revealed significantly decreased neovascularization at the tumor invasion front in treated animals compared to those receiving vehicle control (Eggers, 2011). This may be explained in part by a recent finding in rat vascular smooth muscle cells that inhibition of Cdk5 resulted in significantly increased caspase 3 activation and cleaved PARP after EGF and H2O2 treatment compared to control cells (Wang, 2019). Thus, a fast growing tumor environment producing many reactive oxygen species may be sensitive to CDK5 inhibition of blood vessel growth. CP668863 mechanism of action should be confirmed via protein quantification of conserved CDK5 phosphorylation of FAK S732 and PAK1 T212 to ensure both CP668863 mono and combination therapy recipient tumors express significantly less of these residues than non-inhibitor treated cohorts (Eggers, 2011).

There is a statistically significant correlation between new onset diabetes and development of pancreatic cancer in the three year period before PDAC diagnosis. Diabetes is associated with 45-88% of PDAC patient. Given that CDK5 activity in islets is involved in many pathways regulating glycemic control, the correlation between new onset diabetes and development of pancreatic cancer led us to question whether factors in pancreatic tumors and associated microenvironment influence CDK activity in the islets. Increased CDK5 activity has been shown to increase insulin exocytosis; however, chronically increased glucose levels (48 hours) alter CDK5 localization and increase its hyperactivity in islet □ cells.

Specifically, CDK5 enhances PDX1 translocation from the nucleus to the cytoplasm and causes a decrease in insulin transcript and protein. Furthermore, it has been shown that CDK5 phosphorylates L-type voltage-dependent Ca2+ channel (L-VDCC) which in turn decreases the inward Ca2+ flux that results in decreased insulin secretion in response to increased glucose concentration. This was confirmed in p35 -/- mice, which show decreased CDK5 kinase activity, and glucose challenge increased serum insulin as compared to control mice with higher CDK5 activity. Therefore, stimulation in vitro and in vivo of CDK5 through high glucose results in decreased insulin production and secretion, similar to the phenotype observed in type II diabetes mellitus (T2DM) (Pannala, 2009; Ubeda, 2006; Wei, 2005).

Hence, we hypothesized that inhibiting CDK5 in the context of PDAC may result in better glucose regulation; however, fasting glucose performed at day 30 of treatment showed no significant change in blood glucose for CP668863 with gemcitabine compared to gemcitabine only (data not shown). Additionally, we performed interperitoneally glucose tolerance testing biweekly for the first eight weeks of tumor progression and did not see significant differences between the five cohorts. We did not investigate or characterize the development of diabetes in this orthotopic model system, as we felt this would be better suited for analysis in subsequent studies that will employ mouse models of pancreatic cancer. Thus, the consequences of inhibiting CDK5 in the context of diabetes associated with PDAC progression requires further investigation.

We deemed CDK5 a target of exceptional interest not only based on previously published and promising indications of prolonged cancer survival, but because of the integral role it appears to play in pain signaling and glucotoxicity, both of which commonly lead to comorbidities and worsened outcomes in PDAC patients. As previously stated, inhibitors of CDK5 have been shown in vivo to reduce nocifensive behaviors in response to induced inflammatory state. Since cancer and PDAC are a chronic inflamed state, we wanted to know if CDK5 inhibitor CP668863 would reduce nocifensive behavior in tumor challenged animals.

Seliciclib, or R-Roscovitine,, has been utilized in phase I clinical trials without serious side effects. Thus, CDK5 inhibitors such as CP668863 may be efficacious without serious morbidity or mortality (Benson, 2007). Inhibition of CDK5 kinase has been shown to decrease pain as determined by a decreased response to thermal stimuli (Pareek, 2007; Utreras, 2009). Therefore, CDK5 inhibition may decrease pain in pancreatic cancer. Given the role of CDK5 in neural development, we plan to investigate whether CDK5 plays a role in perineural invasion that is widely observed in pancreatic cancer.

Using a modified version of Sevcik et. al protocol for scoring nocifensive behaviors via posture, exploratory behavior, grooming, and coat texture, we established a baseline nocifensive behavior score for 8 week old female nude mice observed in isolation for five-minute intervals (Sevcik, 2006). We additionally incorporated a grid system for monitoring the animals' degree of movement. These nude mice were orthotopically implanted with 1x10^6 S2013 cells and then individually enrolled into a randomly selected treatment group once they had radiographically obvious tumors greater than 3 mm in any diameter, as described in chapter two. Five mice from each treatment group were randomly selected for weekly behavior scoring. Two blind reviewers scored each mouse. However, our methodology yielded data (not shown) that failed to trend in any pattern from week to week and was statistically insignificant. More robust models of pain modeling will be required to explore the affects of CDK5 inhibition on cancer associated pain.

In summary, we present evidence that inhibition of CDK5 with small molecule inhibitors may have therapeutic potential for pancreatic cancer. Previous studies that inhibited CDK5 activity with a dominant negative construct in combination with small molecule inhibition of ERK 1/2 or Akt also showed a significant and potentially greater reduction in tumorigenesis (Feldmann, 2010). Future experiments should address the ability of CP668863 to slow tumor growth, invasion, and metastasis

advanced stages of disease, and further investigate the capacity of CP668863 to regulate glucose and insulin, and to modulate pain and perineural invasion in the context of pancreatic cancer progression.

Figure One: CP668863 potently inhibits CDK5 kinase activity.

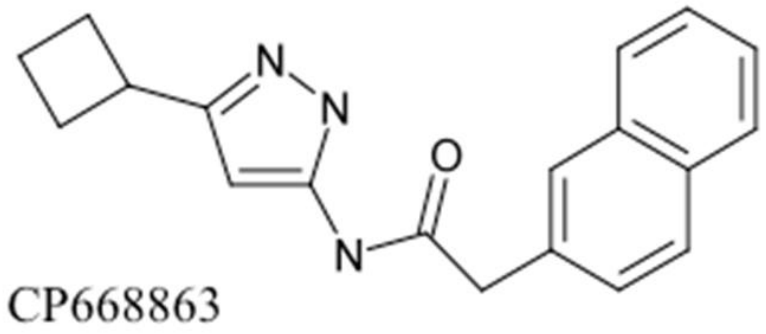

Figure One of the chemical structure of CP668863 a modified 3,5-disubstituted pyrazole.

Figure Two A. represents multiple reaction monitoring (MRM) chromatogram of CP668863 in mouse serum. B. Following a single 8 mg/kg subcutaneous bolus of CP668863, maxillary venous blood was collected at 30 seconds, 15, 30, 60 minutes, 2, 4, and eight hours post injection to evaluate circulating concentration of CP668863 in ng/ml. C. Mice were injected with CP668863 bolus at concentrations of 1, 8, and 16 mg/kg daily. Venous blood was collected two hours post injection daily with serum drug concentrations in ng/ml.

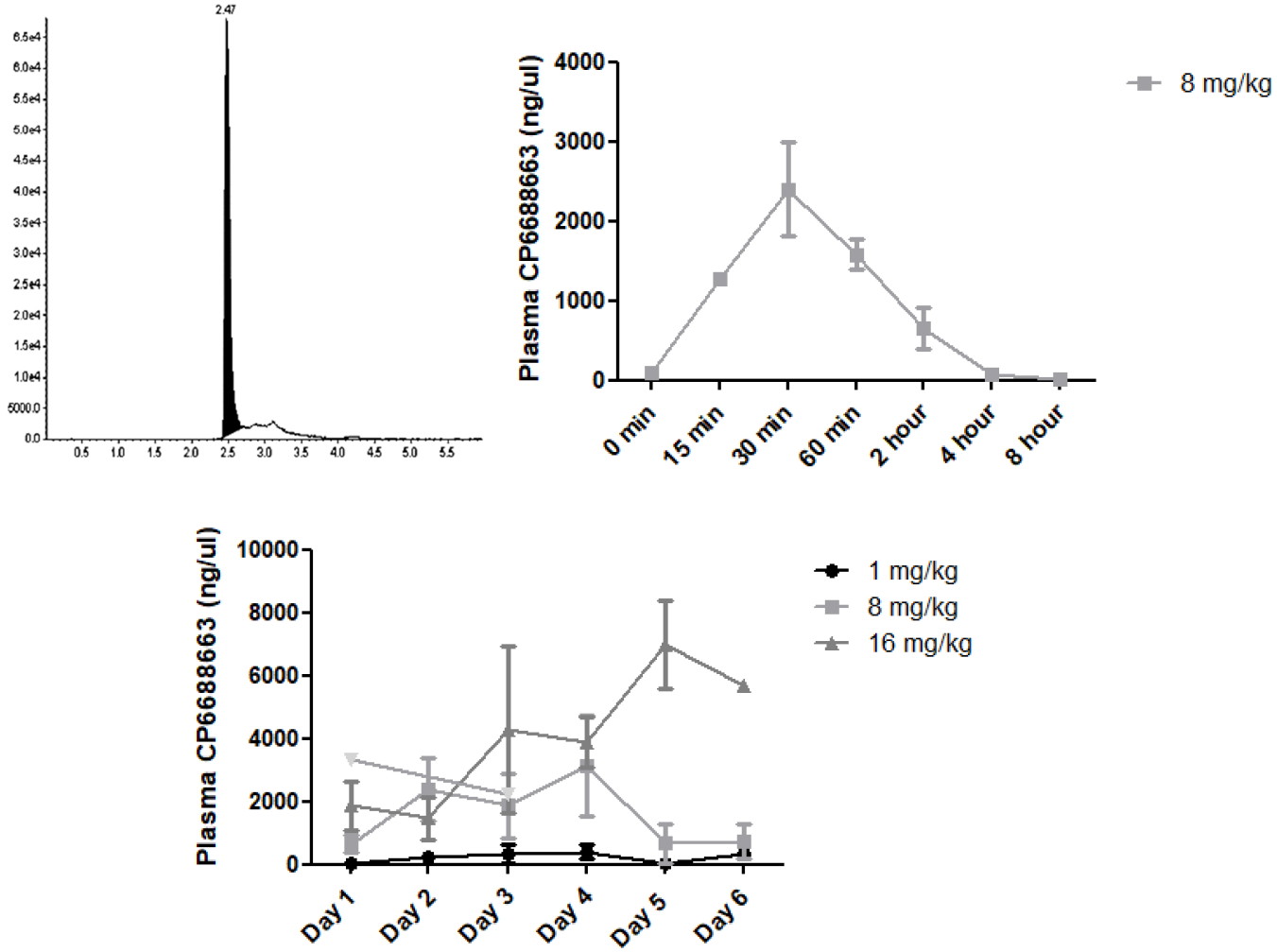

Figure Two: Pharmacokinetic profile of CP668863 in serum

Figure Three: Immune competent C57/Bl6 mice were orthotopically challenged with 6x10^5 syngeneic PDAC cells derived from transgenic KPC tumors and allowed three days to form solid pancreatic tumors (validated via necropsy of randomly selected animal). Animals were randomly enrolled into one of five treatment cohorts (n=8/cohort; i) saline control, ii) CP668863 vehicle, iii) CP668863 monotherapy, iv) standard of care gemcitabine, and v) combination CP668863 and gemcitabine). A) Combination CP668863 plus gemcitabine significantly improved survival over all other cohorts (Wilcoxon test: $p=0.0054$ compared to gemcitabine alone). B) CP668863 alone and in combination with gemcitabine significantly reduced tumor burden, and ($p<0.0005$ compared to gemcitabine), C) and inhibited metastatic lesions to the spleen, gastric lymphatics, intestine, stomach, diaphragm, liver, and peritoneum.

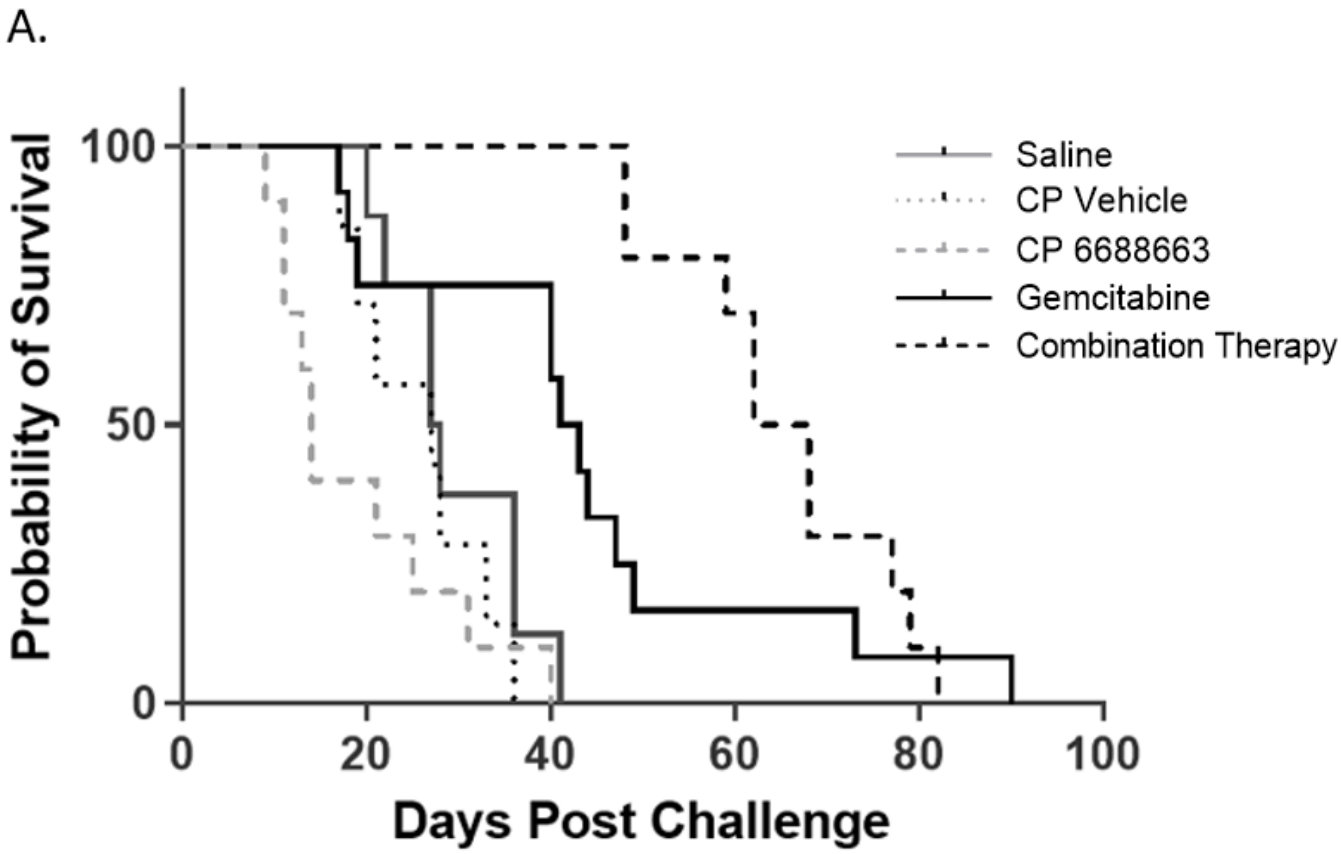

Figure Three: A) CP 6688663 significantly improves survival time in immune-competent orthotopic mouse model of invasive syngeneic KPC cells

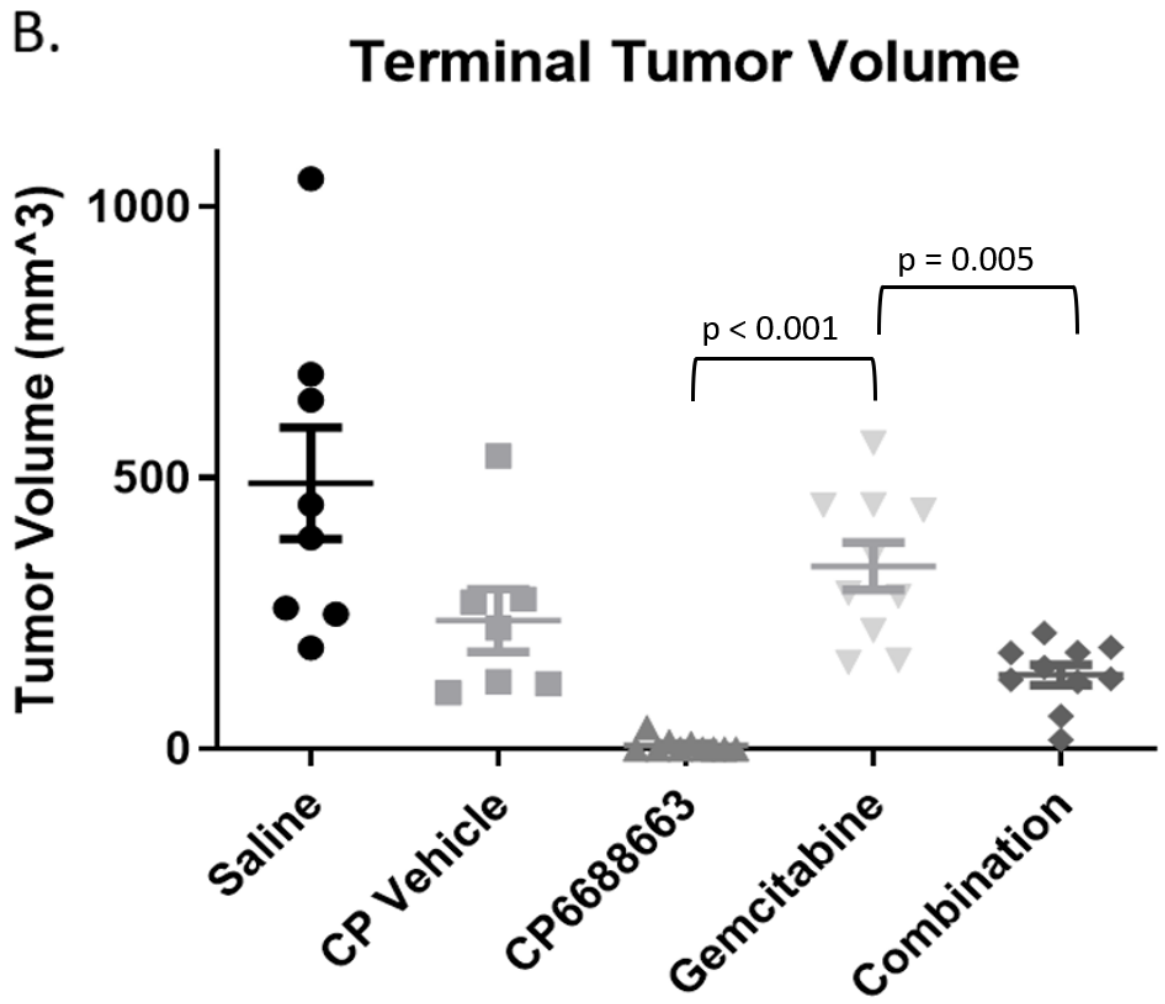

Figure Three: B) CP 6688663 significantly reduces tumor burden in immune-competent orthotopic mouse model of invasive syngeneic KPC cells.

