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Crissy L. Tarver

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MOLECULAR ROLE OF ANGIPOIETIN-LIKE 4’s CARBOXY-TERMINAL DOMAIN IN PANCREATIC DUCTAL ADENOCARCINOMA PROGRESSION

Crissy L. Tarver

A Dissertation

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in The Biotechnology Science and Engineering Program to The School of Graduate Studies of The University of Alabama in Huntsville

Huntsville, Alabama

2019
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Submitted by Crissy L. Tarver in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology Science and Engineering and accepted on behalf of the Faculty of the School of Graduate Studies by the dissertation committee.

We, the undersigned members of the Graduate Faculty of The University of Alabama in Huntsville, certify that we have advised and/or supervised the candidate on the work described in this dissertation. We further certify that we have reviewed the dissertation manuscript and approve it in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology Science and Engineering.

[Signatures and dates of the committee members]

[Program Chair's signature and date]

[College Dean's signature and date]

[Graduate Dean's signature and date]
Abstract
The School of Graduate Studies
The University of Alabama in Huntsville

Degree  Doctor of Philosophy  Program  Biotechnology Science and Engineering

Name of Candidate  Crissy Lynette Tarver

Title  Molecular Role of Angiopoietin-like 4’s Carboxy-Terminal Domain in Pancreatic Ductal Adenocarcinoma Progression

Pancreatic ductal adenocarcinoma (PDAC) represents 85% of pancreatic cancers. With early stage diagnosis, PDAC has a relative five-year survival rate of 34% but drops to 3% with metastasis. There is a lack of distinct symptoms and dependable biomarkers which delays diagnosis, leading to poor prognosis. Angiopoietin-like 4 (ANGPTL4) protein has been indicated in the progression of several types of aggressive cancer. ANGPTL4 is natively present in two fragments, an amino-terminus coiled-coil domain (nANGPTL4) and a carboxy-terminus fibrinogen-like domain (cANGPTL4), which have demonstrated diverse biological functions. Biochemical studies have been performed but a full structural understanding of functions and interactions is not available.

Overexpression of ANGPTL4 has been found in pancreatic tumor tissues of all stages. Its complexity and ability to target various cancer cell microenvironments, endothelial cells, and metabolic activities makes it a prime candidate for therapeutic intervention. The goal of this research was to acquire knowledge of cANGPTL4’s molecular role in PDAC progression by comprehending its mechanisms of action through structure-function studies that would enable the development of therapeutics. In order to accomplish this goal, differential expression of hypoxia induced ANGPTL4 was compared
between hypoxia-induced expression of ANGPTL4 between common pancreatic and breast cancer cell lines. Furthermore, crystal structures of cANGPTL4 was investigated in complex with various ligands such as carbohydrates, fatty acids, and glycerol.

Hypoxia-induced expression of ANGPTL4 was shown to be largest in Panc-1 (pancreatic cancer) and MDA-MB231 (breast cancer) cell lines. Both of these forms of cancer have highly invasive phenotypes. Capan-1, pancreatic cancer cells that are not well differentiated, showed the third largest increase in ANGPTL4 expression. cANGPTL4 was shown to be colocalized with integrin-β1 in Panc-1 cells with immunofluorescence and confocal imaging. Previous research revealed cANGPTL4 interacts with integrin β1, resulting in reduced cell-cell contact mediating metastasis.

Finally, three high resolution crystallographic structures were determined of cANGPTL4 complexed with a different ligand. A crystal structure of cANGPTL4 in complex with glycerol was solved to a crystallographic resolution of 2.11Å, cANGPTL4/palmitic acid complex to 1.75 Å, and cANGPTL4/myristic acid complex to 2.37 Å. The binding of palmitic acid was revealed in crystal structures, cell culture assays with fluorescence, and a thermal shift assay using a Tycho NT.6 from Nanotemper. This combined data reveals that cANGPTL4 aids in pancreatic cancer cell progression by providing both energy and biological molecules.

Abstract Approval: Committee Chair

Program Chair

Graduate Dean
Acknowledgments

First and foremost, I want to thank my supervisor Dr. Marc Pusey. He has taught me how to perform good experimental research. I appreciate all contributions of his time, ideas, and project funding to make my Ph.D. experience productive as well as stimulating. His joy and enthusiasm for research was very contagious for me, even during tough times.

The members of Argonne National Lab SBC-CAT and SSRL at Stanford have contributed immensely to my time on the beamline. These groups have been a source of friendships, camaraderie, good advice, and collaboration. I would like to acknowledge Dr. Krzysztof Lazarski, Dr. Randall Alkire, Dr. Kemin Tan, and Ms. Michelle Radford at Argonne National Lab, Dr. Aina Cohen at SSRL, and Dr. Edward Snell at Hauptman-Woodward Medical Research Institute for all of their assistance. Dr. Lazarski worked countless hours keeping 19BM and ID beamlines running at optimal capacity. Dr. Alkire and Dr. Tan taught me how to collect, process, and use diffraction data for Sulfur-SAD phasing. Ms. Radford worked around my class and teaching schedules to make data collections possible. I very much appreciated Dr. Aina Cohen’s friendship, confidence in my abilities, enthusiasm, and willingness to work with me doing whatever it took to accomplish a goal. I am grateful for Dr. Snell’s guidance and direction in protein crystallography and scientific writing.

Dr. John Rose, Dr. Scott Pegan, and Ms. Caroline Langley at the University of Georgia in Athens were very gracious and helpful with the collection of ITC data. I was able to collect ITC data as needed, and Caroline assisted me in ITC data processing with the Malvern MicroCal system. Dr. Laurel Karr, Dr. Eric Fox, and Mr. David Donovan at
NASA/ Marshall Space Flight Center kindly allowed me to use their facility and cell culture equipment for this project. Mr. Donovan was amazing at maintaining the equipment and sterility of the cell culture area. Additionally, cell culture experiments were performed in the lab of Dr. Gordon MacGregor at the University of Alabama in Huntsville. Thank you, Dr. MacGregor for all of your advice pertaining to cell culture.

I have appreciated the camaraderie and local cell culture expertise of Dr. Debra Moriarity and Timley Watkins. Thank you, Dr. Moriarity, for teaching me the art of performing cell culture and for providing some of the cell lines used in this project. Thank you, Timley, for the late nights you worked on the confocal microscope with me imaging cells and podocytes.

My time at UAH was made enjoyable in large part to the many friends that became a part of my life. I am grateful for the friendship of Judy Cooper, Debbie Carswell, Jana Whittle, Brandi Wilson, James Wolfsberger, and Scott Strange whom endured this journey with me. Judy Cooper, our music has bonded us for life!

I would like to thank each committee member for their guidance and patience throughout this project: Dr. Roger Cruz-Vera (Chair), Dr. Sara Cooper, Dr. Robert McFeeters, Dr. Edward Snell, and Dr. Marc Pusey.

Lastly, I would like to express my gratitude to my family for all their love and support. My husband, Roy, whose encouragement during the final stages of this Ph.D. is very appreciated. To Ethan, Madeleine, and Victoria Tarver: thank you for exhibiting extreme patience with me during this entire process. I love you 3000!
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ABBREVIATIONS and ACRONYMS

aa Amino acid
ACh Acetylcholine
ACS American Cancer Society
Akt/PKB Protein kinase B
Ala Alanine
ANGPTL Angiopoietin-like
cANGPTL4 Angiopoietin-like 4, C-terminal domain
fANGPTL4 Angiopoietin-like 4, Full length
nANGPTL4 Angiopoietin-like 4, N-terminal domain
Apaf-1 Apoptosis protease-activating factor 1
ApoB Apolipoprotein B
ATP Adenosine triphosphate
CA19-9 Carbohydrate antigen
Ca\(^{2+}\) Calcium
CT Computed tomography
DAG Diacylglycerol
DMEM Dulbecco's modified eagle's medium
ECM Extracellular matrix
E-cadherin Epithelial cadherin
EDTA Ethylenediaminetetraacetic acid
EGFR Epidermal growth factor receptor
EMT Epithelial-mesenchymal transition
<table>
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<th>Abbreviation</th>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>ETC</td>
<td>Electron transport chain</td>
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<tr>
<td>FABP</td>
<td>Fatty acid-binding protein</td>
</tr>
<tr>
<td>FADH2</td>
<td>Adenine flavin dinucleotide (reduced)</td>
</tr>
<tr>
<td>FATP</td>
<td>Fatty acid transport protein</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>GC</td>
<td>Golgi complex</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293</td>
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<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
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<tr>
<td>HIF-1</td>
<td>Hypoxia inducible factor-1</td>
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<tr>
<td>IPN</td>
<td>Intraductal papillary neoplasm</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase pathway</td>
</tr>
<tr>
<td>MCN</td>
<td>Mucinous cystic neoplasm</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced)</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
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<tr>
<td>NET</td>
<td>Neuroendocrine tumors</td>
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<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PDA</td>
<td>Pancreatic Ductal Adenocarcinoma</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
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<td>PIPLC</td>
<td>Phosphatidylinositol phospholipase C</td>
</tr>
<tr>
<td>PPARs</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>RAF</td>
<td>Rapidly Accelerated Fibrosarcoma</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
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<td>TCA</td>
<td>Tricarboxylic acid</td>
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<td>Tie</td>
<td>Tyrosine kinase</td>
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<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
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<td>VLDL</td>
<td>Very low-density lipoprotein</td>
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Chapter 1