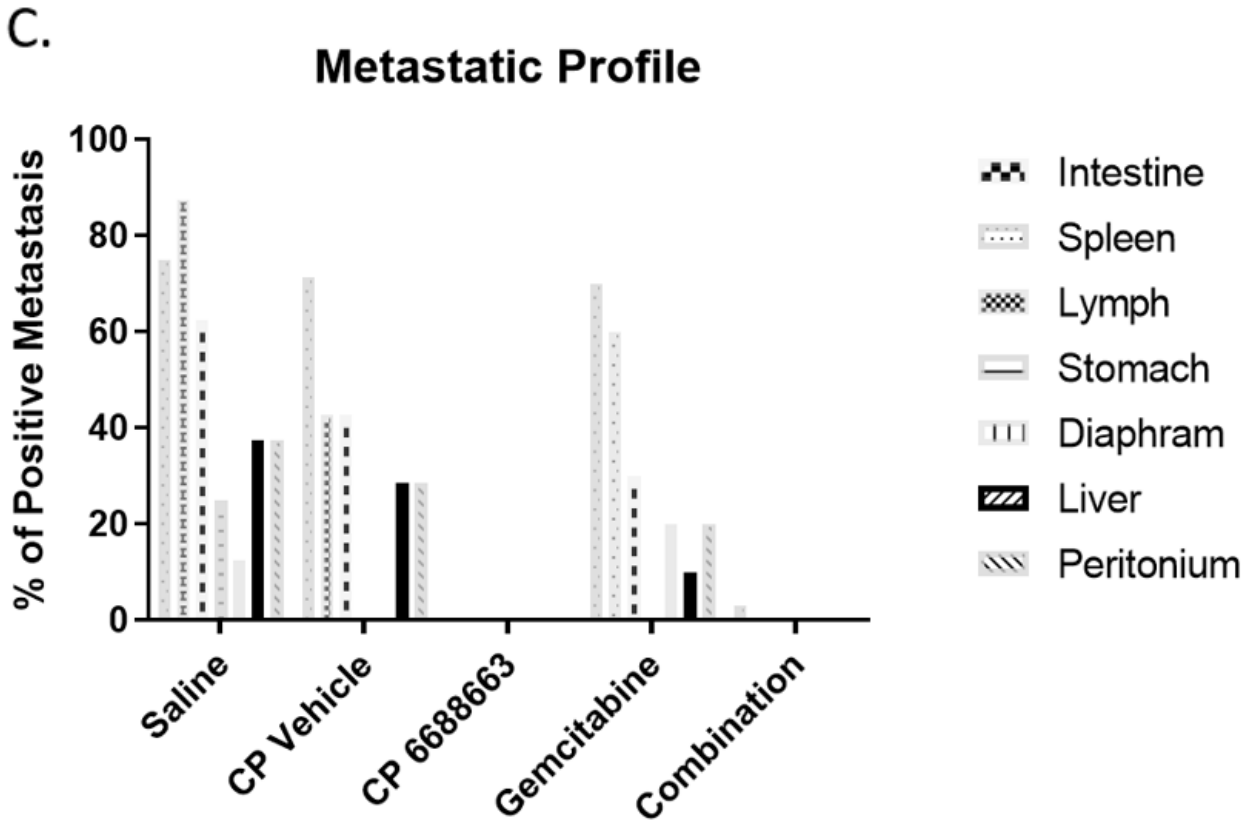

Figure Three: C) CP 6688663 significantly reduces metastasis in immune-competent orthotopic mouse model of invasive syngeneic KPC cells.

Figure Four: Athymic mice were orthotopically challenged with 1x10^6 human derived S2013 cells and monitored biweekly via high resolution ultrasound for solid tumor formation. Pancreatic tumor masses with a diameter greater than 3 mm in any axis were randomly enrolled into one of five treatment cohorts cohort; i) saline control, ii) CP668863 vehicle, iii) CP668863 monotherapy, iv) standard of care gemcitabine, and v) combination CP668863 and gemcitabine). A) Combination CP668863 plus gemcitabine significantly improved survival over all other cohorts (Wilcoxon test: p=0.0433 compared to gemcitabine alone). B) CP668863 alone and in combination with gemcitabine significantly reduced tumor burden, (p=0.0146, and p=0.0012 respectively, when compared to gemcitabine alone), and C) in all but one case inhibited metastatic lesions to the spleen, gastric lymphatics, intestine, stomach, diaphragm, liver, and peritoneum. Animals radiographically free of tumor burden for two consecutive weeks were removed from therapy (n=5 combination therapy recipients).

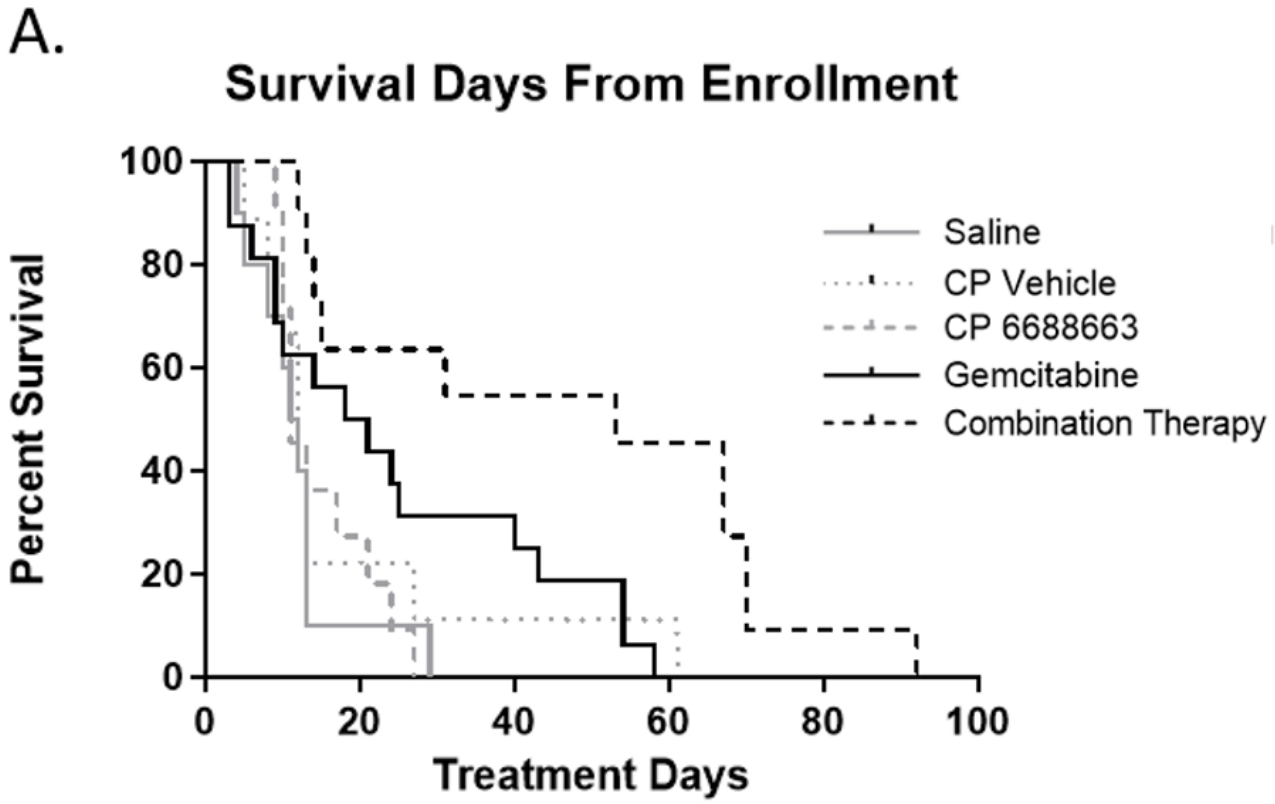

Figure Four: A) CP 6688663 significantly improves survival time.

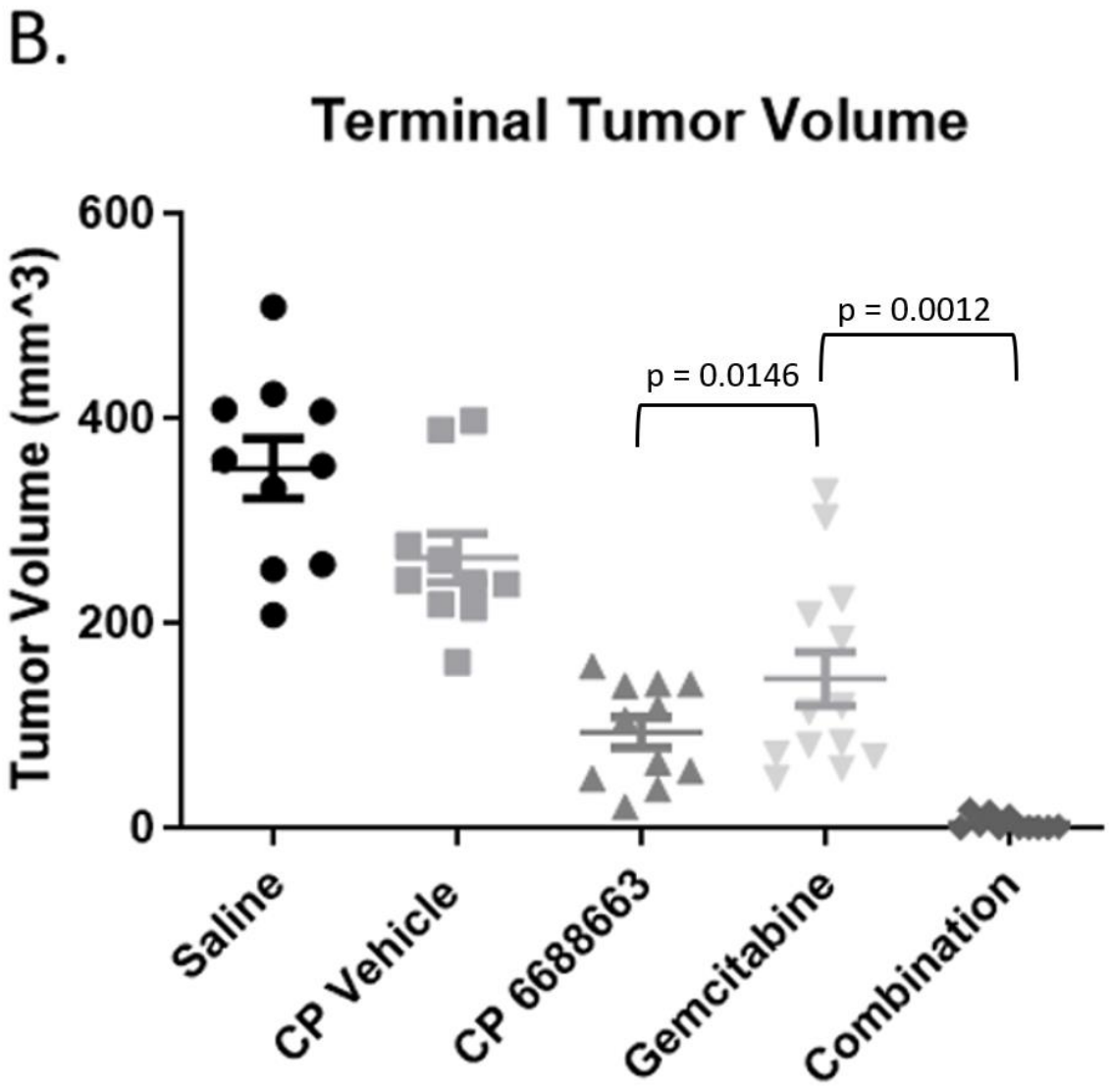

Figure Four: B) CP 6688663 significantly reduces tumor volume in orthotopic model using human PDAC cell line S2013

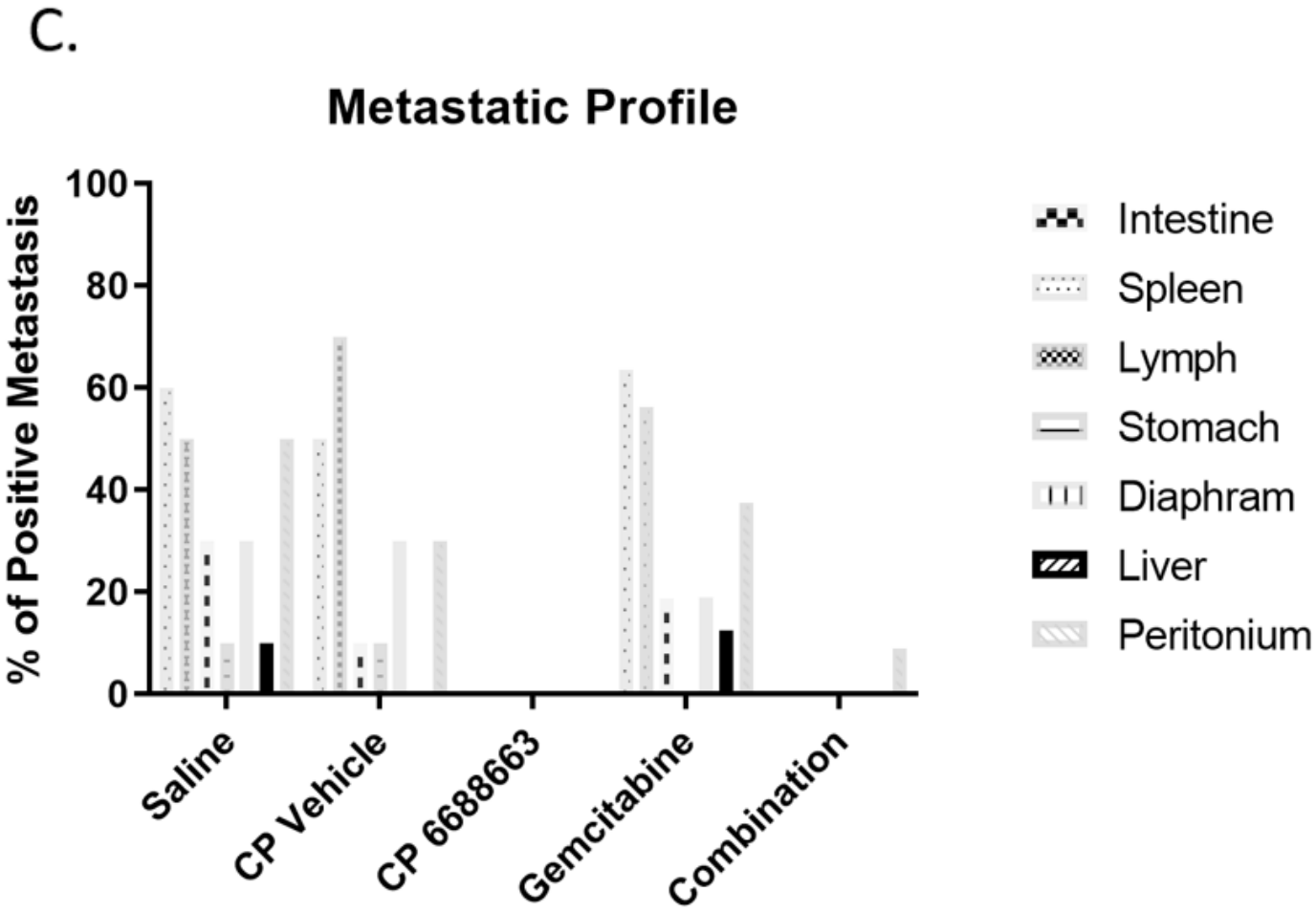

Figure Four: C) CP 6688663 reduces metastatic lesions in orthotopic model using human PDAC cell line S2013.

Figure Five details treatment information for individual animals numbered on the x-axis. “Treatment Days” columns indicate the number of days the individual spent enrolled in the study. “Change in Tumor Volume” panels indicate the change in tumor volume from the individual’s enrollment compared to necropsy. Combination treatment was CP668863 plus gemcitabine. One animal treated with combination therapy showed progression of tumor size by 19% while on treatment for 31 days before requiring euthanasia. Combination recipients 2-5 were in remission at the time of euthanasia and all combination animals that tolerated treatment for more than 32 days had no detectable tumor mass. One monotherapy CP 6688663 recipient went into remission, but only tolerated 10 days of treatment. All other tumors progressed under all other treatment arms.

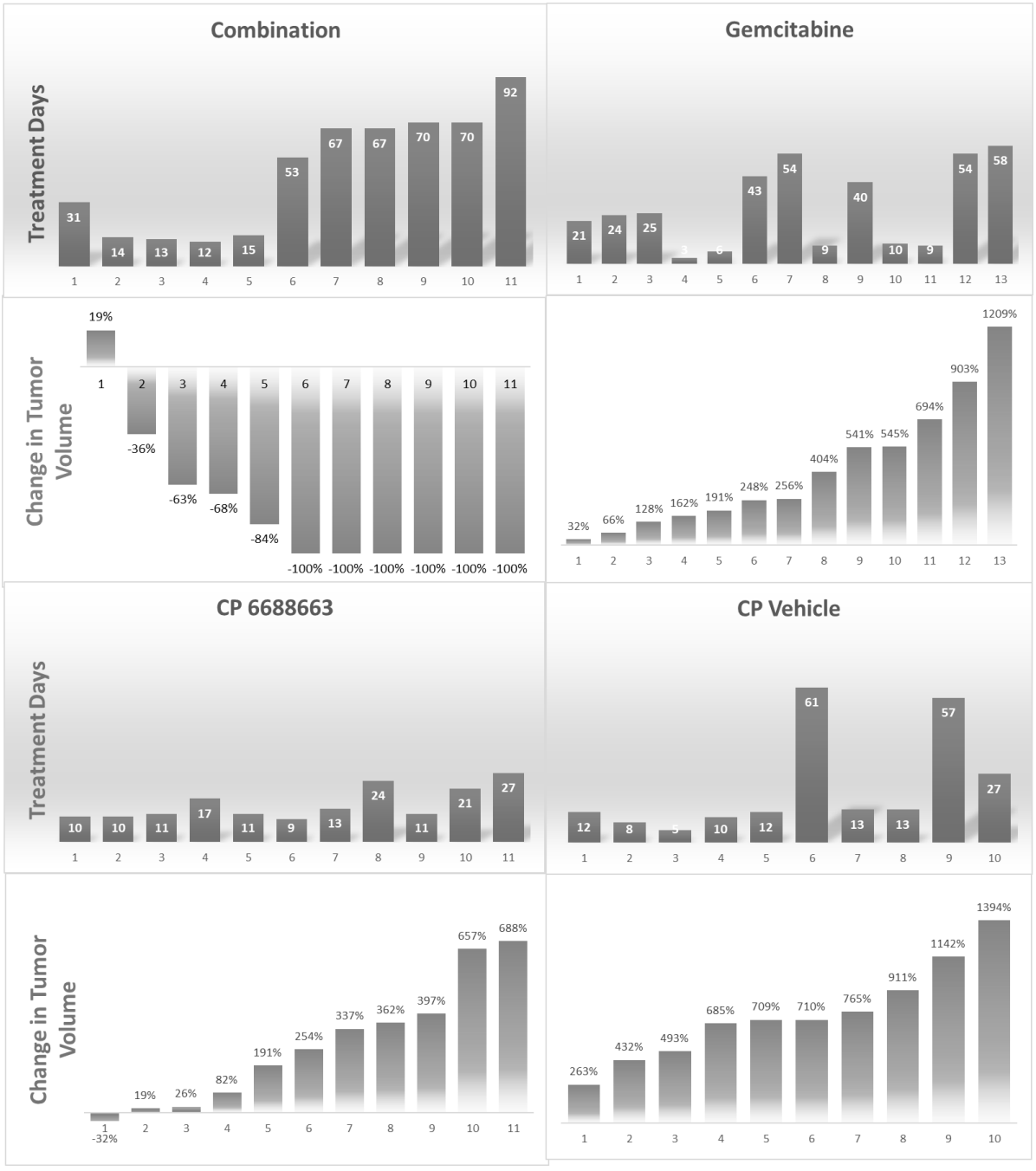

Figure Five: Combination and monotherapeutic use of CP 6688663 reduces tumor burden in orthotopic model using human PDAC cell line S2013.

In Figure Six Bliss Independence approach to quantify synergy between therapies with independent mechanisms of action is used to evaluate combination effectiveness of CP 6688663 and gemcitabine, with a CI of 90.1% (CI=EA + EB – EA EB / EAB).

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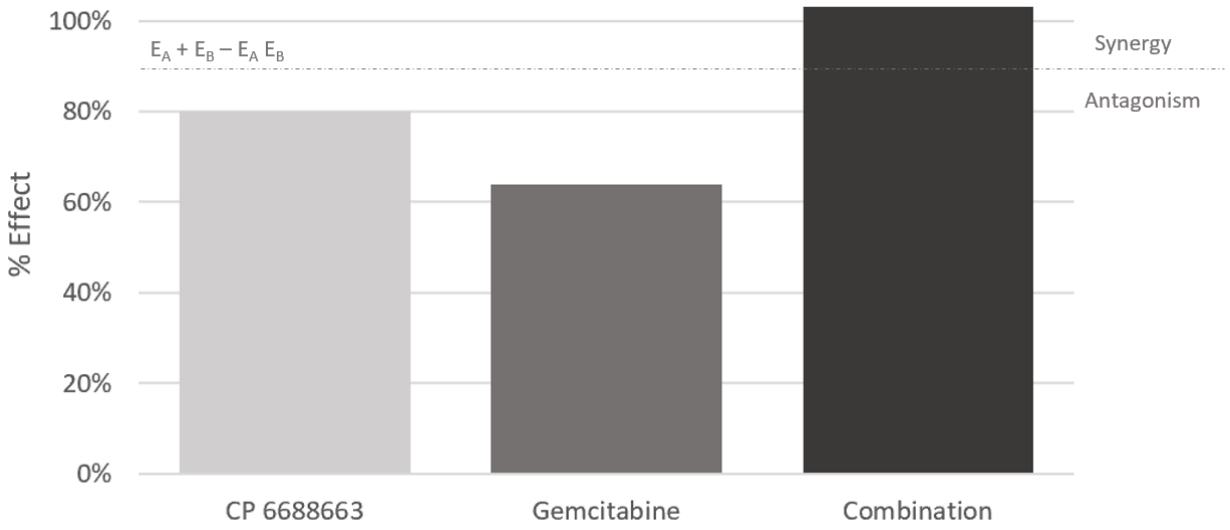


Figure Six: CP 6688663 and gemcitabine have synergistic efficacies in vivo

CHAPTER TWO:

Patents: A tool for translating research from bench to bedside

Contains excerpts from:

Qi, B., Crawford, A. J., et al. (2020). Tuned near infrared fluorescent hyaluronic acid conjugates for delivery to pancreatic cancer for intraoperative imaging. Theranostics, 10(8), 3413.

Qi, B., Crawford, A. J., et al. (2019). Surgical imaging of pancreatic cancer using polysaccharide-delivered near infrared fluorophores. In Molecular-Guided Surgery: Molecules, Devices, and Applications V (Vol. 10862, p. 108620O). International Society for Optics and Photonics.

Qi, B., Crawford, A. J., et al. (2018). Indocyanine green loaded hyaluronan-derived nanoparticles for fluorescence-enhanced surgical imaging of pancreatic cancer. Nanomedicine: Nanotechnology, Biology and Medicine, 14(3), 769-780.

Patents and technology transfer

Since the 1980's, legislatures have invested immense effort into strategies to capitalize on the United States' global lead in basic and translational research. This is largely motivated by the discrepancy the US faces in converting its world leading research discoveries and innovations into commercial products, relative to other countries. This truncated productivity leaves the US out of substantial GDP growth and reduces public access to new innovations. Proponents of government and university owned patents reason that enhanced public private partnerships narrow the gap between the technology transfer of basic research discoveries and accessible commercial products.

With these concepts of technology transfer in mind, we set out to develop translationally relevant technologies to improve PDAC patient outcomes. First, we engineered a research tool, our Abdominal Imaging Window (AIW), which facilitates in vivo longitudinal deep tissue microscopy of pancreatic tumors and microenvironment throughout disease progression. Second, we collaborated with experts in the field of fluorescent imaging to develop tumor specific contrast agents that could be used in real time intraoperatively to improve surgical resection outcomes. We then partnered with pharmaceutical scientists to evaluate novel small molecule inhibitors and combined therapeutic approaches to treating PDAC. Lastly, we investigated tools for strategic Science, Technology, Engineering, and Mathematics (STEM) outreach programing for not only recruiting future scientists, but for building a community of trust and understanding of scientific practices in the next generation.

Abdominal Imaging Windows

3D printing is changing the landscape of biomedical research by providing quick access to low-cost prototyping of custom research tools. This ease of customization is optimal for researchers working in medical and biological fields where subject variability is high. To meet the need for longitudinal intravital imaging of organs in a live animal, we developed a 3D printed mount for securing a microscopy cover

slip over any visceral tissue for reliable, safe, and efficient iterative intravital imaging. These customizable windows fit imaging fields of 8-15 mm and reduce artifact by gravity suspending the subject during imaging on upright microscopes.

Introduction

Intravital microscopy gives researchers a rare look into how living cells behave in their complete and native environment. While much of what we know about cellular processes and pathways has been discovered in static and biologically isolated conditions, deep tissue intravital microscopy facilitates a whole picture view of cell-to-cell interactions, real time responses to signaling and stimuli, and the probing questions that cannot be interrogated via cell culture or fixed tissue.

This technology is exceptionally important in tumor biology. Much like the uniqueness of patients, even the best animal models of cancer have disease heterogeneity from animal to animal. Tumor heterogeneity within a tumor and between individuals makes assumptions about disease progression risky and may obscure profound effects on specific cellular populations.

The caveat to this powerful technology remains the mechanical ability to present the tissue of a live animal to the microscope objective and light source in a stable enough fashion to perform deep tissues scans over minutes and hours. Skin flap models simply remove the skin and peritoneum obstructing the tissue of interest for direct viewing. Protocols for skin flap models exist for up to 8 hours of imaging but are typically terminal. And they do not address the obstacles of microscopy like forming a meniscus or mitigating the artifact created by the animal's respiration.

Imaging windows are surgically implanted brackets that hold a cover slip over the tissue of interest for intravital imaging of a live animal. There are commercially available AIWs for brain and skin imaging, but not for visceral organs (e.g., liver, kidney, spleen, pancreas). Several research groups have published on their lab generated abdominal imaging windows (AIW) (Ritsma, 2012; Sobolik, 2016; Alieva, 2014). Most of these devices are composed of metal and can be difficult to have built by machinists. While they do provide an imageable view of tissue, they do not account for mounting the animal on the microscopy stage.

Materials and Methods

IACUC approved methods

Prior to surgery, hair is removed by commercially available depilatory cream (Nair). The printed window will be disinfected by soaking for 5 minutes in glutaraldehyde, Betadine, Chlorhexadine, or other suitable disinfectant, and rinsed with 70% ethanol immediately prior to use. To implant window, a ~0.5cm incision is made in both the skin and the abdominal muscle wall, and the spleen is removed to prevent visual obstruction of the pancreas during imaging. The splenic vasculature is pressure cauterized with Chromic gut 4.0 suture on both sides of the cut site. A sterile coverslip is glued to the inside of the round portion of the window with veterinary grade 2-octyl cyanoacrylate (super glue), as is the abdominal muscle wall glued to the outside of that round portion to create a biological seal (see images below). Alternatively, the abdominal muscle wall may be sutured to the indented portion of the window using a purse string suture (Ethilon Black Monofilament). The skin is then fitted into the concavity of the ring, and it is also held in place with a purse string suture (Ethilon Black monofilament). The rectangular portion of the window 'frame' fits into the adaptor in the two-photon microscope for imaging. The apparatus is placed laterally on the abdomen and between the ribs and the pelvis such that the frame does not impair movement of the animal.

Results

By engineering not only a 3D printable abdominal imaging window, but one that mounts directly onto a customizable microscopy stage mount, we were able to longitudinally image tumor bearing mice on an upright multiphoton microscope with minimal respiratory artifact.

The abdominal imaging window facilitates stable presentation of visceral organs as seen in Figure 7. The 13.5 mm immersed well ergonomically fits small animals, with truncated ends to accommodate ambulatory hip and rib movements. The beveling along the well secures the surgically placed purse-string suture through the dermis, ensuring permanent placement. The customizable 4 mm well depth enables imaging-objective positioning within microns of the tissue of interest, maximizing tissue imaging focal length, while alternatively providing a deep meniscus reservoir for superficial imaging. On the anterior well side is an adhesion lip for securing the imaging cover slip. The well is surrounded by a 19 x 23 mm four cornered mounting bracket, which attaches to the stage mount insert (Figure Two) for stable animal and tissue presentation. The opening bellow the well is covered on both ends with repeat-puncture safe rubber, allowing for blunt probe manipulation of tissue within the imaging field, or direct tissue injection, while maintaining a sterile environment.

In figure 8 the post surgically implanted animals are mounted via customizable stage inserts which gravity suspend the animal to reduce artifacts secondary to respiration and motility. The window mounts to the region indicated in yellow, presenting the tissue of interest into level field of the microscope objective. The customizable mount conveniently accommodates animals of various sizes, tissue presentation, and microscope stages. Openings within the bracket facilitate continuous monitoring of the animal as well as support devices such as oxygen, isoflurane, and heating apparatus. Solid superficial material ensures animal is protected from rogue light/laser sources and mounting media.

Various organ features including changes to vascular formation, macroscopic metastasis, cyst formation, and other feature can be observed macroscopically or with aid of dissecting microscope as shown in Figure 9. Variable surgical placement allows for visualization of various regions of interest. A. reveals the pancreas body, including fluid suspension of tumor cells, major pancreatic vasculature, and liver lobes. B. captures pancreas head and body, duodenum, and portal duct.

Once mounted, the windowed subject's tissue is imageable via any epifluorescent light or excitation source. Animals can be heavily or lightly sedated or habituated to the stage mount for sedation free imaging (Figure 10).

The researcher can easily image the same tissue location over the course of hours, days, or months through various mapping techniques (Figure 11). Large vasculature and collagen branches can be used as fiduciary markers to guide the user to their region of interest (Figure 12). The same can be done by marking on coverslip or using gridded coverslips, especially for multiple regions of interest. Endogenously expressing fluorescent proteins can also make unique landmarks, although it is worth while to consider that any number of conditions can affect repeated use of exogenous labels such as injected labeled antibodies or nanoparticles.

Discussion

Advances in multiphoton microscopy allow for deep tissue cellular imaging. To study biological cancer processes at the cellular level, longitudinally, we have engineered a permanent, surgically implanted abdominal imaging window for multiphoton imaging of developing visceral tissues and tumors. Highly focused light energy can be used to stimulate emission shifts of specific fluorescently labeled cellular components, allowing us to not only visualize the cellular landscape of a tumor, but even the sub-cellular features and markers of individual cells (allowing for robust differentiation of each cell and monitoring of its individual responses). The functional limitation of photon microscopy is that the skin absorbs this energy to protect our visceral organs from damage. Parting the skin to facilitate photon penetrance causes surgically-induced changes to the tumor progression upon closure (a cascade of inflammatory regulators, clotting factors and immune cells all flood the area and incidentally alter tumor progression). These pathological responses can be mitigated by allowing for recovery time post-surgical placement of the device followed then by orthotopic challenge.

The markets for brain and skin longitudinal imaging devices continue to expand, but without market penetrance into applications for visceral organs despite being the larger proportion of research. Given the current demand for novel research technologies, the market size of the cancer research field, and the highly differentiated value of this invention, further investigation into the strategy of bringing this device to market is warranted. The continual expansion of federal research initiatives into this area, including the recent surge of funding to support the Cancer Atlas program, indicates potential for continual economic growth. The adaptive design of the imaging device has many potential avenues of expansion and further differentiation, as well as the potential for a line of accessory products.

Figure Seven: The abdominal imaging window facilitates stable presentation of visceral organs. The well is surrounded by a 19x23 mm four cornered mounting bracket, which attaches to the stage mount insert (Figure Two) for stable animal and tissue presentation. The opening bellow the well is covered on both ends with repeat-puncture safe rubber, allowing for blunt probe manipulation of tissue within the imaging field, or direct tissue injection, while maintaining a sterile environment.

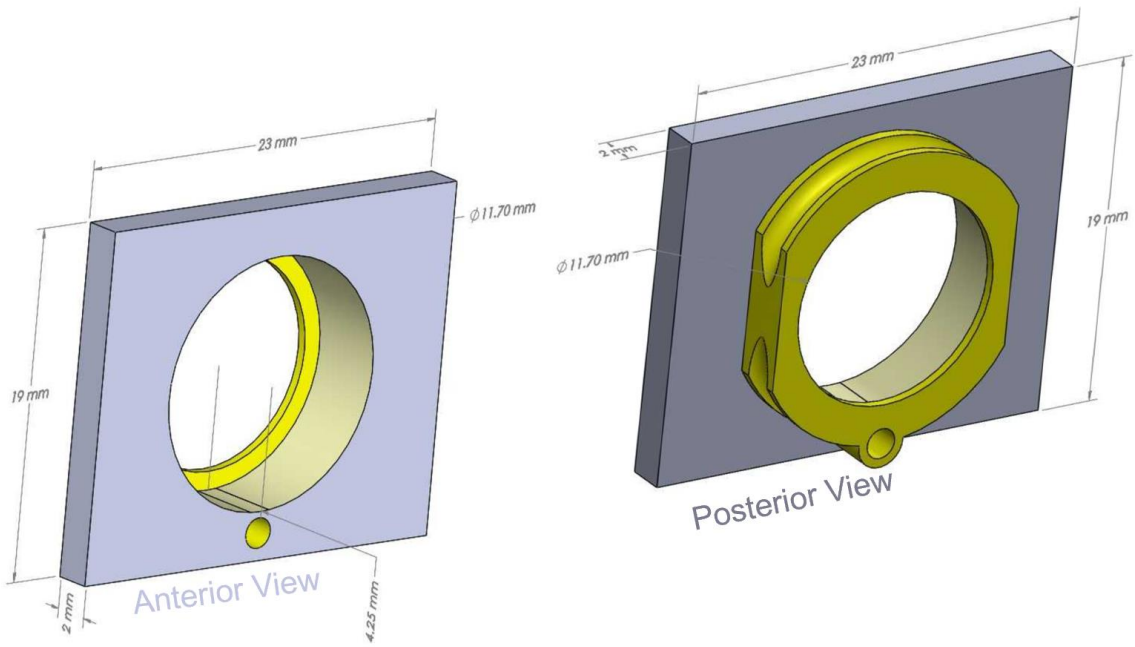


Figure Seven: The abdominal imaging window facilitates stable presentation of visceral organs.

Figure Eight: Customizable microscope stage mount gravity suspends window bearing animal. Solid superficial material ensures animal is protected from rogue light/laser sources and mounting media.

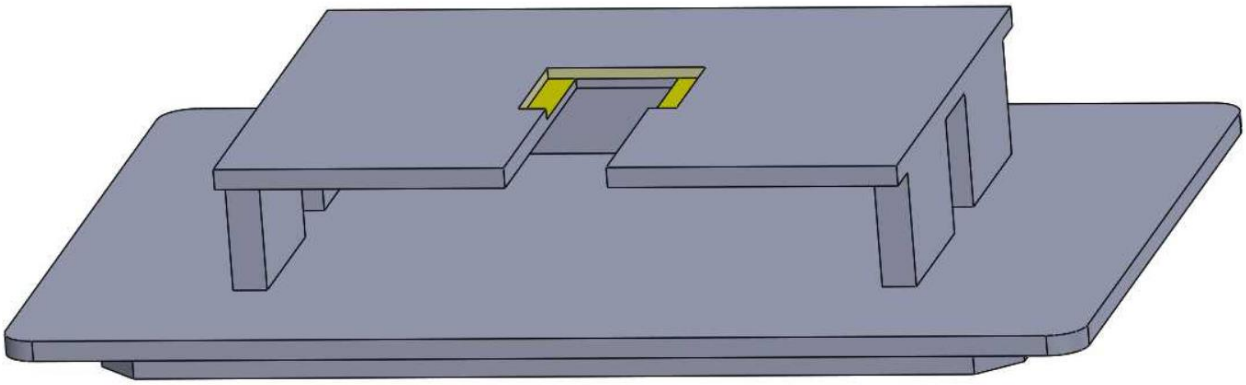


Figure Eight: Customizable microscope stage mount gravity suspends window bearing animal.

Figure Nine: Various organ features including changes to vascular formation, macroscopic metastasis, cyst formation, etc. can be observed macroscopically. A) reveals the pancreas body, including fluid suspension of tumor cells, major pancreatic vasculature, and liver lobe 3. B) captures pancreas head and body, duodenum, and portal duct.