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive form of pancreatic cancer that is accompanied by a low probability of survival (Noone et al., 2017). The American Cancer Society (ACS) estimates that approximately 45,750 people will succumb to this disease in 2019. PDAC has a relative five-year survival rate of 34% with early stage diagnoses that drops to 3% with metastasis (Noone et al., 2017). The deficiency of a meticulous diagnostic exam makes early stage diagnoses a serious challenge. A poor prognosis is also due to a lack of comprehension of PDAC’s complex molecular processes. This complexity is due to intrinsic factors, such as genetic and epigenetic heterogeneity within a tumor, and extrinsic factors, such as tumor microenvironment. These factors result in large variations in the degree to which cancer cells invade and metastasize (Gupta and Massagué, 2006; Prasetyanti and Medema, 2017; Assenov et al., 2018).

Angiopoietin-like 4 (ANGPTL4) has been indicated in the progression of several types of aggressive cancer (Padua et al., 2008; Hu et al., 2011; Nakayama et al., 2011; Kirbya et al., 2016), but its structure and function are poorly understood (Zhu et al., 2012; Tan et al., 2012). This lack of knowledge impedes the comprehension of this
protein’s basic biology. PDAC’s high mortality rate is due to its inclination to metastasize. Several studies found increased expression levels of ANGPTL4 in excised pancreatic tumor cells, and studies have suggested potential roles of this protein’s carboxy-terminal fibrinogen-like domain in tumor progression (Tan et al., 2012). For example, the carboxy-terminal fibrinogen-like domain of ANGPTL4 has been shown to facilitate metastasis by disrupting endothelial barrier integrity when interacting with integrin β1, VE-cadherin, and claudin-5. The binding affinity of these proteins was previously determined by surface plasmon resonance (SPR), but the interacting residues are not known (Huang et al., 2011). Furthermore, it is unknown if other biological molecules, such as carbohydrates or fatty acids, bind to ANGPTL4’s carboxy-terminal domain.

ANGPTL4 is natively present as a full-length protein and in two fragments, an amino-terminus coiled-coil domain (nANGPTL4) and a carboxyl-terminus fibrinogen-like domain (cANGPTL4), which have shown diverse biological functions (Lei et al., 2011). cANGPTL4 has been associated with angiogenesis, cancer cell invasion, cell migration, endothelial cell functions, and cell adhesion (Tan et al., 2012). Biochemical studies have been performed but a full structural understanding of functions and interactions is not available. ANGPTL4’s complexity and ability to target various cancer cell microenvironments, endothelial cells, and metabolic activities makes it a prime candidate for therapeutic intervention (Tan et al., 2012). The function of a protein can be inferred from its structure, and changes in protein conformations or binding to other proteins can lead to altered functions. The goal of this research was to acquire knowledge of cANGPTL4’s molecular role in PDAC progression by comprehending its
mechanisms of action through structure-function studies that would enable the development of therapeutics. The three specific aims of this study to accomplish this goal were:

> Aim 1: To understand how cANGPTL4 expression drives malignant phenotype.

> Aim 2: To extend the existing structure-function studies from bacterially produced to structure-function studies associated with human cancer cells.

> Aim 3: To determine specific residues of cANGPTL4 that interact with binding partners.

Differential expression of certain proteins has been correlated to phenotypic characteristics such as invasion, migration, tumorigenesis, and chemotherapeutic resistance (van der Horst et al., 2012). The observation that ANGPTL4 is highly expressed in multiple carcinomas and cell lines raised some interesting questions. As part of this study we explore:

> Is ANGPTL4 differentially expressed in pancreatic cancer cell lines? If so, is there a correlation between expression levels and phenotypic characteristics of pancreatic cancer cells?

> Are hypoxic environmental conditions necessary for expression and secretion of ANGPTL4 cancer cells?

> How does the expression levels of ANGPTL4 in pancreatic cancer cell lines compare to expression levels in breast cancer cell lines?

> Which isoforms of ANGPTL4 protein are secreted in pancreatic cancer cells?

We discovered that hypoxia-induced expression of ANGPTL4 was highest in Panc-1 and MDA-MB231 cell lines. Cells from both lines are considered to have highly
invasive phenotypes. Using immunofluorescence and confocal imaging, colocalization of cANGPTL4 and integrin β1 was seen in Panc-1 cells. Previous research revealed cANGPTL4 interacts with integrin β1, resulting in reduced cell-cell contact mediating metastasis. Finally, three high-resolution crystallographic structures were determined of cANGPTL4, each in complex with a different ligand. Palmitic and myristic acid were shown to bind to cANGPTL4, and the same binding site was also found to bind glycerol. This binding was observed in both crystallographic derived structures and inferred from cell culture assays. The binding of palmitic acid to cANGPTL4 was further supported by thermal denaturation assay. Results indicated a Kd ranging from 0.77 to 1.3 µM at a protein concentration of 1.0 µM, which is well within expected physiological concentrations (Abdelmagid et al., 2015). Furthermore, immunofluorescence and confocal imaging studies showed that the cANGPTL4/fatty acid complex were taken up by the cell.

The binding of cANGPTL4 to glycerol, myristic acid, and palmitic acid points to possible mechanisms for function for cANGPTL4. Unliganded cANGPTL4 binds to cell surface proteins, while the cANGPTL4/fatty acid complex does not, but is instead taken up by the cell. cANGPTL4 may offer a route for the delivery of drug molecules into cancer cells. Whether the fatty acid transport is just a means of supplying energy to the cell, or if it also includes a signaling function, remains to be determined.

We present these findings here.
Chapter 2

Background and Significance

2.1 The Pancreas

The pancreas is a retroperitoneal appendage with both exocrine and endocrine functions. A healthy pancreas appears lobular and yellow in color. This organ is divided into four sections: head, neck, body, and tail (Figure 1). The widest part is the pancreatic head, and it is positioned in the C-loop of the duodenum. The protrusion from the lower portion of the head of the pancreas is the uncinate process. It lies between the superior mesenteric vessels and the aorta. The pancreatic head and body are attached by a pancreatic neck that is positioned in front of the portal vein. Extending from the neck to the left is the body of the pancreas, which narrows into a tapering tail. This tail continues in the direction of the splenic hilum. A major pancreatic duct joins the pancreas to the common bile duct, and both ducts drain into the major duodenal papilla. It drains exocrine secretions as it runs across the pancreas from tail to head. An accessory pancreatic duct supplies additional drainage in some individuals. This duct drains separately into the duodenum at the minor duodenal papilla (Longnecker et al., 2018).

Similar to the gastrointestinal tract, blood supply to the pancreas comes from several main arteries. The celiac trunk and superior mesenteric artery supply blood to the pancreas. From the celiac trunk, the common hepatic artery branches into the hepatic and
gastroduodenal artery. The gastroduodenal artery branches into the anterior and posterior pancreaticoduodenal arteries that supply blood to the head of the pancreas. From the celiac trunk, the splenic artery produces the dorsal pancreatic artery, pancreatic magna, and caudal pancreatic artery. These supply blood to the pancreatic body and tail. Vessels of the extensive lymphatic system travel alongside the arteries in the pancreas (Longnecker et al., 2018).