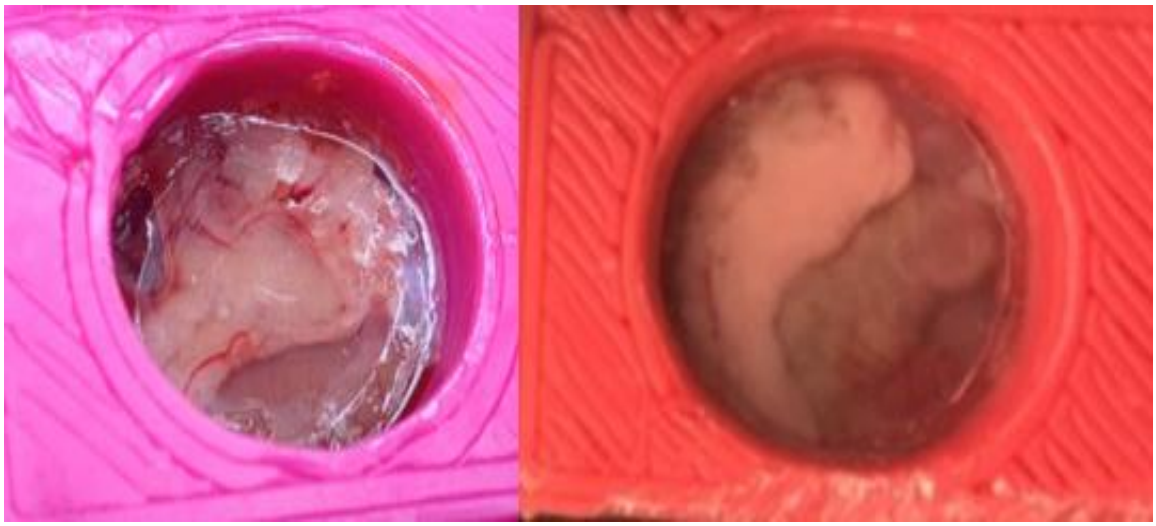


Figure Nine: Various organ features including changes to vascular formation, macroscopic metastasis, cyst formation, etc. can be observed macroscopically.

Figure Ten: Once mounted, the windowed subject's tissue is imageable via any epifluorescent light or excitation source. Animals can be heavily or lightly sedated or habituated to the stage mount for sedation free imaging.

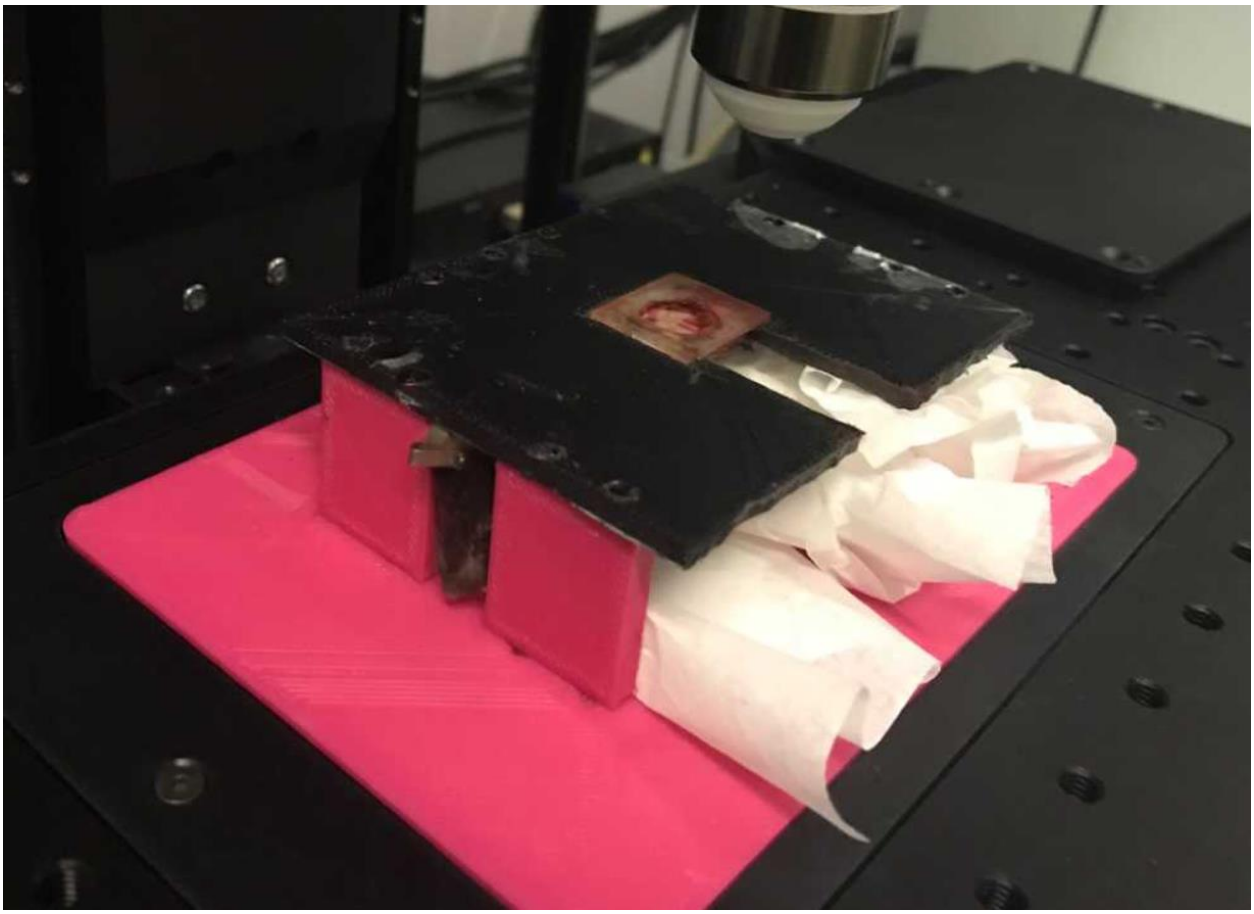


Figure Ten: Once mounted, the windowed subject's tissue is imageable via any epifluorescent light or excitation source.

Figure Eleven: Longitudinal images captured from windowed specimen depict tumor cell congregation between deep groove collagen structures. Figure A was acquired 24 hours post injection, while Figure B was quickly identified and reimaged after 48 hours.

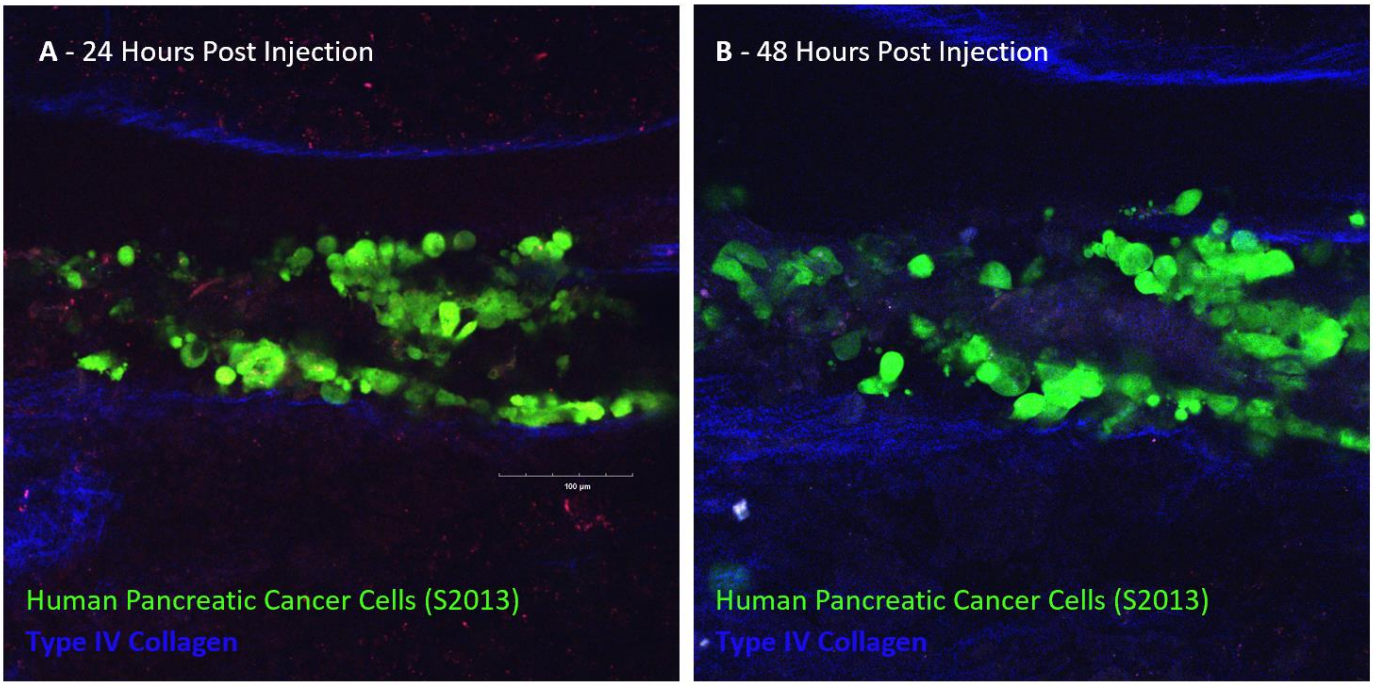


Figure Eleven: Longitudinal images captured from windowed specimen depict tumor cell congregation between deep groove collagen structures.

Figure twelve: in vivo image of myelin directed NP41 labeled neuron (purple) penetrating mass of orthotopically implanted GFP expressing pancreatic cancer cells, acquired via AIW.

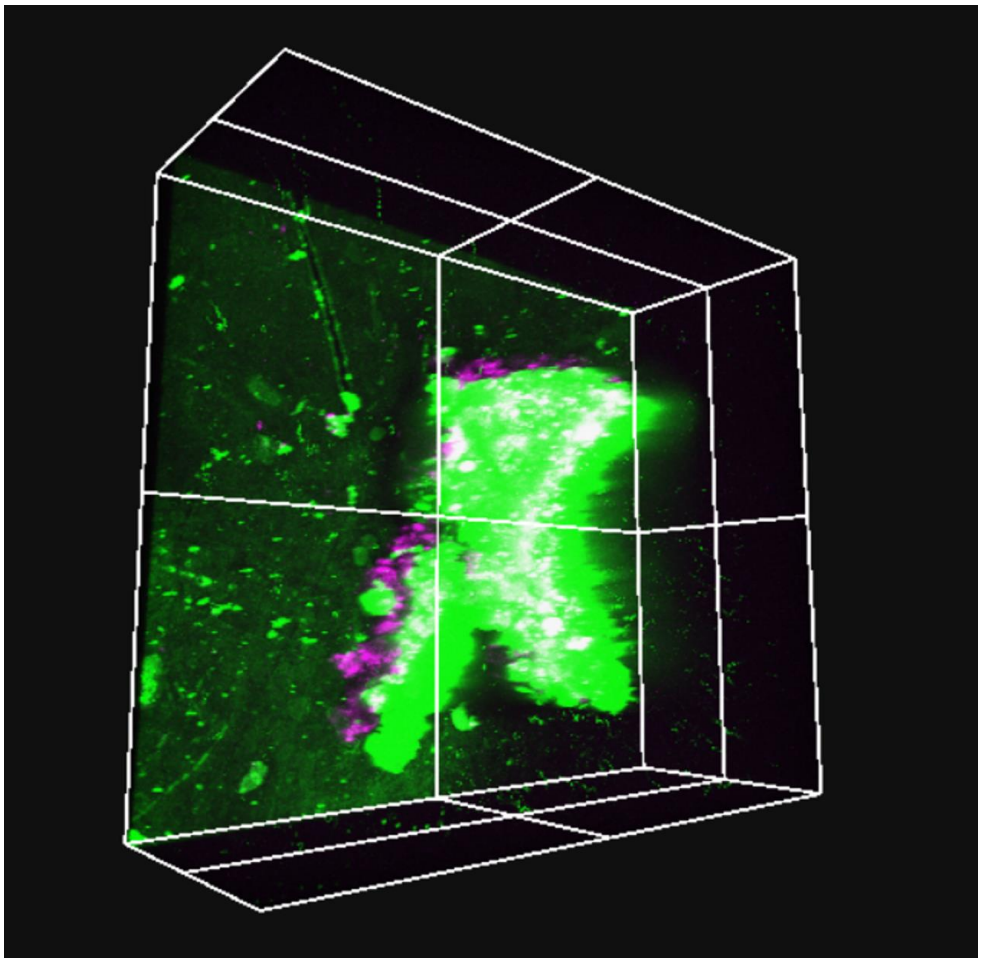


Figure twelve: in vivo image of myelin directed NP41 labeled neuron (purple) penetrating mass of orthotopically implanted GFP expressing pancreatic cancer cells, acquired via AIW.

II. Fluorescent contrast agents for improved surgical resection

Introduction

To date, surgical resection remains the only curative option for those diagnosed with pancreatic ductal adenocarcinoma (Garrido, 2015). These complicated resections require the surgeon to spare as much unaffected tissue as possible while trying to ensure negative margins predominantly by just tactile feel. There are currently no optical contrast agents available for indicating small occult metastases or tumor margins for PDAC resections (Verbeek, 2014). However, non-radioactive near-infrared fluorescent (NIRF) dyes which fluoresce in wavelengths between 700 and 1000 nm have been identified as potential candidates for enhancing the optical contrast between tumor and tumor adjacent tissues. Use of fluorescent probes in surgery (also known as fluorescence image-guided surgery or FIGS) has been a long sought-after technology but remains poorly developed for use in tumor resection. This is largely because of obstacles in developing usable contrast agents with tumor specificity and biocompatibility (Owens, 2016).

Even fluorescent markers capable of conjugation to tumor cell antigens or cell-penetrating peptides in a biocompatible solution must still overcome contrast-to-noise ratios (CNR) and signal-to-noise ratios (SNR) such that the tumor tissue is discernable from adjacent tissue (Owens, 2016). This is exceptionally challenging in the case of PDAC where the diseased tissue sits adjacent to the renal and hepatic tissues which act to clear the contrast agent from the body. However, our collaborators from the laboratory of Dr. Aaron Mohs, including Dr. Bowen Qi, had discovered a recent report that zwitterions where more commonly cleared via renal filtration and thus reduced hepatic noise background (Choi, 2011).

In our initial studies (Qi, 2018), we focused on the optimization of a nanoformulation of indocyanine green (ICG) physiochemically entrapped in self assembling hyaluronic acid (HA), and directly

compared ICG with our collaborators' nanoformulated ICG (NanoICG) as a contrast agent that could be used in real time surgical applications to discern normal from diseased tissue using an orthotopic mouse model of pancreatic cancer. We felt that ICG was an ideal starting place given that our collaborators' nanoformulation would be novel and useful (criteria for intellectual property protection), but would be more readily brought to clinic given ICG's FDA approved status for use in humans (indicating an easier go-to-market strategy) (Ogawa, 2009; Hill, 2016). We hypothesized that by entrapping our fluorophore in hyaluronic acid nanoparticles we could achieve enhanced tumor specificity both because of HA's abundant production throughout all stages of pancreatic tumor progression and ability to bind CD44, but also because of its numerous chemical groups which are highly adaptable to modification including conjugation, including N-acetylglucosamine, hydroxyl, and acetyl moieties (Kim, 2020; Bulpitt, 1999).

In all of our collaborative studies, Dr. Mohs' lab and Dr. Qi performed the chemistry, histology, and immunological studies referenced in our publications. They partnered with our lab to utilize our expertise in preclinical trial design, animal models of PDAC, agent delivery, imaging, and necropsy.

Methods

All materials, reagents, and methods have been previously published (Qi, 2018). All animal studies were performed under a UNMC Institutional Animal Care and Use Committee approved protocol. Briefly, tumor models were induced via orthotopic injection of 10,000 KPC cells into the pancreas body of 6-8 week old female C57BL/6 mice. Tumors were allowed to progress for two weeks at which point tumors were easily palpable in all challenged mice. The tumor bearing animals were intravenously injected with contrast reagent 24 hours prior to imaging. Immediately prior to imaging animals were euthanized. Liver and spleen were removed from the abdominal cavity to prevent obstruction of wide field imaging of the pancreas. The Mohs' lab custom FIGS system was used to model intraoperative imaging of the intact pancreas and adjacent tissues. This system's spectroscopic unit is a handheld pen that emits a 785 nm excitation laser and collects NIR emission, and visible color which get overlaid onto an adjacent monitor for real-time widefield imaging. The excitation laser was regularly calibrated and always used at a power of 80 mW. Two additional wide field imaging platforms were also used: the Lab-FLARE RP1 small animal imaging platform which excites the entire field of view and was used to collect 800 nm emission over 150 ms and 12 ms exposure times, and the Fluobeam 800 imaging system.

After FIGS, the animals were necropsied and their visceral tissues were presented in a consistent fashion with the pancreas oriented so that the head was at the top of the imaging tray and the tail down. Pancreas, femur, lung, kidney, small intestine, liver, quadricep, heart, spleen stomach, large intestine and lymph node were then reimaged without other tissue background by our FIGS system, as well as with a Pearl Trilogy imaging system. Post imaging, tissues were rapidly frozen in OCT and cryosectioned for immunohistology staining.

Results

We found that both in vitro and in vivo studies indicated that NanoICG was a more selective agent that yielded improved tumor tissue contrast than ICG alone. We also found NanoICG to be a safe, noncytotoxic agent with potential use in detection and resection of pancreatic cancer (Qi, 2018). While both NanoICG and ICG were detectable in the pancreas during intraoperative imaging, NanoICG expressed superior contrast enhancement than ICG in the same conditions, and had an overall greater signal intensity of over 200%.

When evaluated ex vivo we found greater fluorescent intensity in the pancreatic tail where the orthotopic injection was made. The average fold-increase in fluorescence between diseased and non diseased tissues were 5.61 (NanoICG) compared to 2.20 (ICG), indicating that NanoICG has enhanced contrast effects for the detection of pancreatic cancer than FDA approved ICG. Our collaborators went on to histologically validate accumulation of NanoICG in lesion positive tissues (Qi, 2018). This enhanced specificity for pancreatic cancer lesions may be due in part to the innate nature of HA derived nanoparticles which include specific binding of CD44, and or through enhanced permeability and retention (EPR) effect (Misra, 2011; Dosio, 2015). These results in combination with similar reports of HA nanoparticle uses in selective tumor imaging led us to believe that further investigation into these contrast agents was warranted.

In our second publication (Qi, 2020)we investigated the role of varied molecular weights of our HA nanoparticles on the rate of tissue clearance. We also transitioned away from using ICG and instead conjugated the nanoparticles to either IRDye800 or Cy7.5 (Qi, 2020). While the NanoICG in our prior studies did enhance tumor contrast over ICG alone, we struggled to over come the signal intensity in the liver and spleen. Our hypothesis was that varied molecular weights of HA conjugated to dyes with

different routes of metabolic clearance would yield a unique peritoneal biodistribution profiles that we could then tune for optimal intraoperative tumor imaging.

We evaluated the biodistribution and tumor uptake of HA molecular weights at 5, 20, and 100 kDa directly conjugated to either IRDYe800 or Cy7.5 with the goal of achieving maximal CNR and SNR. In non-tumor bearing wild type mice, HA-Cy7.5 conjugates were detected 24 hours post injection in the highest concentration in the liver and to a lesser extent the spleen and kidney. HA_{20K}-Cy7.5 had the highest fluorescent intensity and persisted in the liver for up to 4 days, but all three molecular weights shared consistent relative distributions in clearance and reticuloendothelial (RES) tissues (liver, spleen and femur). HA_{5K}-IRDye800 and HA_{20K}-IRDye800 accumulated in the kidneys, but HA_{100K}-IRDye800 was retained to a greater extent in the RES organs.

We than compared these biodistributions to age and gender matched tumor bearing animals and found consistent biodistributions in clearance and RES tissues. As expected when we compared conjugated and unconjugated dye, the pancreas fluorescent signal was on average 18 fold higher for conjugated HA-Cy7.5 dye than Cy7.5 alone, and 11 fold higher for conjugated HA-IRDye800 than IRDye alone.

With a better understanding of typical biodistributions of each of these formulations, we proceeded with profiling each agent using the same methodologies described in the prior study. The HA-Cy7.5 formulations, and in particular HA_{20K}-Cy7.5, had universally higher fluorescence intensity than any of the HA-IRDye800, but also had significant accumulation in the gastrointestinal tract. While less intensely fluorescent, the HA-IRDye800 had greater comparable contrast between tumor and normal adjacent tissue, and nearly no GI accumulation. The fold change between tumor tissue and adjacent tissue fluorescence was as follows: 0.14 (Cy7.5), 2.10 (HA5k-Cy7.5), 3.59 (HA20k-Cy7.5), 9.17 (HA100k-

Cy7.5), as detected at 825 nm, and 0 (IRDye800), 2.17 (HA5k-IRDye800), 663.09 (HA20k-IRDye800), 52.32 (HA100k-IRDye800), as detected at 810 nm.

In our ex vivo analysis we found that all of our agents accumulated in diseased but not healthy or unaffected tissues with HA_{20K} outperforming HA_{5K} and HA_{100k} for both dye conjugations. We further validated our FIGS system by comparing the relative fluorescent intensities detected by our system to the read out from a LI-CORE pearl imaging system. Disease status of the tissues was confirmed by a pathologist.

Using the Curadel Lab-FLARE RPI and FDA approved Fluobeam 800 we further evaluated our two lead candidates and found that HA_{20K}-Cy7.5 has consistently high accumulation in RES tissues, and while HA_{20K}-IRDye800 has significantly less signal in RES tissues, it is evident that is cleared through the kidneys with background signal in the kidney, ureters, and bladder. Both agents were not only able to label lesions smaller than 7 mm, but expressed enhanced contrast of metastatic tissues in the lymphatics that were all confirmed via pathology.

These results confirm that modifying the molecular weight of our HA-NIRF dyes significantly impacts tissue clearance and the properties of contrast agents. The ability to modify biodistributions of such agents broadens a potential toolkit for tailoring intraoperative imaging contrast agents which could yield in more curative procedures and better patient outcomes.

Overall, our collaborative efforts yielded commercially relevant technologies for both basic and translational research of pancreatic cancer development and treatment. Our AIW enables previously unobtainable access to longitudinal changes in a tumor challenged pancreas microenvironment in vivo. While this technology is protectable in the patent scope, our market research and customer discovery has indicated greater access to the technology could be achieved through open access of the CAD files. This will allow any researcher with access to a 3D printer the ability to scale and customize the tool to

their needs. As for our HA derived contrast agents, while commercially promising, fail to avoid the obviousness clause that would bar patentability. As we continue to develop and refine these contrast agents, we will emphasize novel synthesis and fluorescent agents that will enable the patentability necessary to de-risk the associated costs of human clinical trials.

Figure Thirteen: (A) This diagram depicts the FIGs imaging modality used to compare interoperative uses of NanoICG vs ICG. The FIGs system utilized both NIR and brightfield imaging simultaneously. (B) 24 hours post intravenous injection of either agent (NanoICG depicted on right, ICG on left), animals were sacrificed and opened to expose visceral organs. Prior to imaging the spleen and livers were removed to prevent background tissue from saturating the image. Tissues were labeled as follows: Int – Intestine, PT – pancreatic tumor, SI – small intestine, and St – stomach. This particular FIGs system utilizes a handheld spectroscopic pen which acts as an excitation source. In the bottom most panel we see the overlay of the brightfield image from the top and the NIR spectral information from the middle panel.

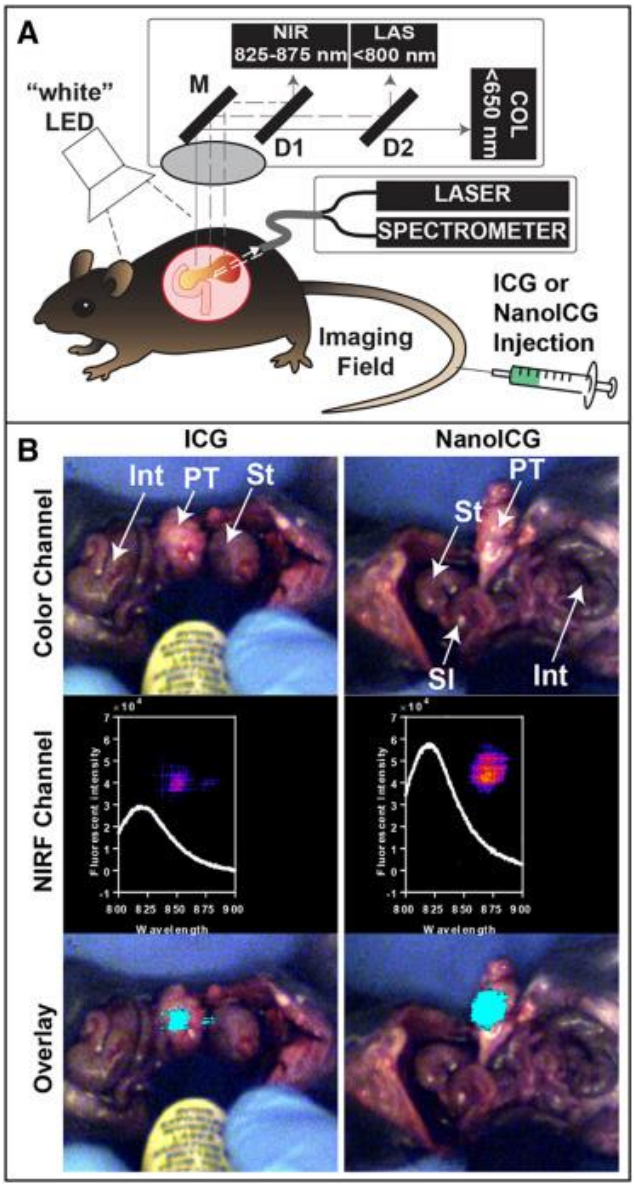


Figure Thirteen: FIGs imaging modality and example interoperative tissue imaging.

Figure Fourteen includes (A) a brightfield image of the pancreas presented head to tail from left to right and (B) NIRF image of the same tissue. The numbers indicate discrete points of spectroscopic acquisition locations as reflected in (C).

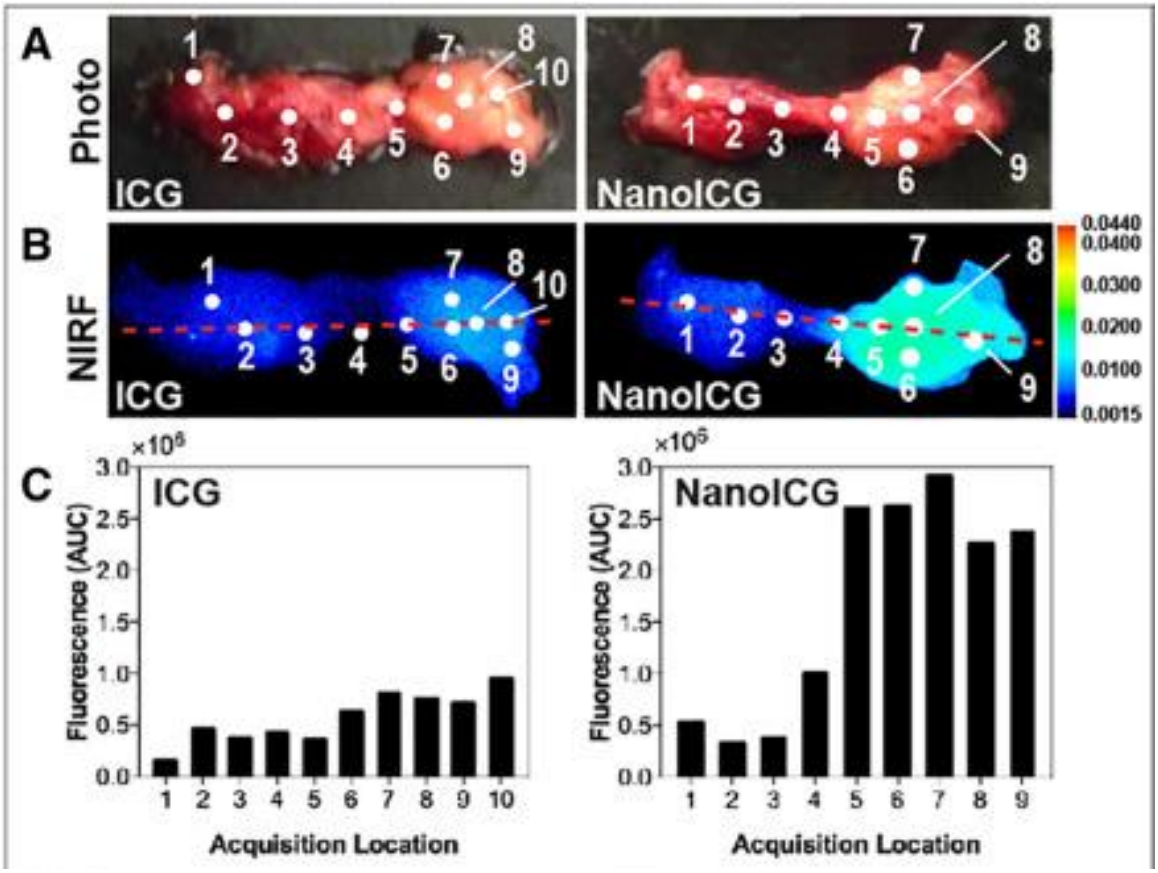


Figure Fourteen: Spectroscopic acquisition of excised tumor bearing pancreas

Figure Fifteen shows the two channel visible and visible with NIR images collected during FIGS imaging. Our handheld spectroscopic pen was directed at PDAC tumor tissue (PDAC) and then adjacent unaffected pancreas (UP) for each of our HA-NIRF groups. The green crosshairs indicate point of spectroscopic excitation. The white dots outline the entire macroscopically evident tumor tissue.

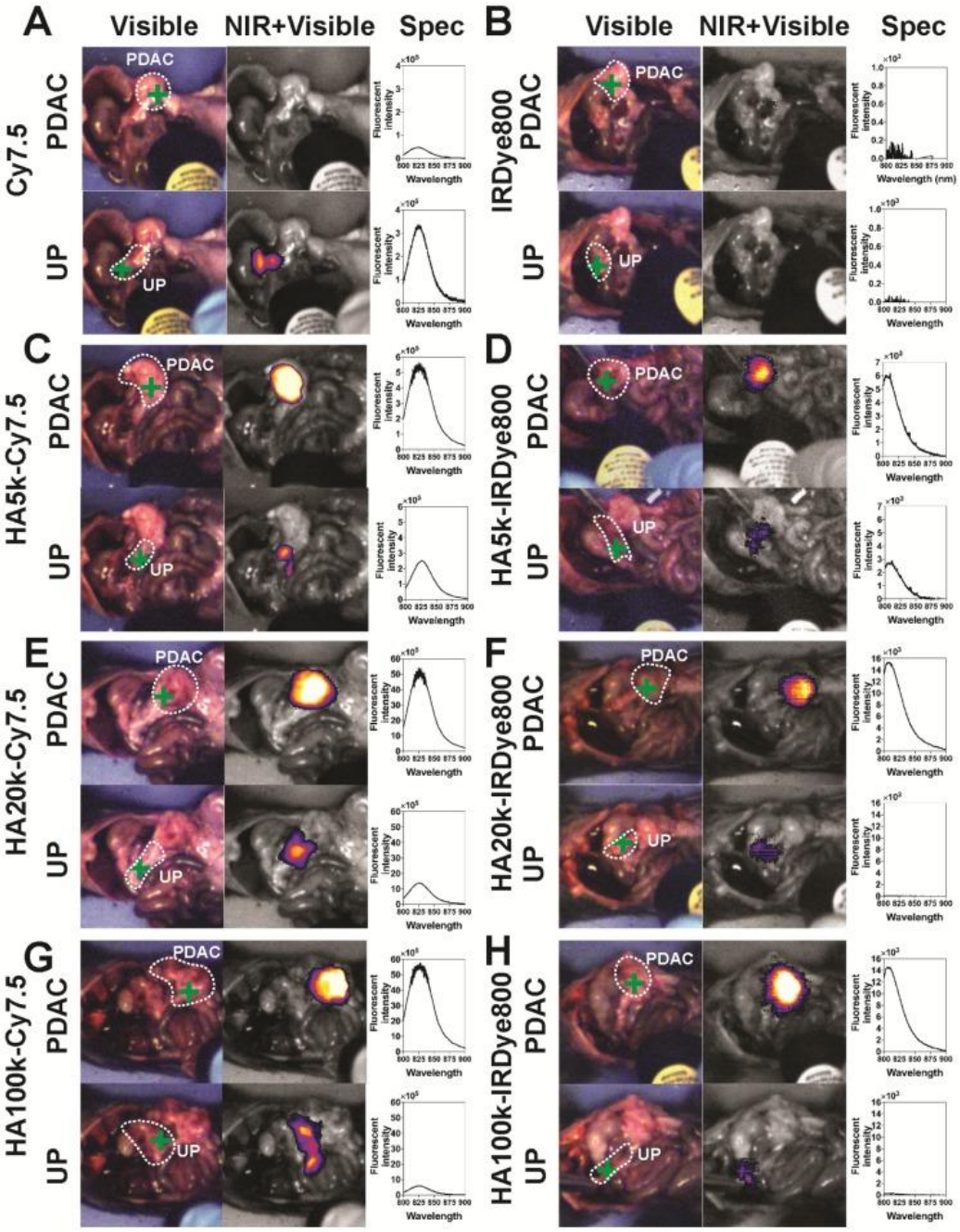


Figure Fifteen: Performance comparison of varied molecular weight HA-NIRF in tumor and tumor adjacent murine pancreatic tissue.

Figure Sixteen A/C Lab-FLARE RP1 whole body images of PDAC bearing mice compared directly to B/D Fluobeam 800 imaging system. NIRF accumulation is indicated in A/C by cyan pseudocolor and in B/D by grayscale. Both systems indicate Cy7.5 conjugates are cleared through liver and RES organs while IRDye800 conjugates are renally cleared. These images also capture the discernable uptake of NIRF into intraperitoneal metastasis. A/C Organs of interest are outline in yellow and labeled as follows: 1- Heart, 2- Lung, 3- Liver, 4- Stomach, 5- Uninvolved pancreas, 6+7- Primary pancreatic tumor and involved spleen, 8- Intraperitoneal tumor, 9- Kidney, 10- Small intestines, 11- Large intestines, 12- Femur.

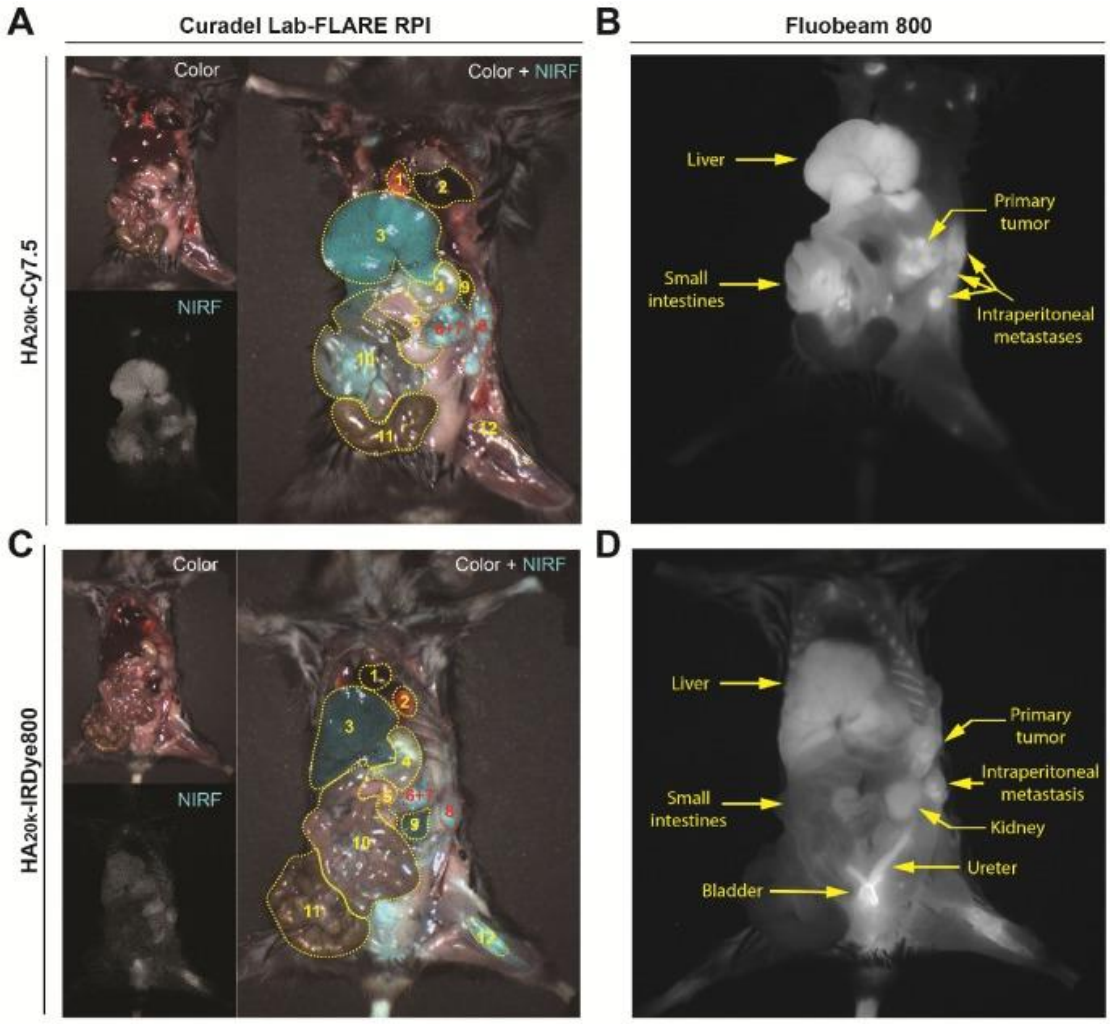


Figure Sixteen: Curadel and Fluobeam 800 whole body images of PDAC bearing murine model

Chapter Four[1]:

Compiled works in expanding community access and appreciation of Science Technology Engineering and Mathematics

[1] The material presented in this chapter have been previously published under the following reference: (Crawford et al, 2021).