The pancreas is both an exocrine and endocrine gland. A majority of the pancreas consists of exocrine, or acinar, cells which form clusters called acinus. Acinar cells
produce, store, and secrete various pancreatic enzyme proteins necessary for the digestion of nutrients (Figure 2) (Gorelick et al., 2018). Digestive enzymes such as amylase and lipase are active upon secretion since they are not known to damage pancreatic cells. Pancreatic proteases, such as trypsin and chymotrypsin, synthesized by acinar cells are stored as inactive proenzymes (trypsinogen and chymotrypsinogen) in zymogen granules to prevent pancreatic cellular damage (Pandol, 2011). Once stimulated by acetylcholine (ACh), cytosolic Ca$^{2+}$ concentrations increase and trigger exocytosis of zymogen granules into the pancreatic ductal system (Lee et al., 2005). These ducts are lined with columnar epithelial cells (abundant in carbonic anhydrase) that secret large volumes of water and bicarbonate ions to facilitate the transport of digestive enzymes into the duodenum (Figure 3) (Petersen, 2018; Nishimori et al., 1999). The alkaline pancreatic secretions neutralize the gastric acid in the small intestine, providing the neutral pH pancreatic digestive enzymes require for optimal activity (Nishimori et al., 1999). Inactive zymogens are converted into active digestive enzymes in the duodenal lumen. Trypsinogen is converted to trypsin after the cleavage of a peptide fragment by the enzyme enterokinase, an enzyme produced by duodenal cells. The active trypsin enzyme then activates chymotrypsinogen (Rinderknecht, 1986).

Pancreatic lipase, or steapsin, is a primary digestive enzyme secreted by the pancreas (Persson, 1989). The main function of this enzyme is the hydrolysis of fat molecules consumed in diet. Triacylglycerol (TAG) is broken down into monoacylglycerol and free fatty acid. Pancreatic lipase, unlike trypsinogen, is secreted in its active form but requires colipase (co-enzyme) for optimal activity. Serum
concentrations of this digestive enzyme increase during conditions such as pancreatitis (Persson, 1989).

Figure 2.2: Electron micrograph image of pancreatic acinar cells showing the endoplasmic reticulum, Golgi complexes, and zymogen granules involved in the synthesis and storage of digestive enzymes (Pandol, 2011). Image from https://www.ncbi.nlm.nih.gov/books/NBK54127.

Figure 2.3: Pancreatic ductal cells secrete an abundant amount of water and bicarbonate while transporting digestive enzymes to the duodenum (Persson, 1989).
Acinar cells have a large number of endoplasmic reticulum (ER), which allow them to produce and process proteins at a higher frequency than all other human cells (Palade, 1975). Digestive enzymes are synthesized in the rough endoplasmic reticulum (RER) (Simon and Blobel, 1993). These newly generated polypeptides undergo modifications such as glycosylation, phosphorylation, and disulfide bridge formation in the ER. Polypeptides must also be accurately folded in the ER to be transported to their correct destination, such as the Golgi complex (GC). Secretion of these stored digestive enzymes into pancreatic ducts is regulated by concentrations of cytosolic calcium, which is stored in and released by the ER (Jamieson and Palade, 1971).

Pancreatic acinar cells are especially vulnerable to ER stress, an accumulation of misfolded proteins in the lumen of ER (Pollard et al., 2017). The processing of large quantities of proteins along with insufficient ER chaperones, alcohol consumption, smoking, genetic and environmental factors induce ER stress (Pandol, 2011). Chronic ER stress can increase cellular reactive oxygen species, induce inflammation, provoke apoptosis, and result in perpetual cell damage. The unfolded protein response (UPR) is a mechanism used by cells to relieve ER stress and reestablish normal cell function. UPR acts to pause protein production, decompose of misfolded proteins, and increase the synthesis of molecular chaperones for proper protein folding (Chong et al., 2017). Sustained ER stress from inhibition of the UPR has been implicated in acinar cell damage leading to acute pancreatitis (Sah et al., 2014), diabetes (Ma et al., 2014), and cancer (Cubillos-Ruiz et al., 2017).

While secretions from the exocrine pancreas are essential for the digestion of nutrients, the endocrine pancreas plays an important role in metabolic regulation.
Endocrine tissue makes up approximately 2% of the pancreas and contains cell clusters called pancreatic islets (known as islets of Langerhans) (El Sayed and Mukherjee, 2019). Pancreatic islets are evenly distributed throughout the pancreas with an abundant network of capillaries, sympathetic, parasympathetic, and sensory nerves (Lacy, 1974). They are composed of approximately 35% alpha (α), 55% beta (β), less than 10% delta (δ), less than 5% gamma (γ), and less than 1% epsilon (ε) cells in humans. Pancreatic alpha cells synthesize glucagon, a hormone that raises blood glucose levels by stimulating the liver to convert its glycogen into glucose. Beta cells produce and secrete insulin in response to high concentrations of blood glucose. Delta cells produce somatostatin, a hormone that suppresses the release of the other hormones made in the pancreas. The gamma cells produce pancreatic polypeptides, which stimulate the secretion of gastric and intestinal enzymes. Epsilon cells contain ghrelin, an amino acid peptide shown to inhibit the secretion of insulin in humans (Lawlor et al., 2017). Endocrine cell heterogeneity complicates the study of diseases.

2.2 Characteristics of Cancer

Cancer is characterized by distinct genetic mutations that result in the cell’s loss of control over vital functions (Hanahan and Weinberg, 2011). Cancer cells have mutated genes with a gain of function or loss of function role. These oncogenes result in defective regulatory mechanisms that work to maintain homeostasis. As normal cells evolve into malignant cells, Hanahan and Weinberg proposed that they acquire six biological capabilities which include: 1) continuing cellular proliferation, 2) escaping growth suppressors, 3) avoiding cell death, 4) enabling replicative immortality, 5)
stimulating angiogenesis, and 6) initiating invasion (Hanahan and Weinberg, 2000). This idea has been supported by studies showing multiple alterations of tumor cell genomes (Kinzler and Vogelstein, 1996).

The most common trait of cancer cells is continuous cellular proliferation. Somatic mutations that result in the constant activation of signaling pathways stimulated by growth factor receptors were revealed by DNA sequencing of tumor tissues. These mutations have been shown to affect the protein structure B-Raf and alter signaling through the Raf to mitogen-activated protein kinase pathway (MAPK). In numerous types of cancer, mutations in phosphoinositide 3-kinase’s (PI3K) catalytic subunit which triggers protein kinase B (Akt/PKB) signal transduction (Hanahan and Weinberg, 2000). Additionally, proliferative phenotype is by enriched by mutations of tumor suppressor genes like PTEN and SMAD4/DPC4. PTEN is a negative regulator of PI3K signaling, and SMAD4/DPC4 is a tumor suppressor gene for pancreatic cancer (Jiang and Liu, 2009; Fang, 2001). SMAD4 facilitates signal transduction and gene regulation of members of the transforming growth factor beta (TGF-β) family (Fang, 2001).

The second biological capability mentioned by Hanahan and Weinberg was the ability to escape growth suppressors, or tumor suppressor genes (Hanahan and Weinberg, 2000). Studies utilizing gain of function/loss of function experiments have identified dozens of tumor suppressor genes (Hanahan and Weinberg, 2000). One such gene is TP53, which receives intracellular signals from stress sensors. TP53 can stop cell-cycle progression in cases of DNA damage, suboptimal growth factor signals, suboptimal glucose levels, or hypoxia. In instances of devastating damage, apoptosis can be activated by TP53. A mutation in TP53 that results in its loss of function is frequently
seen various cancer cells (Levine, 1997). In pancreatic ductal adenocarcinoma (PDAC), overexpression of mutated TP53 (not loss of expression) was the primary genetic alteration in individuals with increased serum levels of carbohydrate antigen 19-9 (CA19-9). Blood serum levels of CA19-9 greater than 1,000 U/mL has been correlated with inadequate responses to pancreatectomy and low survival rates (Xiang et al., 2016).

More commonly known tumor suppressor genes are APC, BRAC1, and BRAC2.

Research has demonstrated the concept of ‘contact inhibition’ suppressing cellular proliferation of normal cells making cell-cell contact when grown in cell culture (Hanahan and Weinberg, 2000). Liver kinase B1 and merlin are tumor suppressor genes involved in management of contact inhibition. Merlin acts to couple adhesion molecules on the cell surface, like epithelial cadherin (E-cadherin), to receptors, like epidermal growth factor receptor (EGFR). This limits the ability of growth factor receptor to produce mitogenic signals (Curto et al., 2007). Liver kinase B1 assists in preserving tissue integrity, and a loss of function results in epithelial cell vulnerability to myc-induced transformation (Partanen et al., 2009; Hezel and Bardeesy, 2008). These systems are important in containing uncontrolled cellular proliferation.