Observational analysis: STEM educational outreach event enhances STEM career preparedness, but not appreciation for scientific methodology

Abstract

Substantial, involved, and expensive efforts to promote the dissemination of scientific knowledge and career interest in the Scientific, Technological, Engineering and Mathematical (STEM) fields are enthusiastically supported by many scientific, federal, and local organizations. The articulated underlying goals for these efforts include an enhanced public understanding of science and science-related policy, increased diversity in STEM careers, and an increase in the future STEM workforce. This effort is primarily driven by the underperformance of the United States in STEM fields, including poor test performance and limited number of students pursuing STEM degrees. Despite this investment, attitudes towards STEM have not notably changed. The goal of this project was to determine the feasibility of evaluating students' attitudes towards STEM in response to a previously established scientific outreach event, which was designed to address three common goals in STEM outreach: STEM literacy, diversity and inclusion, and career preparedness. We found there was a notable difference in the attitudes towards scientific activities and interest in pursuing a "Science Career." Strikingly, interest in hypothesis development, the keystone of all STEM disciplines, was the least liked of all the activities offered during the event. Our data suggest that events designed to enhance interest in pursuing a STEM career may benefit from different elements compared to events designed to increase understanding of STEM literacy concepts, such as hypothesis development.

Introduction

The United States is currently experiencing a long-standing labor shortage of prepared Scientific, Technological, Engineering and Mathematical (STEM) workforce employees and regularly reports some of the lowest STEM program retention rates and percentage of citizens pursuing STEM graduate education compared to other industrialized countries (The National Academies, 2014; Casey, 2012). The President's Council of Advisors on Science and Technology projects a labor market shortfall of nearly 1 million STEM professionals than are predicted to matriculate in the coming years (Olson, 2012). Projections indicate that, in order to fulfill this shortage, an increase of 34% of STEM bachelor's degree holders will be required (Xue, 2015). Given the nature of the industries that rely on STEM graduates, a shortage in the labor market compromises not only the United States' innovation and global economic position, but our health and security as a nation (Anonymous, 2005). Additionally, components of STEM education, including math and critical thinking, are of universal importance in all career fields and contribute to society's ability to reason key issues of policy (Macilwain, 2013)

In order to address this labor shortage problem, significant monetary and temporal resources have been devoted to increasing the pool of STEM graduates by shaping positive attitudes toward STEM subjects. Despite the US government's significant annual financial investment of nearly three billion dollars for the last decade to support STEM education programming, self-reported student data collected as part of the college entrance process by the American College Testing (ACT) indicate there has been no increase in interest in STEM education over the last decade (US Accountability Office, 2018; American College Testing, 2018). While many agencies continue to demand improvement to STEM recruitment strategies, little initiative has been taken to evaluate the effectiveness of current spending strategies (Macilwain 2013; The National Academies Press, 2014).

STEM outreach programs can be structured in a variety of ways, including week-long camps, one-day events, and monthly programs with scientists, commonly with the goal to increase discipline-specific knowledge and encourage students to pursue careers in STEM (Gibson, 2002; Hodges, 2016; Olson, 2012). Typically, inquiry-based activities are designed to provide hands-on experiences and develop critical thinking skills; however, the effectiveness of this approach in achieving the desired goals is rarely assessed or critiqued, despite being necessary to improve future events. Our physiology-based single-day outreach event for middle school students utilized inquiry-based activities to both highlight the scientific method in practice and deepen knowledge of the cardiovascular and visual systems.

STEM Event Goals

To establish evaluation criteria necessary for quality improvement of our STEM outreach event we first identified the common goals conserved across typical outreach programs from federal, state, and privately sponsored groups. Typical program goals fell into three categories, which we identified as STEM literacy, diversity and inclusion, and career preparedness (Gibson, 2002; Hodges, 2016; Mohr-Schroeder, 2014).

Increased Literacy: The first category of program goals consists of building a strong foundation for scientific and STEM literacy. This includes not only an understanding of basic math and science, but also experience with critical thinking and problem solving. These skills are valuable for nearly all citizens, not just those actively working in STEM fields. The emphasis on STEM literacy comes from the transferable skills of problem solving into many decision-making processes including personal health, financial decisions, civic matters, and parenting (Lau, 2011). Higher productivity in any of these key areas are assumed to produce greater economic opportunities for society (Rothwell, 2013).

Increased Diversity: The second common educational goal encompasses increasing diversity and inclusion in STEM. Continued efforts to promote STEM literacy to historically underrepresented groups

in science (including racial and ethnic groups such as African Americans, Latinos, and American Indians, as well as low-income individuals) is emphasized throughout many federal programs (Pandya, 2012). To diversify the future scientific workforce, efforts should be made to ensure minority representation in outreach programs. Inclusion of historically underrepresented groups is expected to increase total levels of innovation (Bell, 2018).

Increased Career Preparedness: Lastly, federal STEM goals tend to include aims for improving students' readiness to fulfill the demand for STEM careers (Anonymous, 2005). Because STEM professionals encompass fields as diverse as agriculture, energy, healthcare, and defense, their economic impact is far-reaching (Langdon, 2011).

We set out to evaluate our annual, established event in the context of the three main goals of STEM outreach and determine if these were being addressed by our event. The evaluated program consisted of activities as outlined in Figure 1. We hypothesized that, with the incorporation of a five-minute post event student survey, we would be able to identify key strengths and weaknesses of our event, thus enabling us to deliver a more effective event in the future.

Methods

In order to evaluate our hypothesis, we assembled a volunteer outreach team composed of faculty and students from the University of Nebraska Medical Center (UNMC) to execute an educational, scientific, and physiology-themed outreach event at an urban, Nebraska public middle school. UNMC has sponsored this event for over five years. The established event was created based on input from the participating school's teachers and focused on physiology-based concepts from the school's curriculum. We placed special emphasis on the steps of the scientific method. Two faculty members from the College of Medicine co-organized the event using a previously established event outline (Clark, 2019).

Three volunteer teams, consisting of three graduate students each, had 48 minutes with a classroom of 25-28 seventh grade students, totaling 265 students that participated on the event day.

Event activities

On the day of the event, each volunteer team was assigned to a classroom. An outline of the 48-minute classroom session is included in Figure 1. Prior to splitting the class into two groups for the hands-on activities, a classroom lead volunteer introduced the group as "scientists". The volunteers then lead the students in a discussion asking, "What is science?" and "Who wants to be a scientist when they grow up?". Classroom leads then presented how scientific discovery is the reason for much of humanity's knowledge of the world, as well as development of new technologies and medical advances; further, classroom leaders then described how this information is often shared with others through what is written in textbooks, such as the ones they use in school. We also talked about the process of how scientists design experiments to answer questions and solve problems. Next, we introduced the class to a famous experiment by Dr. John Snow, who hypothesized that a certain water pump was the source of London's cholera outbreak in 1854. He tested his hypothesis by removing the handle of the specific pump and found the number of local cholera infections and deaths slowed remarkably. We chose this example for its clear representation of the steps of the scientific method to ensure easy comprehension for the seventh-grade students. We then divided students into two groups of 12-14 individuals to partake in hands-on activities on the visual system and cardiovascular physiology as detailed in Appendix I: Instructor's Guide (Crawford, 2021). At the end of each session, we allotted 10 minutes for students to ask the volunteers any questions they had about physiology, accomplishments in the laboratory, and daily activities of scientists.

Visual System Activity

The visual system activity started by reviewing the anatomy of the eye with dissecting a fixed sheep eye (Nebraska Scientific, Omaha, NE), and focused on specific structures such as the cornea, iris, pupil, lens, retina, and optic nerve. We let the students feel the firmness of the sclera and lens and drew special attention to the opalescent tapetum lucidum with its notable appearance.

The latter portion of the visual system activity reviewed the scientific method. We asked the students questions related to each part of the scientific method as detailed in Appendix 1: Instructor's Guide. For example, during the hypothesis step of the scientific method, we asked, "Based on your own experiences in life, do you think both eyes would be required for depth perception?". We performed a simple experiment in which students attempted to drop pennies into a cup to test their hypotheses. For step-by-step instructions for this experiment, please see Appendix I: Instructor's Guide.

We continued to review and carry out the scientific method with students by asking them to draw conclusions about their data and by engaging them in a discussion of their results. Our discussion with the students briefly explained how depth perception works and highlighted the importance of a robust sample size by showing individuals' depth perceptions occasionally deviated from the group as a whole. We also highlighted confounding variables and how the students might address them in future experiments. Finally, to demonstrate the assumptions we often make for a well-designed study, we asked the students in the first activity rotation if they expected similar results from the second half of their class. As an extension of the scientific method, some classrooms performed the X-ray Vision Activity, if time allowed. Individual talking points of the discussion and the X-ray Vision Activity can be found in Appendix 1: Instructor's Guide.

Cardiovascular Activity

The other hands-on activity reviewed cardiovascular physiology, beginning with a dissection of a fixed sheep heart. The brief anatomy lesson focused on the atria, ventricles, valves, aorta, chordae tendineae, and coronary arteries. See Appendix 1: Instructor's Guide for the labeled heart anatomy. In brief, we explained to the students that the heart is able to beat on its own, but the rate of this beating changes in response to stimuli. We asked the students what activities might cause their heart rate to change.

The latter portion of the cardiovascular activity focused on the scientific method as detailed in Appendix 1: Instructor's Guide. Similar to the visual system activity detailed previously, we asked students questions related to each part of the scientific method. To test their hypotheses, we guided students through a hands-on heart rate activity. Following the activity, we continued to review and carry out the scientific method with the students by drawing conclusions and having a discussion. During the discussion, we asked students if their conclusions led to more questions and prompted them to make additional hypotheses about heart rate. We explained that asking new questions based on results and conclusions drives science forward. Individual talking points of the discussion can be found in Appendix 1: Instructor's Guide.

IRB Approval

The participating school's Research Review Committee approved the event. The committee required a letter of intent, a copy of our evaluation survey, and an opt-out parental consent form. The letter of intent detailed the content of the outreach event, introduced the outreach team, and explained the purpose of the evaluation survey. Students' parents received the opt-out parental consent form via email. This document informed parents of the event, the purpose of the evaluation, and gave the option to refuse their child's participation in completing the survey. The approved final survey included

modifications to maintain student anonymity. We were not permitted to ask the students' sex, gender, or ethnicity. UNMC's Institutional Review Board approved this as an exempt study.

Survey

We evaluated our event using student self-reported answers on general knowledge, appreciation, career awareness, and career interest from the survey, which students completed post event. The survey sought to assess the event's effect on STEM literacy and career preparedness. This was accomplished using specific questions that correspond to the conserved STEM outreach goals, which are listed in Table 1. The survey results were analyzed by the Methodology & Evaluation Research Core (MERC) from the University of Nebraska-Lincoln.

Table 1: Survey questions regarding conserved STEM outreach goals

STEM Literacy
"Which one was your favorite [eye] station or activity?"*
"Which one was your favorite [heart] station or activity?"*
"This event increased my appreciation of science."[#]
"This event increased my knowledge of science."[#]
Career Preparedness
"This outreach event helped me to understand what a career in science is like."[#]
"After attending the event how has your interest in careers involving science changed?" [†]

*Options: developing hypothesis; dissection; testing hypothesis; X-ray vision (Visual System Station only)

[#]Likert ratings (1 = strongly disagree; 2 = disagree; 3 = agree; 4 = strongly agree)

[†]Likert ratings (1 = decreased; 2 = no change; 3 = increased)

Results

Event Goals: Improving STEM Literacy

Results from the post-survey support the observations made by the volunteer instructors during an informal debriefing session (Figure 3A); students reported their preferred activity to be the dissections, followed by hypothesis testing. The lowest preferred activity was hypothesis development. When student responses were stratified by station (cardiovascular system vs. visual system), the activity preference remained the same (Figure 3B & 3C, respectively).

At the conclusion of the event, most students responded to the survey question “This event increased my appreciation of science” with “strongly agree” or “agree” (Figure 4A). Additionally, over 80% of students responded to the survey question “This event increased my knowledge of science” with “strongly agree” or “agree” (Figure 4B).

Overall, the instructors anecdotally reported very high student engagement throughout the demonstrations and activities. Observationally, the volunteer instructors also observed higher levels of engagement during the dissection activities, with more hesitant participation during the hypothesis development and experimental design discussions. Students appeared especially engaged with questions about each instructor’s disease model and the progress they were making as scientists.

Event Goals: Improving STEM Inclusion and Diversity

The population of instructors included multiple minority groups that a diverse classroom of students might identify with. Of these scientists, 75% identified as female, 50% identified as underrepresented in STEM fields, and 37.5% were first generation college students. It is of note that our original survey sought to include demographic data about the students partaking in the outreach event. However, the survey was modified due to concerns about student anonymity, and we were unable to

collect such data. Public information about the demographics of students in the [middle school] in 2019-2020 where this activity was presented showed that 24.5% of students were Black or African American, 36.8% were Hispanic or Latino, and 25.6% were white. Each of the other categories, American Indian or Native Alaskan, Asian, native Hawaiian or Pacific Islander, or more than one, had fewer than 10% of the total students. While we could not analyze the student responses with regard to demographic criteria, we were able to extend this science outreach to a diverse population. Therefore, future events are needed to comprehensively address quality improvement for determining inclusion and diversity of this particular established outreach event and will depend in part on community trust in the scientific process to collect potentially identifying information.

Although the lack of data left us unable to stratify impact on individual participants of different backgrounds (thus leaving us unable to identify impact on our diversity and inclusion goal in our survey), our diverse set of instructors were still able to engage students in activities that engage trust in the scientific community – a common goal for inclusion and diversity activities (Kouper, 2010). Unfortunately, our survey lacked questions regarding student attitudes surrounding trust of science and scientists, an area for future consideration.

Event Goals: Improving STEM Career Preparedness

As shown in Figure 5A, approximately 70% of students self-reported that “This outreach event helped me to understand what a career in science is like”. While most students indicated no change in their interest in careers involving science, a greater percentage of the total students reported they were more interested, compared to less interested, in a career involving science after the event (Figure 5B).

Instructors noted that student engagement and questions were highly responsive to familiar disease pathologies that affect them and their families. Thus, numerous focal points and discussions from this outreach event centered around exploring and improving students’ readiness for STEM

careers. As a result, over 70% of students reported that their understanding of science careers was improved, and more than 21% of students said their interest in a science career had increased (Figure 5A and 5B).

Discussion

Event Design and Execution

We conducted an observational study to evaluate the effectiveness of an established outreach event, which has been conducted numerous times over the past five years. Our evaluation examined three well-conserved STEM outreach goals among federal, state, and privately funded scientific outreach efforts. The goals included enhancing STEM literacy, increasing inclusion and diversity, and improving career preparedness (Foster, 2011; Gibson, 2002; Hodges, 2016;). The ability of the event to meet these goals was accessed via indicators of student attitudes towards science including knowledge of science, appreciation of science, career awareness, and career interest. Although our event has been well received by both the teachers, as indicated by their continued request to perform the yearly event, and the students, as indicated by feedback to their teachers, we had not previously evaluated the program in terms of meeting the commonly defined goals of STEM outreach.

Event Goals: Improving STEM Literacy

The first goal of our outreach event was to promote scientific literacy by generating understanding of the scientific method. Thus, we divided both activities (visual system and cardiovascular system) into equal time allotments to focus on both goals. One-time allotment discussed anatomical terminology through dissection, while the other led students through an experiment using the scientific method. Our dissection activities consisted primarily of anatomical terms that the students had previously been exposed to in their anatomy curriculum in the classroom. Examples included the cornea, lens, and tapetum lucidum for the visual activity, and the ventricles and atria for the

cardiovascular activity. This allowed us to introduce new physiological concepts and transition the students into critical thinking activities such as hypothesis development and testing.

The instructors engaged students throughout the discussion and instruction of the scientific method (Figure 2) using guided prompts from a previously produced handout (see Appendix I: Instructor's Guide). During this portion of the activities, students made observations such as "I notice every one of us has two eyes. I wonder if having two eyes instead of one is important". Instructors used these observations to prompt students to generate guesses and speculations. Their guesses and speculation were then identified as hypotheses. While their hypotheses varied, the students had a nearly universal interpretation of the plotted data and were able to identify if their original predictions were supported as correct or incorrect. Discussions following the data interpretation encouraged students to further expand on their results and make additional hypotheses about their follow up questions. This discussion of the scientific method, combined with the dissection activities, allowed us to engage students in general recall as well as critical thinking and problem-solving skills. Thus, we concluded this event met our STEM literacy outreach goal to improve students' confidence in their knowledge of science (Figure 4B).

Student Activity Preferences & Preference Factors

The scientific method requires significant critical thinking, especially in the development of a hypothesis and analysis of results. Thus, critical thinking and the scientific method are highly emphasized aspects in outreach programs. In fact, practicing the scientific method and critical thinking skills are a conserved focus amongst many funding agencies, particularly federal funding agencies (Casey, 2012). However, limited resources exist detailing delivery of the scientific method to students. It is ironic that many of the instructors of outreach events, such as graduate faculty and students, are often individuals who work heavily with the scientific method every day, but who have overwhelmingly

underreported means for collecting data on the results of their outreach event and/or how to reformat event for quality improvement based on their results. Therefore, we utilized an established outreach event to consciously engage students throughout the process of the scientific method, particularly forming and testing a hypothesis (Figure 1 & 2). However, students reported an overwhelming lowest preference for the hypothesis generating activities and highest preference for dissection activities (Figure 3). These preferences held true for both the cardiovascular and visual system activities (Figure 3B and 3C, respectively), indicating that the responses were not biased by a particular instructor or station.

In our post analysis of the event, we considered a few factors that may provide insight towards improving the quality of future outreach events. Students' majority preference for the dissection may be a result of the hands-on nature of the dissection activity. This is supported by the fact that other hands-on activities, such as hypothesis testing, were preferred over activities that were not as hands-on, such as developing hypotheses. The hands-on approach of the dissection and hypothesis testing activities provides an opportunity for critical thinking and intellectual engagement for kinesthetic and visual learners. Students may have preferred the hands-on activities because they required easier, lower order thinking, such as general recall and recitation of information. Thus, future plans include requiring students to properly identify an anatomical feature before being allowed to touch the tissues. Having students apply what they have previously learned in the didactic lecture from their schoolteachers to the inquiry-based dissection provides an opportunity to incorporate critical thinking and intellectual engagement with the outreach instructor. This discussion and application of material has been previously shown to maximize learning and enhance critical thinking abilities (Freeman, 2014; Peters, 2002).

Of the select students who did not prefer the dissection activities, their questionnaire responses indicate this was largely due to them being off put by the specimens and their odors (formaldehyde from preserved specimens) and/or disinterested in the subject material. It is of note that, given the time

limitations of the outreach event, we were only able to engage with students in the context of biological and physiological sciences. We may have been able to capture more student interest if we have been able to present on a broader array of topics.