Normal or programmed cell death, apoptosis, eliminates unhealthy somatic cells without discharging harmful materials into neighboring cells (Elmore, 2007). Influences such as cell stress, a lack of nutrients, DNA damage, or uninhibited cell growth induce apoptosis. Resistance to apoptosis in a quality of most cancer cells. Apoptosis is induced by extracellular signals (extrinsic pathway) or intracellular signals (intrinsic pathway). The extrinsic pathway entails transmembrane receptor-mediated interactions. Apoptotic signaling is activated by cell surface receptors binding to their ligands, such as tumor
necrosis factor (TNF) or FasL (also known as CD95). Somatic mutations of Fas in cancer cells have resulted in a loss of Fas-mediated apoptosis (Elmore, 2007).

The intrinsic pathway is non-receptor mediated and initiated in the mitochondria in response to cellular stressor, heat, DNA damage, and radiation (Wang and Youle, 2009). These stimuli change the inner mitochondrial membrane, and pro-apoptotic proteins are released into the cytosol. One such protein is Cytochrome c, which binds to apoptosis protease-activating factor 1 (Apaf-1) and complexes with procaspase-9. The complex activates caspase-9 and commits the cell to apoptosis. However, a decrease in expression of Apaf-1 has been seen in melanoma. Cancer cells also escape cell death through the loss of TP53 function. This results in the loss of apoptosis-inducing damage sensors (Wang and Youle, 2009).

Enabling replicative immortality involves telomerase activity. Numerous studies have indicated that telomeres protecting the ends of chromosomes allow unlimited proliferation (Jafri et al., 2016). In nonmalignant cells, telomeres shorten and lose their capability to protect the ends of chromosomal DNA as cells divide. The consequences of the eventual loss of telomeres is chromosomal end to end fusion and cell crisis. Telomerase, an enzyme that aids in the maintenance of telomere length, is not found in nonmalignant cells but overexpressed in human cancer cells. Cancer cells that obtain the benefit of telomerase activity acquires replicative immortality (Shay and Wright, 2000).

The vascular system provides cells with a means of gas and metabolic exchange. During embryogenesis, new endothelial cells form the vasculature system. This system stops developing in adulthood, but it can be ‘turned on’ during certain physiological conditions such as wound healing. In cancer cells, angiogenesis is activated and remains
on to sustain cell growth. Analyses of premalignant lesions have revealed the angiogenic switch to be on at this early stage. Variation in tumor microenvironments will result in different angiogenic signals (Hanahan and Folkman, 1996).

Tumors formed from epithelial tissues develop modifications in their attachment to other cells and to the extracellular matrix (ECM) (Frantz et al., 2010). A reduction in epithelial cadherin (E-cadherin) expression, an adhesion molecule, has been associated with cell migration. Transformed epithelial cells have primarily attained invasion ability through a process called epithelial-mesenchymal transition (EMT) (Yilmaz and Christofori, 2009; Klymkowsky and Savagner, 2009). EMT is a biological process that changes polarized epithelial cells into a mesenchymal phenotype. Some of the cells undergoing this process will present with differentiated characteristics while others have characteristics of stem cells. This contributes to heterogeneity within tumor tissues (Polyak and Weinberg, 2009). Various factors in the tumor microenvironment such as hypoxia, tumor necrosis factor alpha (TNFα), and cytokines are able to induce EMT (Maier et al., 2010). These cells exhibit reduced expression levels of E-cadherin, occludin, and claudin. However, there is an increase in expression of N-cadherin, vimentin, and fibronectin. Transcription factors inducing EMT are able to organize invasion and metastasis (Barriere et al., 2015).

2.3 Pancreatic Cancer

Tumors that originate in the endocrine pancreas are neuroendocrine tumors (NET) and occur in approximately 2.2 per 1,000,000 individuals. A relative five-year survival rate of 30% for non-functioning PNETs and 97% for functioning PNETs has been
reported by the National Cancer Institute (NCI) (Ro et al., 2013). A majority of these tumors are non-functioning, and they are not associated with excessive secretion of hormones. Functioning PNETs are associated with increased levels of hormones and produce hormone-related syndromes, such as insulinomas. Individuals with insulinoma experience hypoglycemic symptoms with high blood glucose levels (Ro et al., 2013). The prognosis of endocrine tumors is generally higher than exocrine tumors.

PDAC, cancer originating in the pancreatic ducts, has a high mortality rate and often detrimental to most individuals. The ACS reports a relative five-year survival rate of 34% with early stage diagnoses, and this rate drops to 3% with metastasis (Noone et al., 2017). It is estimated that approximately 45,750 people will perish due to PDAC in 2019, and this disease will occur more frequently in men than women (Noone et al., 2017). Chronic pancreatitis (Etemad and Whitcomb, 2011), diabetes mellitus (Sharma and Chari, 2018), alcohol and tobacco use (Zhang et al., 2017), and genetics (Cowan and Maitra, 2014) have been identified as risk factors. An increased risk of developing PDAC has been linked to mutations in multiple genes including KRAS, BRAC2, p16/CDKN2A, PRSS1, and SK11. However, the poor prognosis and low survival rate of individuals suffering from this disease can be attributed to a lack of information underlying its molecular processes and complex biology. This complexity is due to intrinsic factors, such as genetic and epigenetic heterogeneity within a tumor, and extrinsic factors, such as tumor microenvironment. These factors result in large variations in the degree to which cancer cells invade and metastasize (Gupta and Massagué, 2006).
PDAC consists of pancreatic epithelial tissue that transitioned to a precursor lesion (Distler et al., 2014). There are three distinct noninvasive precursor lesions of PDAC. Among these precursors are intraductal papillary neoplasms (IPNs), mucinous cystic neoplasms (MCNs), and pancreatic intraepithelial neoplasia (PanINs). While MCNs are mucin-producing cyst-forming neoplasms, IPMNs are mucin-like epithelial tumors. PanINs are the most common. They are classified according to their ductal epithelial tissue of origin and degree of abnormality. PanINs are small epithelial tumors found in small pancreatic ductal tissue. Genes in advance PanIN lesions become unpredictable, proliferate, and establish a dense stromal microenvironment. The stromal tissue becomes more developed as the precancerous lesion progresses (Distler et al., 2014).

Genetic mutations in genes the KRAS (90%), CDKN2A/p16 (90%), TP53 (70%), and mothers against decapentaplegic homolog 4 (SMAD4) (55%) genes occur throughout the development of PDAC (Lu et al., 2017). Transcription of the mutant KRAS gene is present in 90% of all cases of PDAC, and the most prevalent instigating factor in precursor lesions. KRAS mutations frequently occur at codons 12, 61, and 13. KRAS is involved in multiple important cellular functions including cell survival, cell differentiation and cellular proliferation. KRAS triggers Ras and activates uncontrolled proliferation, or inactivation of CDKN2A (Waters and Der, 2018). With this inactivation, there is a loss of p16 protein (cell cycle’s G-1 to S regulation) resulting in uncontrolled cellular proliferation (Li et al., 2011). Cells escape apoptosis with inactivation of TP53, and SMAD4 occurs frequently in later stages of development. In many forms of cancer, an accumulation and coexistence of genetic mutations. In PDAC,
hypoxia inducible factor-1 works with oncogenic KRAS and loss of p53 to increase upregulation of glucose transporters (Prunier et al., 2011).

Currently, there are no precise diagnostic exam or testable biomarker for PDAC. Carbohydrate antigen 19-9 (CA 19-9) is the only tumor marker available for pancreatic cancer. It has been successful in 75-85% of individuals positive for PDAC (Poruk et al., 2013). Since a highly successful laboratory test for PDAC is not available, the primary diagnostic approach is imaging. An abdominal computed tomography (CT) scan is the most common test performed to look for a mass, which is a signature of the tumor. However, other available methods include magnetic resonance imaging (MRI) with or without cholangiopancreatography (MRCP) and ultrasonography (Lee and Lee, 2014).

Previous research suggests PanIN lesions progress to PDAC tumors by undergoing a lengthy process (Valkenburg et al., 2018). First, an oncogenic mutation transforms pancreatic cells. Next, clonal and tissue expansion occurs when the mutant cells are the dominant phenotype. Finally, a loss of function (tumor suppression) makes invasion possible (Ying et al., 2016). A dense desmoplastic reaction surrounds the malignant cells. This stroma is made up off various types of cells (endothelial, immune, pericytes), fibroblasts, and extracellular proteins. TGFβ and EGF increase stromal fibrosis as a response to wound healing. This strengthens the stroma allowing it to act like a tumor shield. This results in a hypoxic microenvironment, drug resistant PDAC, and immune suppression (Valkenburg et al., 2018).

A hypoxic tumor microenvironment is typical for many malignant tumors (Muz et al., 2015). In order to maintain adequate nutrient levels, PDAC enables metabolic reprogramming. Hypoxia inducible factor-1 (HIF-1) is the transcription factor induced
under hypoxic conditions. It works with oncogenic KRAS and loss of p53 to increase upregulation of glucose transporters. In addition, increased levels of lactate are secreted by the hypoxic cells to acidify the microenvironment. Lactate can be used by more oxygenated cells in the area, encouraging a symbiotic relationship (Abhishek et al., 2018).

PDAC tumors overexpress epidermal growth factor receptor ligands (EGFR), like transforming growth factor-alpha (TGF-α). Autocrine EGF signaling is present in PaINs and growth inhibition was seen in PDAC cells after blocking EGFR with antibodies, so this signaling pathway is known to play a part in tumorigenesis (Narayanan and Weekes, 2016). EGF signaling is the result of phosphatidylinositol-3-kinase (P13K) pathway. P13K induces Akt and mTOR signaling and activates metabolic pathways enhancing macromolecular biosynthesis. This increases expression of nutrient transports and glycolysis with Akt stimulating the production of lipids (Fruman et al., 2017).

2.4 Cancer Cell Metabolism

Metabolism and metabolic adaptation were the first areas studied in cancer cells (DeBerardinis and Chandel, 2016). Many metabolic pathways are altered in cancer cells, in comparison to normal cells, for them to support proliferation, evade apoptosis, and stimulate metastasis. Glycolysis, the stepwise breakdown of glucose from carbohydrates to produce pyruvate with the production of adenosine triphosphate (ATP), is a means cells can harness ATP for energy. An additional enzymatic reaction occurs in the absence of molecular oxygen (hypoxia) which converts pyruvate to lactate. Research has shown that cancer cells preferentially metabolize glucose by glycolysis to lactate, even with sufficient oxygen to support mitochondrial function (Kim and Dang, 2006; Dong et
al., 2017). This is known as aerobic glycolysis or the ‘Warburg Effect’. This metabolic switch provides an organized effect with varying components to cancer cells (Dong et al., 2017).

Mitochondrial ATP production is maintained by fatty acids and amino acids that provide substrates to the tricarboxylic acid (TCA), or Krebs, cycle in cancer cells. Acetyl-CoA (coenzyme A) is generated by the breakdown of fatty acids by beta-oxidation in the mitochondria. When acetyl-CoA is oxidized, high energy electrons are released forming reduced nicotinamide adenine dinucleotide (NADH) and reduced adenine flavin dinucleotide (FADH2). In the electron transport chain (ETC), electrons from these high energy molecules are transferred to low energy molecules such as O2. This results in the formation of mitochondrial ATP. Nutrient and O2 availability to cells within a solid tumor is dependent on each cell’s proximity to blood vessels. While cells located near blood vessels can nourish anabolic pathways, cells distant from blood vessels may use another form of metabolism due to limited access to nutrients and O2. Cancer cells often turn to alternate forms of metabolism such as oxidation of fatty acids and use of amino acids, as well as autophagy (Figure 2.4). Additionally, branched-chain amino acids that are often higher in blood plasma of pancreatic cancer patients (isoleucine, valine, and leucine) can be converted into acetyl-CoA. Various contributions into the TCA cycle allows cancer cells to utilize the nutrients that are accessible in their microenvironment (DeBerardinis and Chandel, 2016).
Angiopoietin-like 4 (ANGPTL4)

Angiopoietin-like proteins (1-4) are modulators of angiogenesis and maintain the integrity of the vascular system. Members of this protein family bind to receptor tyrosine kinase (Tie2) expressed on endothelial and hematopoietic cells. Angiopoietins are proteolytically cleaved into an amino-terminal coiled-coil domain (nANG) and a carboxyl-terminal fibrinogen-like domain (cANG). The amino-terminal domain promotes the formation of higher-order multimers while the carboxyl-terminal domain contains the binding site for Tie2. Angiopoietin1-Tie2 signaling is essential in the regulation of angiogenesis and vascularization (Hato et al., 2008; Zhu et al., 2012).

Angiopoietin-like protein 4 (ANGPTL4) is one of eight members in a family of angiopoietin-like proteins (ANGPTL1-8). ANGPTL4 was detected during a probe for targets of peroxisome proliferator-activated receptor alpha (PPAR-α). ANGPTLs have a similar structure as angiopoietins and play a role in angiogenesis, but do not bind to Tie2.
The human ANGPTL4 gene is located on chromosome 19p13.3, and the gene encodes a 45–65 kDa protein. Inconsistencies in reported molecular masses are due to tissue related isoforms and glycosylation (Yang et al., 2008).

This full-length protein is cleaved into an amino-terminal coiled-coil domain (nANGPTL4), and a carboxy-terminal fibrinogen-like domain (cANGPTL4) when pro-protein convertases recognize the major cleavage site between Lys168 and Leu169. A minor cleavage site is located between Lys170 and Met171. However, the cleavage of ANGPTL4 is contingent upon the expressing tissue. fANGPTL4 and the individual domains have been found in blood plasma (Lei et al., 2011).

Human ANGPTL4 is expressed at notable levels in adipose, heart, muscle, liver, lung, and endothelial cells (Tjeerdema et al., 2014). Adipose tissue is believed to express copious amounts (Yoon et al., 2000; Kim et al., 2000). Studies have shown that expression levels of ANGPTL4 are managed by peroxisome proliferator-activated receptors (PPAR), transcription factors of nuclear hormone receptors (Inouse et al., 2014). Biological conditions such as fasting, chronic caloric restriction, fatty acid signaling, hypoxia, and hypoxia inducible factor-1α (HIF-1α) can induce the expression of ANGPTL4 (Kim et al., 2011). While glucocorticoids stimulate expression in liver and adipose tissue, insulin suppresses it in adipose tissue (Kuo et al., 2014). Adipocytes express the post-translationally modified fANGPTL4 (65 kDa), whereas liver tissue will express both cANGPTL4 (47 kDa) and nANGPTL4 (26 kD) (Yang et al., 2008; Lei et al., 2011).

ANGPTLs 3, 4, and 5 seem to be involved in maintaining processes such as lipid metabolism, glucose metabolism, and energy homeostasis. ANGPTL4 inhibits the activity of lipoprotein lipase (LPL), which breaks down circulating triglyceride into fatty
acids and glycerol to be taken into cells. LPL is expressed and secreted by adipocytes and muscle cells. Once ANGPTL4 is cleaved, two conserved cysteine residues (Cys76 and Cys80) of nANGPTL4 form a disulfide bond mediating oligomerization. This is essential for nANGPTL4 binding to lipoprotein lipase (LPL), converting the catalytically active dimer into an inactive monomer (Sukonian et al., 2006; Lichtenstein et al., 2007).

In order to maintain cellular activities, the production of free fatty acids for beta-oxidation is stimulated during fasting. Free fatty acids induce expression of ANGPTL4, and there is a decrease in LPL activity (Lichtenstein et al., 2007).

Simultaneously, ANGPTL4 plays a role in intracellular lipolysis. Studies have shown a blunt increase of free fatty acids in blood plasma when the ANGPTL4 has been silenced (Aryal et al., 2018). Additionally, findings have indicated that ANGPTL4 plays a key role in fat metabolism. Mice overexpressing ANGPTL4 protein in white adipose tissue harbored elevated levels of triglycerides and free fatty acids (Kersten et al., 2009).

ANGPTL4 has been implicated in glucose metabolism. In adipocytes, insulin has been shown to down regulate ANGPTL4 levels through the PI3K/FOXO1 signaling (Kuo et al., 2014). In another study, feeding subjects after a period of starvation resulted in elevated insulin levels with decreased levels of ANGPTL4 (Xu et al., 2005). Finally, overexpression of ANGPTL4 was correlated with improved glucose tolerance (Xu et al., 2005).

Angiopoietin-like 4 (ANGPTL4) was shown to be overexpressed in human pancreatic cancer cells that are resistant to gemcitabine chemotherapy (Kirbya et al., 2016), expressed in human colorectal cancer (Nakayama et al., 2011), to mediate the endothelial migration of breast cancer cells resulting in lung metastasis (Padua et al.,
2008), and plays a key role in Kaposi’s sarcoma (Hu et al., 2011). Expression levels were found to be associated with survival time from diagnosis in individuals suffering from gemcitabine resistant pancreatic cancer. Individuals with higher expression levels fell into the “short survival” category. Results revealed that elevated ANGPTL4 expression is present in all epithelial tumor samples, and its expression is correlated with the tumor progression (Kirbya et al., 2016).