Impact on Student Attitudes Towards Science

Given the time limitations of only interacting with the students one time and for less than an hour (48 minutes scheduled), we were satisfied with the impact our scientist instructors had on students, as evidenced by the increase of self-reported student appreciation for science (Figure 4A). Considering eighth grade students who express interest in STEM are three times more likely to ultimately pursue STEM careers later in life (22), our event will likely positively affect the chances of these students pursue careers in STEM. Additionally, previous studies indicate that short-term inquiry-based approaches to outreach, such as the outreach approach we took, result in improved attitudes toward science compared to traditional lecture-type instruction (Gibson, 2002). However, it is still unclear whether this change in appreciation is long-lasting.

Impact on Scientific Knowledge

Most agencies sponsoring STEM outreach events recognize that all citizens should have science knowledge even if they are not interested in STEM or STEM careers (American College Test, 2018; National Career Development Association, 2020). With nearly 80% of our students self-reporting an increase in science knowledge following our event, we have evidence indicating our outreach program is establishing and enhancing scientific knowledge for the students. Furthermore, the self-reported increase in science knowledge supports the idea that this established outreach event is meeting some components of the STEM Literacy goal regarding students' understanding STEM career jargon (see Figure 5A).

Event Goals: STEM Inclusion and Diversity

The second goal of the evaluated outreach event was to differentiate the impact on multiple underrepresented student populations including minority, female, and students from families without degree holders. We had 9 instructor scientists present for the activities that represented multiple minority groups that a diverse classroom of students might identify with.

While IRB limitations prohibited us from collecting response data from students regarding these demographics, other components of inclusion were still included. Our outreach event provided an opportunity to build trust among the students with the scientists through interactive "Question and Answers" at the conclusion of the activities. For students who do not pursue careers in science, trust in scientists, including one-on-one interactions with advanced level scientists, contributes to an increased willingness to engage with and be responsive to social issues relating to STEM fields (Kouper, 2010). To build this trust, instructors led the conversation by telling students about the grade school experiences that led them to college, experiences during college, and how they leveraged these experiences to attend graduate school. This discussion included examples of resources available to first generation college students, college readiness programs, and alternative career paths in STEM. Instructors also designated time for briefly explaining their current research and career goals.

Event Goals: Improving Career Preparedness

The third goal of our evaluation was to determine the impacts of our event on students' understanding and interest in science careers. To this end, our efforts began with real world examples of major scientific discoveries and a discussion on the logical rationale leading to these discoveries, such as the aforementioned telling of Dr. John Snow's cholera experiment (see Appendix 1: Instructor's Guide). Instructors revealed how the rationale that led to those discoveries was part of the scientific method that they use every day as scientists. This discussion was expanded upon as instructors detailed how the

scientific method applies to their current research in cancer, hypertension, immunology, microbiology, and regenerative medicine. Importantly, students also inquired about alternative careers in STEM not represented by the instructor group.

Impact on Student's Understanding of Science Careers

We directed the volunteers to introduce themselves using the broad term of "scientist", as opposed to a narrow identifier of their specific career interest such as physiologist, biochemist, or cancer biologist, to place emphasis on STEM fields as opposed to a specific niche. This appeared to synergize well with the rest of our approach; we were satisfied with our impact on student's understanding of STEM careers (Figure 5), as students showed a greater than 70% agreement that the event increased their understanding of careers in science (combined agree and strongly agree responses). The National Career Development Association (2011) recommends middle school guidance counselors emphasize helping students develop increased self-understanding of how career interest, aptitudes, and work values apply to themselves. We were able to increase students' self-understanding of science careers, so the career is seen as an approachable opportunity to pursue (Figure 5).

Impact on Student's Interest in Pursuing a STEM Career

One of the main goals of agencies sponsoring STEM outreach events is to fulfill the demand for STEM careers by increasing students' interest in pursuing a STEM career (National Academies Press, 2014). Not unexpectedly, as our program was a one-time event, most students reported no change (68.2%) in their interest in careers involving science. These results enhance the importance that our event influenced 21.2% of students to be more interested in science careers. This is not surprising, as informal learning environments, like the ones used in our event, are successful in motivating and increasing students' interest in science careers and disciplines (Hodges, 2016; Macilwain, 2013; Yanowitz, 2016).

Study Limitations

We identified limitations to this study. First, as an observational study with limited time and interactions with the students, we were unable to make long-term conclusions about how our event affected students. Next, the approved questionnaire did not include any questions regarding demographic information, one of the major STEM outreach goals (diversity and inclusion). This information can help inform the analysis of our questionnaire results, as individuals from different backgrounds, such as socioeconomic factors, may be predisposed to have more positive or negative experiences with introductory science (6). This data set would also aid us in analyzing our impact on underrepresented populations – a major theme throughout STEM outreach program goals.

Lack of a control group- it could be plausible that any activity (not just the ones presented) would have the positive self-reports

Conclusions

Effective STEM education has immense impacts on the social and economic status of a nation, which is a large part of why the US spends nearly three billion dollars every year with the goal of improving student attitudes towards STEM. Educators and scientists need the best available tools to accomplish this feat. Establishing mechanisms for continual event improvement should be an essential component to any regularly scheduled program. In this study, we demonstrated that, while our event was intentionally focused on activities that would engage students in the scientific method and critical thinking skills, students reported being least interested in these activities. Additionally, we found that, while only a secondary emphasis of our event, student increase in awareness and interest in STEM careers were increased greater than we anticipated.

By placing the common goals of STEM outreach we found in the literature and funding agency websites in several “buckets”, we were able to differentiate what criteria we most wanted to focus on when evaluating their impacts (Figure 6). From there, we evaluated what activities from our event might contribute most to student attitude changes. By asking students to rank their activity preferences and provide feedback on their attitudes towards STEM as a result of the event, we were able to identify clear strengths and weaknesses of our event.

All these valuable data will guide changes in our event design and evaluation methods, which we will continue to expand upon and use with future events. We plan to adapt our event to engage students differently with the scientific method, including game-based play as opposed to mere discussion of experimental design, and continue to evaluate how the event impacts student attitudes. We will also expand upon our survey questions to capture broader influences of STEM attitudes including criteria such as trust. As we modify our event to address student feedback, we will continue to

survey students and reassess the strengths and weaknesses of our event for continued quality improvement.

Acknowledgments

The authors are immensely grateful for the contribution of time and effort from all the additional volunteers who participated in this project: Safwan Elkhatib, Cassandra Moshfegh, and Sarah Hortman. Additional thanks to the MERC Core at the University of Nebraska-Lincoln for analyzing the survey results.

Figure Seventeen: Workflow of introductory (blue), hands-on (purple), and wrap-up (green) activities for the day of the STEM outreach event. Observational evaluation (orange) of the event occurred at a later date.

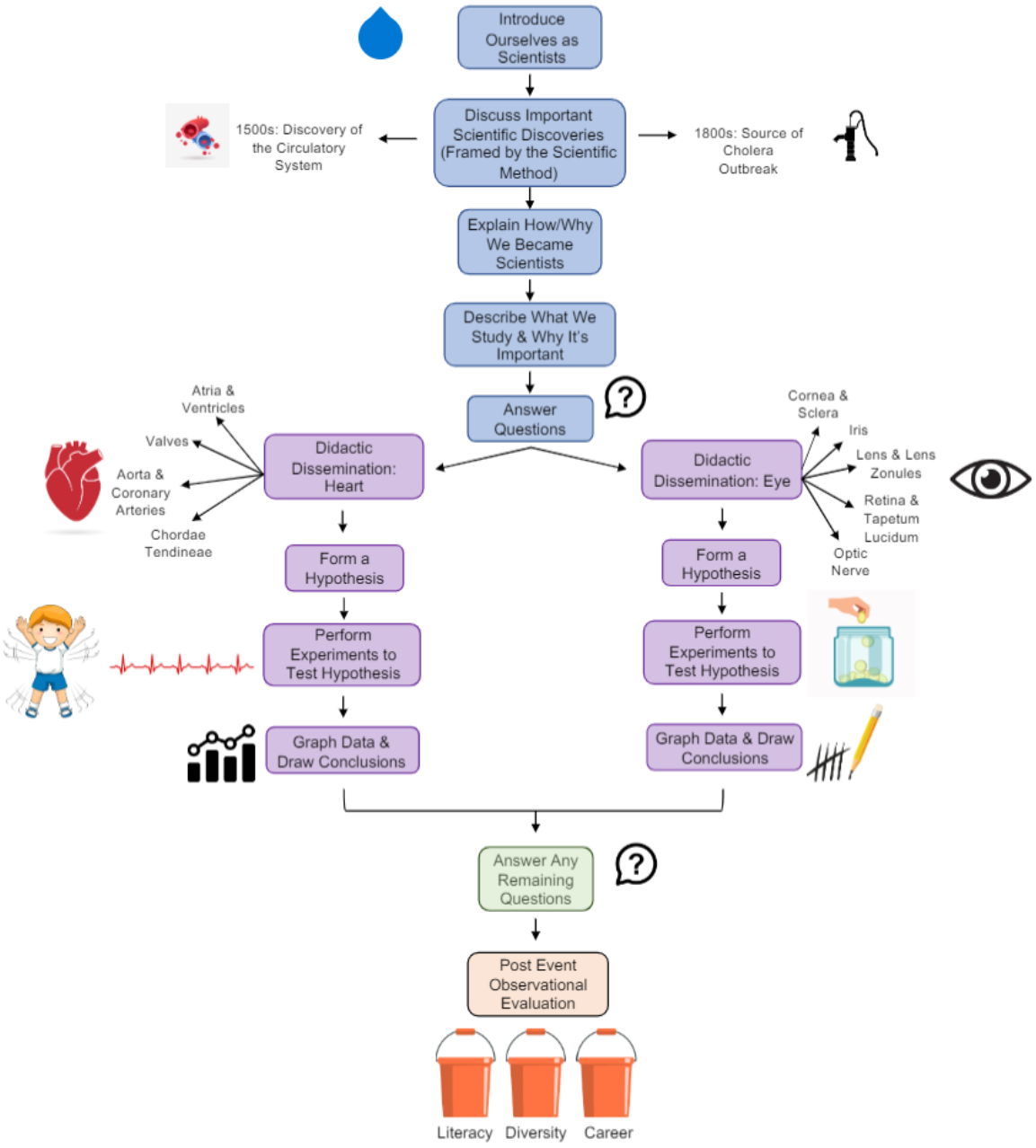

Introduce Ourselves as Scientists
Discuss Important Scientific Discoveries (Framed by the Scientific Method)
1500s: Discovery of the Circulatory System
1800s: Source of Cholera Outbreak
Explain How/Why We Became Scientists
Describe What We Study & Why It's Important
Answer Questions
Didactic Dissemination: Heart
Atria & Ventricles
Valves
Aorta & Coronary Arteries
Chordae Tendineae
Form a Hypothesis
Perform Experiments to Test Hypothesis
Graph Data & Draw Conclusions
Didactic Dissemination: Eye
Cornea & Sclera
Iris
Lens & Lens Zonules
Retina & Tapetum Lucidum
Optic Nerve
Form a Hypothesis
Perform Experiments to Test Hypothesis
Graph Data & Draw Conclusions
Answer Any Remaining Questions
Post Event Observational Evaluation
Literacy
Diversity
Career

Figure XX

Figure Eighteen: Overview of scientific method instruction. The outreach activity began with an introduction to the whole class. Students were divided into two groups and completed one hands-on activity followed by the other. The class came back together for open forum questions about the didactic material, science as a career, and the scientific method.

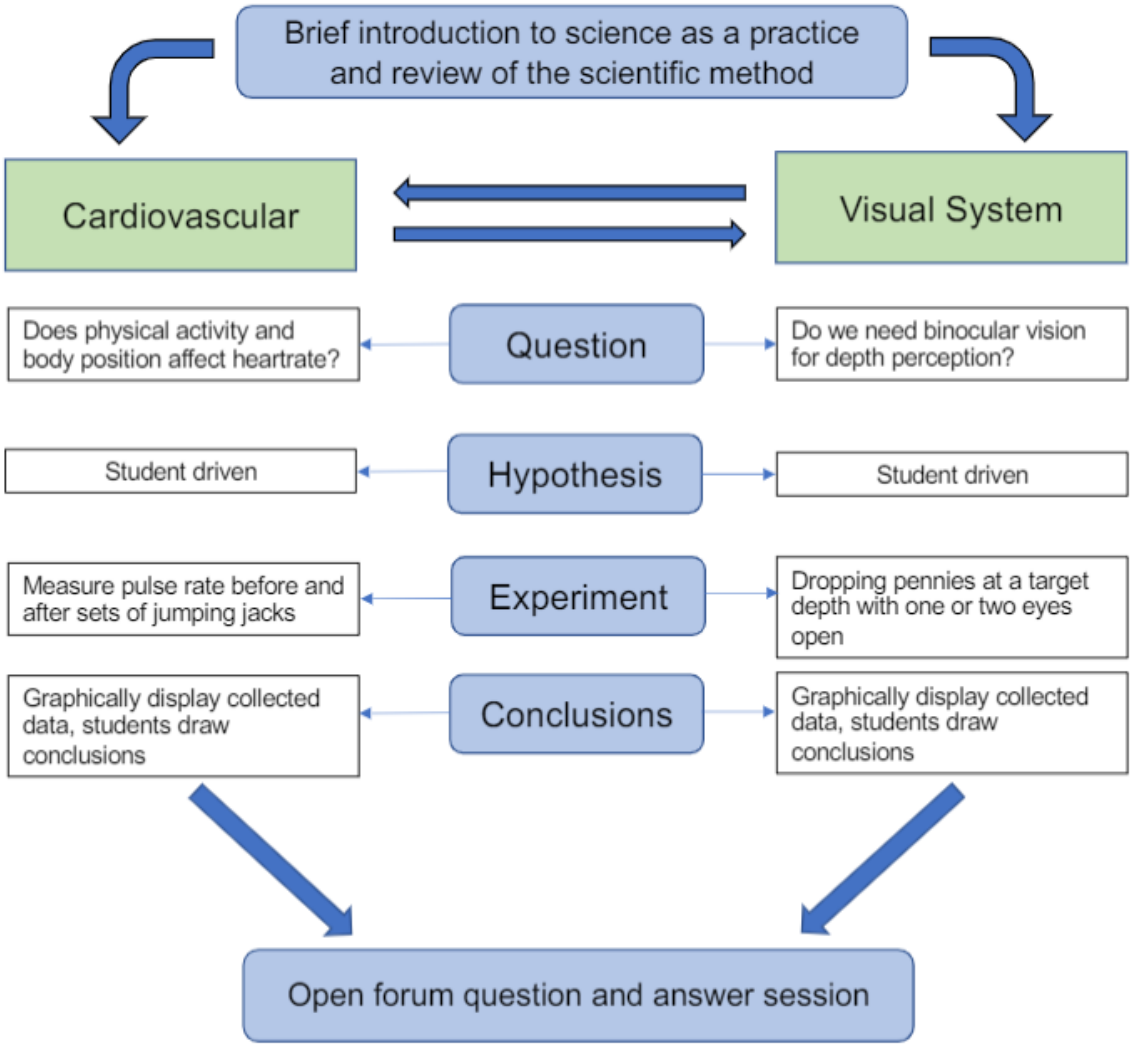

Brief introduction to science as a practice and review of the scientific method
Cardiovascular
Visual System
Does physical activity and body position affect heartrate?
Question
Do we need binocular vision for depth perception?
Student driven
Hypothesis
Student driven
Measure pulse rate before and after sets of jumping jacks
Experiment
Dropping pennies at a target depth with one or two eyes open
Graphically display collected data, students draw conclusions
Conclusions
Graphically display collected data, students draw conclusions
Open forum question and answer session

Figure Nineteen: Students preferred dissection activities. As a measure of STEM literacy, students reported (A) their preferred activity, and were asked (B) Which one was your favorite [eye] station or activity?" and (C) "What was your favorite [heart] station or activity?" (n=247).

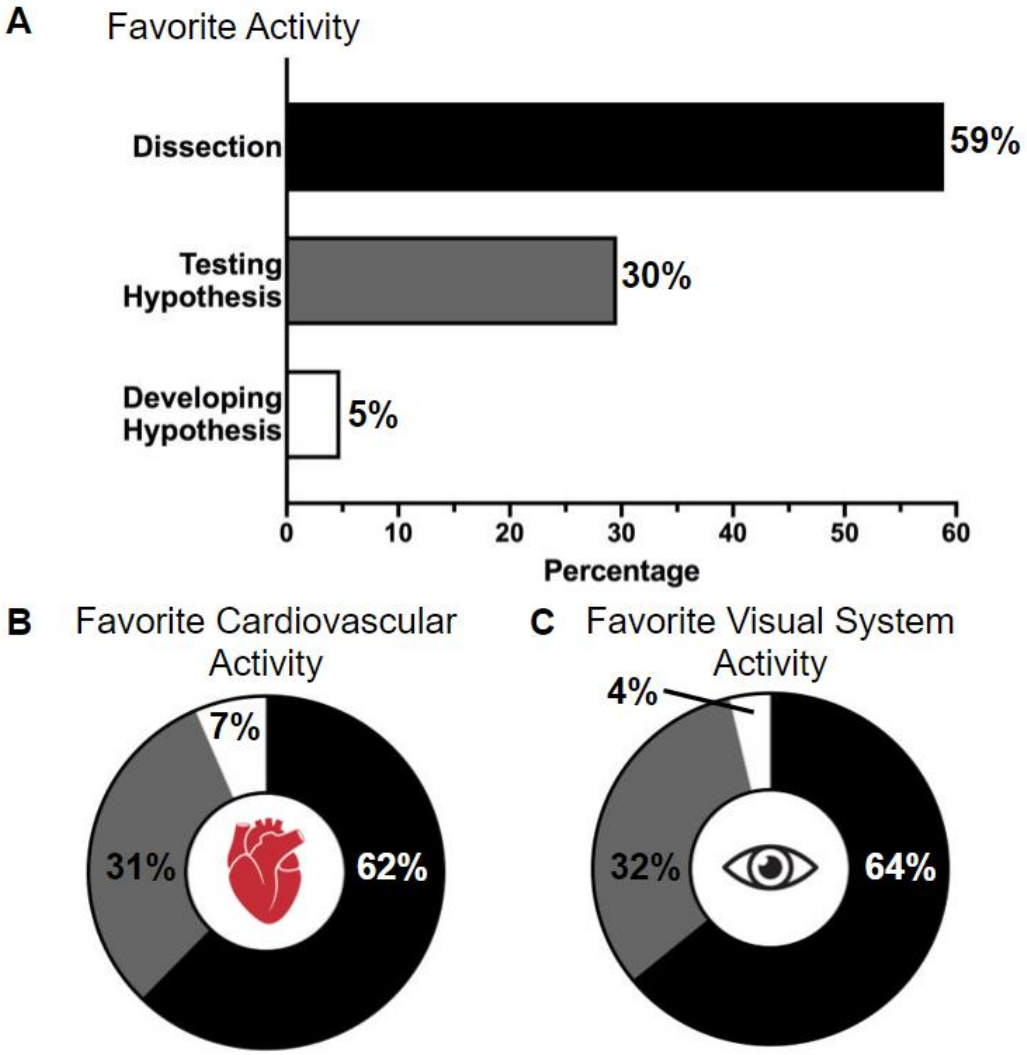
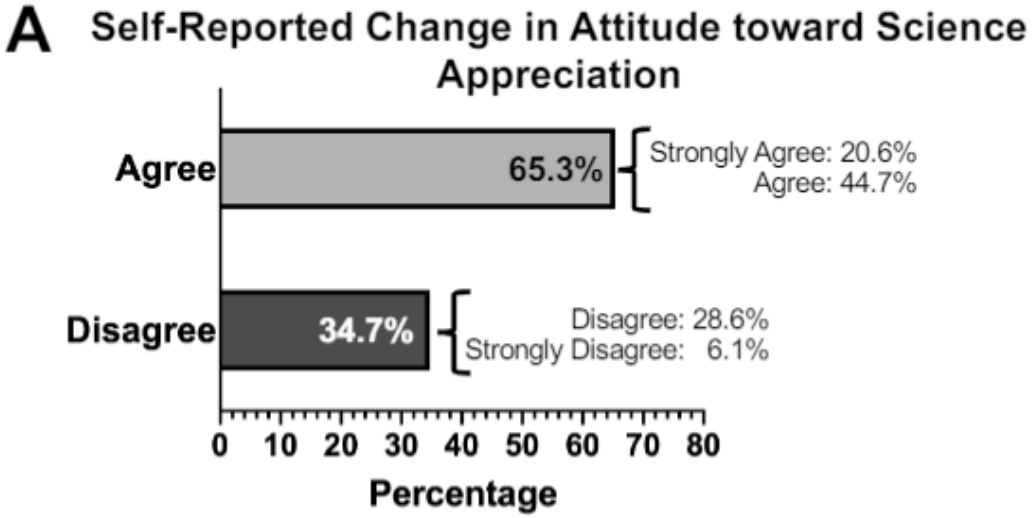

A
Favorite Activity
Dissection
59%
Testing Hypothesis
30%
Developing Hypothesis
5%
0
10
20
30
40
50
60
Percentage
B
Favorite Cardiovascular Activity
7%
31%
62%
C
Favorite Visual System Activity
4%
32%
64%

Figure Twenty: Majority of students self-reported a gain in appreciation and knowledge of science following physiology-based outreach event. As a measure of STEM literacy, students were asked if (A) "This event increased my appreciation of science" and (B) "This event increased my knowledge of science," Agreement or disagreement of the statements are reported with more detailed breakdown of responses. (N=262, 265).