The role of ANGPTL4 in cancer progression remains poorly understood. Research has focused on elucidating the role of ANGPTL4 in angiogenesis. ANGPTL4 has been found within several perinecrotic tumors under hypoxic conditions (Kim et al., 2011). Studies have shown evidence of ANGPTL4 involvement in other roles during cancer progression. ANGPTL4 was indicated in enabling cancer cell survival maintaining elevated levels of oxygen radicals (Zhu et al., 2011). Further research revealed cANGPTL4 interacts with integrin α5β1, VE-cadherin and claudin-5 to disrupt endothelial stability through integrin-mediated PAK/Rac signaling (Huang et al., 2011). Additional studies that determine the interacting residues of cANGPTL4 to each binding partner would provide better insight and determine the role of cANGPTL4 in cancer progression. Studies that include X-ray crystallography, Cryo-EM, ITC data, and site-directed mutagenesis would examine these key interactions providing an increased mechanistic understanding. Furthermore, investigations into the interactions of cANGPTL4 with both cell surface and intracellular proteins, as well as interactions with potential physiological ligands, are needed to provide better insight into the full scope of the role of ANGPTL4 in cancer progression.
Proteomics is the broad study of proteins in biological samples and some examinations include their structure-function, abundance, post-translational modifications, and interactions (Chandramouli and Qian, 2009). Protein-protein and protein-ligand binding are essential for many biological functions. Therefore, protein research is heavily focused on protein binding sites and their properties. For example, many binding sites are prone to be more hydrophobic. Some binding sites demonstrate structural flexibility by binding to multiple proteins or ligands and contain highly conserved residues (Patil et al., 2010). Structural studies involving cANGPTL4, several carbohydrates, and various fatty acids will be performed in this project to investigate possible binding sites of ANGPTL4.

Angiopoietin-like 4 (ANGPTL4) is indicated in the progression of several types of aggressive cancer. Biochemical studies have been performed but a full structural understanding of functions and interactions is not available. The goal of this project is to gain insight into structural interactions that result in the progression of PDAC in order to enable therapeutic development. Overexpression of cANGPTL4 has been found in pancreatic tumor tissues of all stages. Its complexity and ability to target various cancer cell microenvironments, endothelial cells, and metabolic activities makes it a prime candidate for therapeutic intervention. The primary function of a protein depends on its structure, and changes in protein conformations or binding to other proteins can lead to altered protein functions. We provide significant insight into the actions of cANGPTL4 through structure-function studies. This research is significant in that previously unknown properties of cANGPTL4 is shown for the first time. The crystallographic
derived structures elucidated in this work were deposited on the protein data bank to enable future structural studies.
Chapter 3

Differential Expression and Secretion of Hypoxia-Induced ANGPTL4 in Cancer Cell Lines

3.1 Introduction

The relative five-year survival rate for individuals with pancreatic ductal adenocarcinoma (PDAC) drops to 3% with metastasis (Noone et al., 2017). PDAC is responsible for approximately 6% of cancer related deaths annually. Due to vague symptoms and late stage diagnosis, only 10-20% of newly diagnosed patients will have operable tumors (Noone et al., 2017). Historically, chemotherapy with gemcitabine has been the standard treatment, but PDAC tumors have an intrinsically high resistance to chemotherapeutics (Amrutkar and Ivar, 2017). A complete system approach using genomics to look for mutations, epigenomics for chemical modifications, transcriptomics for quantification of RNA transcripts, proteomics for analyses of proteins, and metabolomics to quantify metabolites has the potential to overcome chemo-immunotherapy resistance in PDAC.

Angiopoietin-like 4 (ANGPTL4), a secreted signaling protein, has been linked to the progression of several types of aggressive cancer. This protein has been shown to mediate the endothelial migration of breast cancer cells resulting in lung metastasis (Padua et al., 2008), over-express in human colorectal cancer (Nakayama et al., 2011),
and play a key role in Kaposi’s sarcoma (Hu et al., 2011). In a previous study, differentially expressing gene transcripts were detected from pancreatic tumor tissues of individuals with different life expectancies. ANGPTL4 was identified as a transcript with greater expression in individuals with a shorter life expectancy and pancreatic cancer cells resistant to gemcitabine (Kirbya et al., 2016). However, the role of ANGPTL4 in cancer progression remains poorly understood.

Human ANGPTL4 is one of eight members in a family of angiopoietin-like proteins (ANGPTL1-8) (Yang et al., 2008). Native full-length ANGPTL4 (fANGPTL4) is cleaved into an amino-terminal coiled-coil domain (nANGPTL4), and a carboxy-terminal fibrinogen-like domain (cANGPTL4) by proprotein convertases. The cleavage site is contingent upon the expressing tissue. Adipocytes secrete the post-translationally modified fANGPTL4 (65 kDa), whereas liver tissue secretes nANGPTL4 (26 kDa) (Lei et al., 2011). After cleavage, nANGPTL4 regulates lipid metabolism by inhibiting activity of lipoprotein lipase (LPL), which breaks down circulating triglyceride into fatty acids and glycerol to be taken into cells (Lichtenstein et al., 2007). Numerous studies have indicated cANGPTL4 plays multiple molecular roles in cancer cell progression, but its mechanisms of action are poorly understood.

Differential expression of proteins has been correlated to phenotypic characteristics such as invasion, migration, tumorigenesis, and chemotherapeutic drug resistance (Tan et al., 2012). Cell culture experiments are widely used to study normal biological processes (like metabolism), cellular effects of various chemicals, mutagenesis, and oncogenesis. Cancer cell lines were selected based on phenotypic characteristics such as anoikis resistance, invasion, proliferation rate, metastasis, and
tumorigenesis for this study. Additionally, the four most frequently altered genes in pancreatic tumors (KRAS, p53, p16, and SMAD4) were also considered (Deer et al., 2010). These mutations are found at similar percentages in the pancreatic cancer cell lines used in this project. The pancreatic cancer cell lines used in this study were Capan-1, MiaPaCa2, and Panc-1 cells. Capan-1 cells were derived from an adenocarcinoma found in the head of the pancreas (Deer et al., 2010). Cells had metastasized to the lymph nodes. MiaPaCa2 cell were found in an adenocarcinoma located in the body and tail of a pancreas, and cells had invaded the periaortic region (Deer et al., 2010). Panc-1 cells originally removed from an adenocarcinoma located in the head of the pancreas with invasion into the duodenal wall and metastasis into a peripancreatic lymph node (Deer et al., 2010). Proliferation rate differences have been reported depending on culture conditions (Deer 2010).

Relative phenotypic differences in behaviors such as adhesion, migration, tumorigenicity, and invasion have been previously evaluated for each cell line used in this study (Deer et al., 2010). Panc-1 cells have shown the greatest mobility. They exhibited a five-fold greater mobility than BxPC-3 cells. Invasive properties of cells used in this study were previously determined and rank from most to least invasive as follows: MiaPaCa2>Capan-1>Panc-1. Angiogenic potential is assessed by expression levels of pro-angiogenic cytokines and enzymes, such as cyclooxygenase-2 (COX2). Capan-1 cells have steadily presented high levels of pro-angiogenic factors while MiaPaCa-2 cells have shown consistently low levels. A cancer cell’s propensity to generate a tumor is called tumorigenicity. Capan-1 tumorigenicity is greater than MiaPaCa2 cells, while MiaPaCa2 cells have higher tumorigenicity than Panc-1 cells (Deer 2010).
The microenvironment of solid tumors is known to have low oxygen levels, hypoxic, compared to normal tissues of the same organ (Vaupel et al., 1989). Blood vessels in tumor tissue can elongate, disintegrate, or become compressed. This reduces the exchange area for oxygen and other products. When this occurs, normal cells die, and cancer cells adapt to the hypoxic environment through metabolic reprogramming. An example of this can be seen in PDAC. A median pO2 of 24.3-92.7 mmHg (3.2-12.3%) has been reported for normal pancreatic tissue, with pancreatic tumor tissue at pO2 0-5.3 mmHg (0-0.7%) (Vaupel et al., 1989). Normal arterial blood pO2 is 100 mmHg (13.2%), and typical room air pO2 is 160 mmHg (21.1%) (Vaupel et al., 1989). Hypoxia induces multiple intracellular signaling pathways, such as hypoxia inducing factor (HIF), involved in apoptosis, proliferation, metabolism, and metastasis (Vaupel et al., 1989). In this study, pancreatic cancer cells were cultured in both normoxic and hypoxic conditions to mimic tumor cell microenvironment.