A
Self-Reported Change in Attitude toward Science Appreciation
Agree
65.3%
Strongly Agree: 20.6%
Agree: 44.7%
Disagree
34.7%
Disagree: 28.6%
Strongly Disagree: 6.1%
0 10 20 30 40 50 60 70 80
Percentage

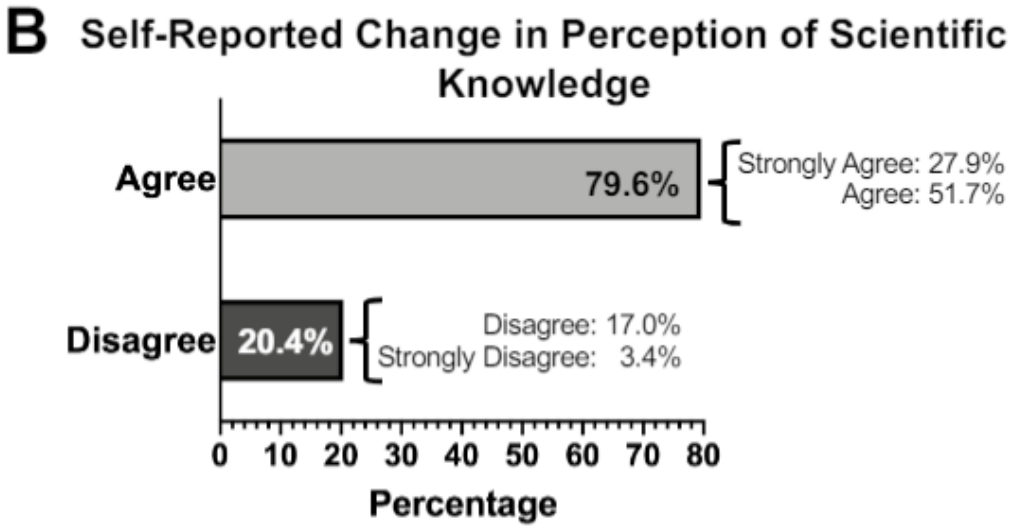
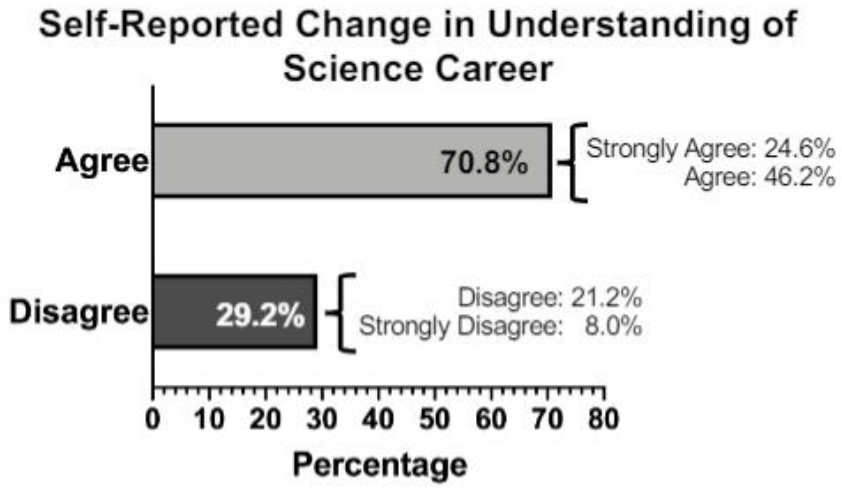

B
Self-Reported Change in Perception of Scientific Knowledge
Agree
79.6%
Strongly Agree: 27.9%
Agree: 51.7%
Disagree
20.4%
Disagree: 17.0%
Strongly Disagree: 3.4%
0 10 20 30 40 50 60 70 80
Percentage

Figure Twenty-one: Majority of students self-reported a gain in understanding of science careers. As a measure of assess career preparedness, students were asked if (A) "This outreach event helped me to understand what a career in science is like" and (B) "After attending the event how has your interest in careers involving science changed?" Agreement or disagreement of the statements are reported with more detailed breakdown of responses. (N = 264, 265).

A


Self-Reported Change in Understanding of Science Career
Agree
70.8%
Strongly Agree: 24.6%
Agree: 46.2%
Disagree
29.2%
Disagree: 21.2%
Strongly Disagree: 8.0%
0 10 20 30 40 50 60 70 80
Percentage

B

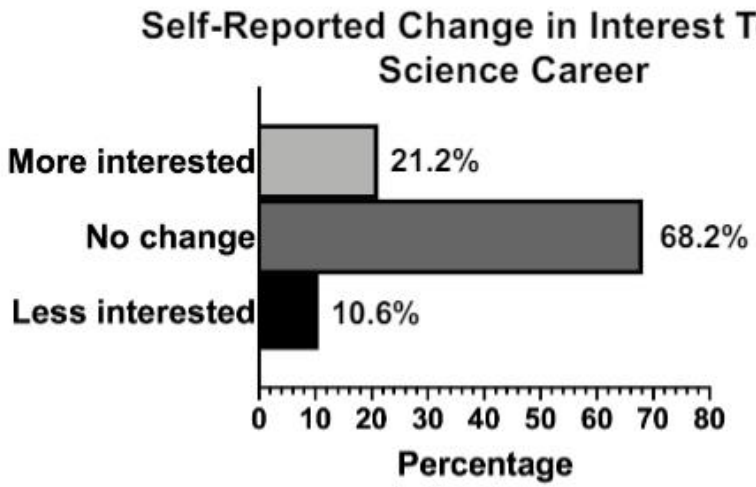
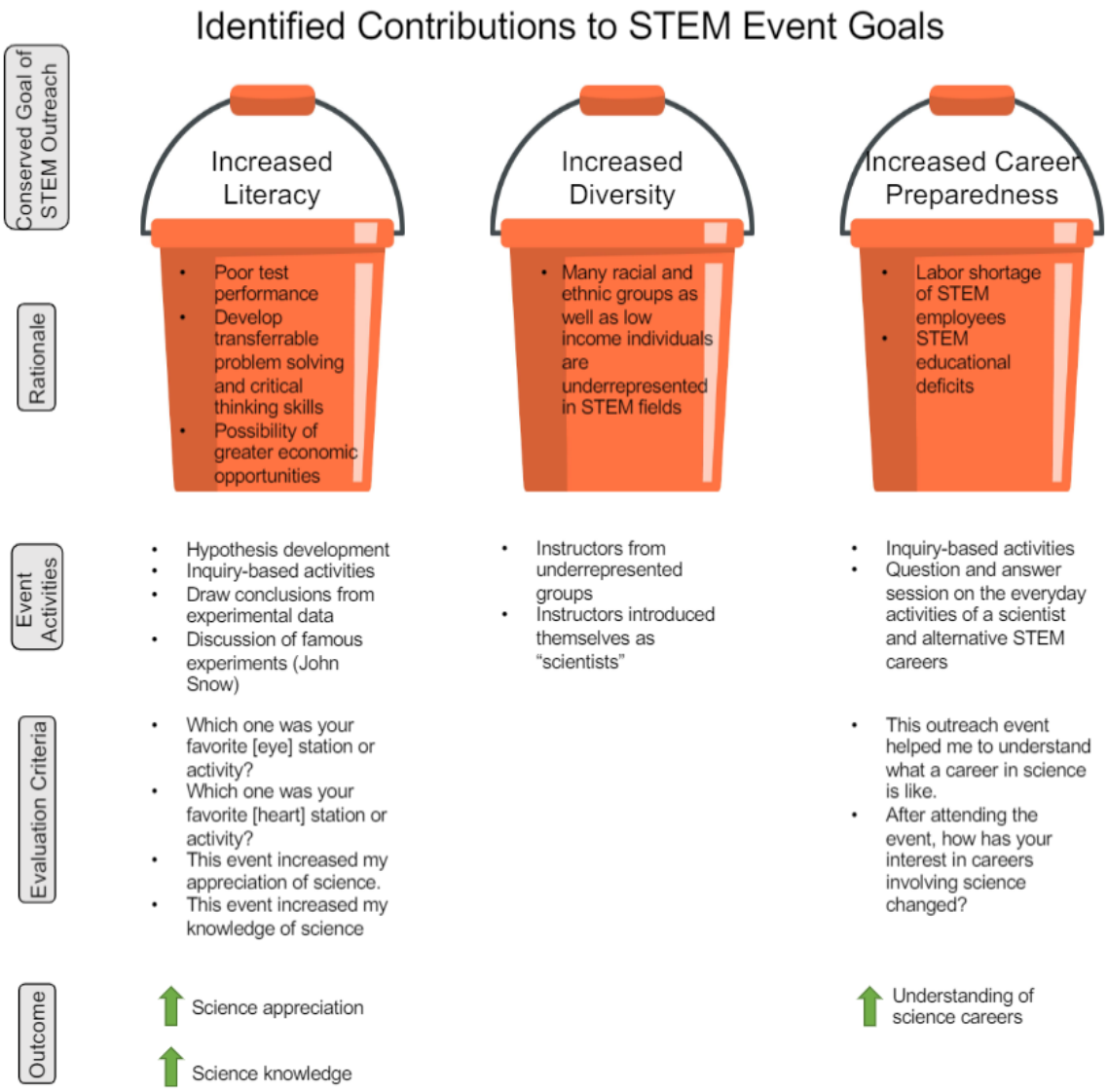

Self-Reported Change in Interest Towards Science Career
More interested
21.2%
No change
68.2%
Less interested
10.6%
0 10 20 30 40 50 60 70 80
Percentage

Figure Twenty-two: Summary of our STEM outreach event. For each STEM event goal, we consider the issues addressed by each goal (rationale), the steps our event took to address these issues (event activities), how we evaluated our event (evaluations criteria), and results of the student surveys (outcomes).


Identified Contributions to STEM Event Goals
Conserved Goal of STEM Outreach
Increased Literacy
Increased Diversity
Increased Career Preparedness
Rationale
Poor test performance
Develop transferrable problem solving and critical thinking skills
Possibility of greater economic opportunities
Many racial and ethnic groups as well as low income individuals are underrepresented in STEM fields
Labor shortage of STEM employees
STEM educational deficits
Event Activities
Hypothesis development
Inquiry-based activities
Draw conclusions from experimental data
Discussion of famous experiments (John Snow)
Instructors from underrepresented groups
Instructors introduced themselves as "scientists"
Inquiry-based activities
Question and answer session on the everyday activities of a scientist and alternative STEM careers
Evaluation Criteria
Which one was your favorite [eye] station or activity?
Which one was your favorite [heart] station or activity?
This event increased my appreciation of science.
This event increased my knowledge of science
This outreach event helped me to understand what a career in science is like.
After attending the event, how has your interest in careers involving science changed?
Outcome
Science appreciation
Science knowledge
Understanding of science careers

Chapter Five:

Overall Discussion and Future Directions

In chapter one we showed that pharmacological inhibition of CDK5 significantly prolonged survival and reduced tumor burden of multiple pancreatic adenocarcinoma mouse models. Given the highly conserved rates of CDK5 over expression in human pancreatic adenocarcinoma samples, CDK5 remains a compelling therapeutic target to progress PDAC and other solid tumor treatment options. We have consistently provided evidence through our studies that there are therapeutic benefits to the combination use of CDK5 inhibitor CP668863 with nucleoside analog gemcitabine.

As cell cycle regulators, CDKs have been a long sought-after target for pharmacological inhibition. However, most pharmacological targeting techniques have been focused on generating small molecule inhibitors that target the ATP binding site. This binding site is highly conserved amongst CDKs rising the challenges of generating unique inhibitors for the family of CDKs. While CP668863 has a greater inhibitory effect on CDK5 than CDK2, its potential inhibition of CDK2 warrants further investigation into its therapeutic impact. This is of particular importance as CDK inhibitors advance from 1st to more selective 2nd generation inhibitors like CDK4/6 inhibitor Palbociclib (1).

Given CDK5's role in neuronal recruitment and organization, we set out to interrogate the effects of CDK5 inhibition on perineural invasion of PDAC tumors in vivo. Because the current methods for investigating neuronal organization throughout solid tumor formation were so limiting, we sought a novel approach to longitudinally following neural formation throughout tumor progression. Our 3D printed abdominal imaging windows and microscope platforms enable regular imaging of key tumor structures and microenvironment changes throughout tumor progression.

We deemed CDK5 a target of exceptional interest not only based on previously published and promising indications of prolonged cancer survival, but because of the integral role it appears to play in pain signaling and glucotoxicity, both of which commonly lead to comorbidities and worsened outcomes in PDAC patients. As previously stated, inhibitors of CDK5 have been shown in vivo to reduce nocifensive

behaviors in response to induced inflammatory state. Since many cancers including PDAC exist in a chronic inflamed and painful state, we wanted to know if CDK5 inhibitor CP668863 would reduce nocifensive behavior in tumor challenged animals.

Using a modified version of the Sevcik et. al protocol for scoring nocifensive behaviors via posture, exploratory behavior, grooming, and coat texture, we established a baseline nocifensive behavior score for 8 week old female nude mice observed in isolation for five-minute intervals (Sevcik, 2006). We additionally incorporated a grid system for monitoring the animals' degree of movement. These nude mice were orthotopically implanted with 1x10^6 S2013 cells and then individually enrolled into a randomly selected treatment group once they had radiographically obvious tumors greater than 3 mm in any diameter, as described in chapter two. Five mice from each treatment group were randomly selected for weekly behavior scoring. Two blind reviewers scored each mouse.

To improve this study, the effects of survival and tumor burden need to be evaluated with separate mouse cohorts than pain and glucose tolerance. Because the mice are individually enrolled into treatment groups on different days and the treatment schedule varies by day, the treatment schedule cannot be synchronized. This means that the randomly selected mice from a single treatment group may have received different treatments on different days. For instance, of the five combination therapy recipients evaluated at any timepoint, mouse one may be recovering from the prior day's gemcitabine injection, mouse two a CP668863 injection, and the other three may have had a treatment free day.

Based on the plasma concentration pharmacokinetic data from chapter two, CP668863 recipient mice would be evaluated 30-60 minutes post injection. Additionally, a non-opioid analgesic control group could add context to the study, although non-opioid analgesics can have confounding and adverse effects on patients (Munir 2007).

In chapter two we demonstrated functionality of lab created tools for longitudinal in vivo imaging. This imaging modality's improvement on previous technologies include compositions that can be 3D printed quickly and cheaply, and a design that facilitates deep tissue imaging on an upright microscope while greatly reducing the imaging artifacts that result from the imaged animal's breathing. AIW bearing mice were viable and maintained normal behavior (no changes in weight, ambulation, posture, or grooming practices) for six months, and in the case of female subjects, even when housed together.

Previous protocols for investigating changes in neuronal organization in response to tumor challenge were limiting. Options included digitally reconstructing large numbers of histological sections into a 3D rendering so that the viewer could reconstruct the neural organization throughout numerous z stacked slide scans. A more efficient option was to probe tumor lysates for protein expression which is an effective means to understanding changes in type and density of neuronal fibers present, limits understanding the spatial relationship of neural organization patterns. Optical tissue clearing provides an option for understanding both the type and density of neuronal fibers with the context of spatial arrangement, however we have found optically clearing tumor bearing pancreases to be exceptionally challenging in part because of the dense necrotic tissue which is not easily perfused with clearing or labeling agents. Additionally, none of the aforementioned techniques could facilitate longitudinal imaging of an individual.

We also identified diverse options for modifying hyaluronic acid nanoparticles conjugated to fluorescent agents for use as intraoperative contrast agents. While physiochemically entrapped ICG enhanced tumor specific uptake as compared to free ICG, there was fluorescence in clearance organs that would limit its use for pancreatic resection. However, through modification of the molecular weight of our collaborator's HA nanoparticles, we were able to tune our agents for uptake and clearance from RES to renal tissues.

In chapter four we established a protocol for surveying and analyzing student perspectives on STEM event impacts on conserved goals of outreach events. Establishing mechanisms for continual event improvement should be an essential component to any regularly scheduled program. In this study, we demonstrated that, while our event was intentionally focused on activities that would engage students in the scientific method and critical thinking skills, students reported being least interested in these activities—particularly hypothesis generation. Additionally, we found that, while only a secondary emphasis of our event, student increase in awareness and interest in STEM careers were increased greater than we anticipated.

Overall, the studies and tools presented in this dissertation support a productive ecosystem of resources available to PDAC researchers and other translational researchers and scientists engaged in STEM outreach. Our studies resulted in numerous publications and a provisional patent application.

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