The aim of this study was to examine the molecular role of ANGPTL4 in PDAC progression. A relationship was shown between expression levels of ANGPTL4 and phenotypic characteristics of pancreatic cancer cells. In this chapter, the various ANGPTL4 isoforms were detected and identified to help reveal a structure-function relationship. Additionally, cellular location of cANGPTL4 and integrin-β1 was examined by immunofluorescence and confocal imaging.

3.2 Material and Methods

Antibodies and reagents used included mouse monoclonal anti-human fANGPTL4 (26-406) antibody (LSBio), rabbit polyclonal anti-human cANGPTL4 (184-
336) antibody (Abcam), rabbit polyclonal anti-human nANGPTL4 (26-166) antibody (Abcam), integrin β1 antibody (Abcam), E-cadherin antibody (Cell Signaling Technologies), HIF1α (Santa Cruz Biotechnology); Alexa Fluor 488-conjugated secondary antibodies (Molecular Probes), Alexa Fluor 594-conjugated secondary antibodies (Molecular Probes), Alexa Fluor 647-conjugated secondary antibodies (Molecular Probes), Alexa Fluor 680-conjugated secondary antibodies (Molecular Probes); Actin-red 555 (Molecular Probes), and Nuc-blue (Molecular Probes); 5-hexadecanoyl fluorescein (Molecular Probes) and 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-hexadecanoic acid (BODIPY-FL C16) (Molecular Probes).

Pancreatic cancer cell lines were purchased from Sigma-Aldrich, and breast cancer cell lines were a generous gift from Dr. Debra Moriarity.

3.2.1 Cell lines and cell culture

Human pancreatic cell lines Panc-1, MiaPaCa2, Capan-1, HEK293, and SKBR3 cells were cultured in complete Dulbecco’s Modified Eagle’s Medium (DMEM/Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 100 U/ml penicillin with 0.1 mg/ml streptomycin (Gibco). Human breast cancer cell lines MCF7 and MDA-MB231 were cultured in RPMI 1640 (Sigma-Aldrich) medium supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 100 U/ml penicillin with 0.1 mg/ml streptomycin (Gibco). MCF7 cell medium was supplemented with 0.01mg/mL insulin. All cell lines were grown in 75 cm² sterile flasks at 37°C in a humidified atmosphere at 5% CO₂ and 20% O₂ (normoxia). For hypoxia, cell culture medium was replaced with serum-free medium and grown in a humidified atmosphere at 1% O₂, 5% CO₂, and 94%
N₂. All cell lines were periodically tested to be mycoplasma free using an assay kit by Lonza (MycoAlert Mycoplasma Detection Kit #LT07-418) using the protocols provided with the kit.

### 3.2.2 Protein secretion analysis

Cells (~2 X 10⁷) were cultured in serum-free medium in a 25 cm² sterile flasks at 37°C. The serum-free conditioned medium was collected from each flask of cells after incubation in normoxia or hypoxia for 48 hours. Each flask of conditioned medium was filtered through a 0.22 µm filter for debris removal. A 10 kDa spin column was used to concentrate the conditioned medium. Protein concentrations were quantified (50 ng/µL), and approximately 5 µL of each were added to three different gradient SDS gels for immunoblots, and two SDS gels for analysis using Coomassie blue. Proteins from three gels were transferred to nitrocellulose membranes, while two SDS-PAGE underwent staining with Coomassie blue and de-staining. The membranes were blocked in 5% BSA overnight at 4°C. The membranes were washed 3 times for 10 minutes in 1X TBST solution. One membrane was incubated in primary antibody for fANGPTL4 (diluted 1:1000 in blocking solution + 0.1% Tween-20); the second membrane was incubated in primary antibody for nANGPTL4 (diluted 1:1000 in blocking solution + 0.1% Tween-20) and then in primary antibody for cANGPTL4 (diluted 1:1000 in blocking solution + 0.1% Tween-20) overnight at 4°C. The third membrane was incubated overnight at 4°C in primary antibody for β-actin for a positive control. Each membrane was washed 3 times for 10 minutes in 1X TBST and incubated with HRP-conjugated anti-species secondary for one hour. Then, each membrane was developed using an enhanced
chemiluminescence kit (Pierce 32106). These membranes were also stripped and re-probed for HIF1α to confirm hypoxia. Protein bands for HIF1α were seen on immunoblots but results are not shown due to problems with imaging.

3.2.3 RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

RNA was extracted with TRIzol according to manufacturer’s instructions (Invitrogen). cDNA reverse transcription was performed for cANGPTL4 using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Concentrations of cDNA samples were determined by nanodrop, and equal amounts were used for real time qPCR (ABI Prism). Primer sequences for genes of interest are as follows: ANGPTL4 (sense, 5’-TCCAACGCCACCCACTTAC-3’; antisense, 5’-TGAAGTCATCTCACAGTTGACCA-3’), β-actin (sense, 5’-ACGCCTCTGGCCGTACCACT-3’; antisense, 5’-TAATGTCACGCACGATTTCCC-3’), and 18S rRNA (sense, 5’-CTACCACATCCAGGAAGCA-3’; antisense, 5’-TTTTTCGTCACTACCTCCCCG-3’. All samples were run in triplicate and normalized to β-actin.

3.2.4 Cell proliferation assay

Cells (1 X 10^5) from each cell line listed above were grown in appropriate growth medium in 24-well plates in normoxia and hypoxia for 24, 48, and 72 hours. One plate of each cell line was collected, trypsinized, and counted using trypan blue stain (Sigma) at each time interval in triplicate.
3.2.5 Immunofluorescent Confocal Imaging

Cells (~3 X 10^5) from each cell line listed above were grown in appropriate growth medium to approximately 85% confluency on hydrophilic, tissue culture treated Ibidi microchamber plates in normoxia and hypoxia. Growth medium were removed, and cells were washed with PBS prior to fixation with 4% paraformaldehyde for 10 minutes. Cells were washed three times in PBS, briefly washed with 0.1M ammonium chloride, washed again with PBS, and blocked with 3% normal goat serum) for 1 hour at room temperature. Following three washes in PBS, cells were incubated overnight at 4 °C with either anti-human fANGPTL4, nANGPTL4, cANGPTL4, Inteigrin β1, E-Cadherin, or HIF1α antibody. After three washes with PBS at room temperature, cells were incubated with Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 647, or Alexa Fluor 680 secondary antibodies for 2 hours. Actin-red 555 and Nuc-blue were added for actin and nuclear staining. Imaging was performed with staining but without presence of a primary antibody, as well as without staining but with a primary antibody as controls. Images were acquired using a Zeiss LSM710 confocal microscope.

3.2.6 Statistical analysis

Statistical analyses were performed in triplicate with two-tailed Mann-Whitney test and graphs created with GraphPad Prism (GraphPad Software). A statistically significant difference was considered p < 0.05.
3.3 Results

3.3.1 Protein secretion analysis

To identify the secretion of ANGPTL4 protein isoforms, pancreatic and breast cancer cell lines were cultured in normoxia and hypoxia. No protein bands were detected with immunoblot analysis of serum-free conditioned medium from cells grown under normoxia for 48 hours. However, secretion of ANGPTL4 protein was elevated under hypoxia in each cell line. Samples of concentrated serum-free conditioned medium from cell lines cultured under hypoxia for 48 hours were analyzed by SDS-PAGE stained with Coomassie blue and immunoblots. A protein band was seen at the expected molecular weight for fANGPTL4 from Panc-1, Capan-1, MDA-MB231, MCF7, and SKBR3 conditioned medium, but not MiaPaCa2 on the SDS-PAGE and confirmed on the immunoblot (Figure 3.1). A sample of recombinant fANGPTL4 was added to the gel and immunoblot as a control. Also, a protein band was also seen at the expected molecular weight for nANGPTL4 from MiaPaCa2 and Capan-1 conditioned medium on the same SDS-PAGE. Proteins from another SDS gel were transferred to a nitrocellulose membrane that was probed for both cANGPTL4 and nANGPTL4, but not fANGPTL4. Primary antibodies specifically for cANGPTL4 and nANGPTL4 were used with this immunoblot to prevent any possible interference from fANGPTL4. The internal marker β-actin was a positive control. This immunoblot was ran with samples from the same serum-free conditioned medium, but on a separate SDS gel and nitrocellulose membrane in order to avoid stripping and re-probing the membrane.
Figure 3.1: Immunoblot and SDS-PAGE analysis of ANGPTL4 protein secretion from pancreatic and breast cancer cell lines under hypoxia for 48 hours. A) In the top panel, a protein band was seen for fANGPTL4 from Panc-1, Capan-1, MDA-MB231, MCF7, and SKBR3 serum-free conditioned medium, but not MiaPaCa2. A sample of recombinant fANGPTL4 was added as a control. In the middle panel, a protein band for nANGPTL4 was seen only from MiaPaCa2 and Capan-1 conditioned medium. The cANGPTL4 isoform was not seen from any of the cell lines, and the internal marker β-actin is shown in the bottom panel. B) SDS-PAGE stained with Coomassie blue showing protein bands at expected molecular weights for fANGPTL4 and nANGPTL4. Each sample was obtained from serum-free conditioned medium from cells grown in hypoxia for 48 hours. Lane 1 contains a molecular weight ladder. The remaining lanes are organized as samples from cells as follows: Panc-1, MiaPaCa2, Capan-1, MDA-MB231, MCF7, SKBR3. The last lane contains fANGPTL4 control.
3.3.2 ANGPTL4 expression under normoxia and hypoxia

In order to study differential expression of ANGPTL4 in various cancer cell phenotypes, various pancreatic and breast cancer cell lines were grown in normoxia and hypoxia for 48 hours prior to RNA extraction. Both cancer cell lines under normoxia for 48 hours expressed minimal RNA as revealed by RT-qPCR (Figure 3.2). Under hypoxia, ANGPTL4 RNA expression was significantly elevated in all cancer cell lines but not in HEK293 cells. The most significant increase was seen in Panc-1 and MDA-MB231 cell lines. Capan-1 cells exhibited the third largest increase in expression under hypoxia. Overall, these results show ANGPTL4 expression levels are significantly increased in cancer cells under hypoxic conditions as found within a tumor microenvironment (Vaupel 1989).

Figure 3.2: Analysis of ANGPTL4 expression by RT-qPCR in pancreatic and breast cancer cell lines cultured under normoxia and hypoxia for 48 hours. Relative expression was determined as the expression ratio of ANGPTL4 RNA/β-actin RNA. Data were collected in triplicate and presented as mean±SD. ANGPTL4 expression was significantly increased in all cell lines grown in hypoxia in comparison to cells grown in normoxia, except HEK293 cells. The most significant increase was seen in Panc-1 and MDA-MB231 cell lines. Capan-1 cells exhibited the third largest increase in expression under hypoxia. p values <0.05 indicate a significant difference.
3.3.3 Cell proliferation assay

To investigate the effects of increased ANGPTL4 expression on cellular proliferation, Panc-1, MiaPaCa2, and Capan-1 cells were grown in normoxia and hypoxia. Cells from each line were collected and counted after 24, 48, and 72 hours in triplicate. While expression levels of ANGPTL4 did significantly increase, cellular proliferation rates did not increase for any of the cell lines (Figure 3.3). In fact, proliferation of each cell line was greater under normoxic conditions. However, fewer dead cells were noted in cultures grown in hypoxia.

Figure 3.3: Cellular proliferation of (A) Panc-1, (B) MiaPaCa2, and (C) Capan-1 cells grown in normoxia and hypoxia. Cells from each line were collected and counted after 24, 48, and 72 hours from initial seeding. While expression levels of ANGPTL4 did significantly increase in each cell line, cellular proliferation rates did not increase for any of the cells in hypoxia.
3.3.4 Cellular localization of ANGPTL4 isoforms

Immunofluorescent labeling and confocal imaging were used to examine cellular localization of fANGPTL4, nANGPTL4, and cANGPTL4. This protein plays multiple molecular roles in cancer cell progression, but its mechanisms of action are poorly understood. Three cleavage isoforms of this protein with independent and multiple cellular functions complicates things (Tan et al., 2012). The expression levels were elevated under hypoxia, as shown by RNA analysis. However, only fANGPTL4 and nANGPTL4 protein isoforms were found in conditioned medium. Since cleavage of fANGPT4 produces both n- and cANGPTL4, it was hypothesized that cANGPT4 bound to the cell surface.

Confocal images show that none of this protein’s isoforms were detected in pancreatic or breast cancer cell lines cultured in normoxia. In hypoxia, cANGPTL4 (green fluorescence) was identified on the extracellular surface of Panc-1 and MDA-MB231 cells (Figure 3.4). This fluorescent signal was also seen with less intensity in the other cancer cell lines. Autofluorescence was accessed by DIC imaging of each cell line, and no autofluorescence was detected (Supplemental 1). A fluorescent signal was not seen in control groups incubated with cANGPTL4 primary antibody but no secondary antibody or vice versa. Furthermore, a signal was not seen in cells incubated with fANGPTL4 or nANGPTL4 antibodies. This indicates that while cANGPTL4 associate with cell surface proteins, fANGPTL4 and nANGPTL4 do not associate with cell surface proteins. These results explain why fANGPTL4 and nANGPTL4 but not cANGPTL4 were found in the conditioned medium in the protein secretion analysis.
Figure 3.4: Cellular localization of cANGPTL4 in pancreatic and breast cancer cells using confocal microscopy.  A) MDA-MB231 cells grown in hypoxia for 24 hours and labeled with cANGPTL4 antibody; nuclei (blue) were stained with Nuc-blue, F-actin (red) was stained with Actin-red 555, and cANGPTL4 (green) was stained with Alexa Fluor 488.  A merged image revealed cANGPTL4 secreted to the outside the cells.  B) Panc-1 cells grown in hypoxia for 24 hours and labeled with cANGPTL4 antibody; nuclei (blue) were stained with Nuc-blue, F-actin (red) was stained with Actin-red 555, and cANGPTL4 (green) was stained with Alexa Fluor 488.  A merged image revealed cANGPTL4 secreted to the outside the cells.  Scale bar = 100µm.
3.3.5 cANGPTL4 and integrin-β1

To gain insights into the interaction between cANGPTL4 and integrin β1, the cellular location of each protein was examined using immunofluorescence with confocal imaging. Once secreted to the cell surface, cANGPTL4 has been shown to facilitate metastasis by disrupting endothelial barrier integrity when interacting with integrin α5β1 (Huang et al., 2011). The binding affinity of cANGPTL4 to integrin β1 was determined by surface plasmon resonance (SPR), but little is known about this interaction (Huang et al., 2011). Immunofluorescence and confocal imaging of Panc-1 cells grown under hypoxia shows colocalization of these proteins (Figure 3.5). Previous studies have shown that cANGPTL4 first bind to integrin β1 before subsequently binding to VE-cadherin and claudin-5 (Huang et al., 2011). These results are supportive showing that cANGPTL4 is interacting with integrin β1 on the cell surface.

Figure 3.5: Cellular localization of cANGPTL4 and integrin-β1 in Panc-1 cells using immunofluorescence and confocal imaging. Panc-1 cells were grown to confluency under hypoxia, then labeled with cANGPTL4 and integrin β1 antibody. Nuclei (blue) were stained with Nuc-blue, cANGPTL4 (green) was stained with Alexa Fluor 488, and integrin β1 was stained with Alexa Fluor 690. The last image is merged and shows colocalization of cANGPTL4 with integrin β1. Scale bar = 100µm.
3.4 Discussion

ANGPTL4 has been suggested to inhibit leaking in the vascular system, which in turn inhibited extravasation of cancer cells (Ito et al., 2003). The molecular mass for ANGPTL4 was not reported in the study so it is unknown if the full-length protein or one of the two fragments was detected (Ito et al., 2003). In another study, fANGPTL4 and cANGPTL4 were overexpressed in melanoma cells (Galaup et al., 2006). ANGPTL4 was shown to inhibit cancer cell metastasis, but the activity was not differentiated for the f- and c form (Galaup et al., 2006)

Contradicting studies have shown that ANGPTL4 plays essential roles in the different stages of proliferation, anoikis resistance, and metastasis (Zhu et al., 2011; Tan et al., 2012). ANGPTL4 has been suggested to promote breast cancer metastasis to lung tissue by increasing leaking in the vascular system. A knock-down of ANGPTL4 expression resulted in a significant decrease of breast cancer cell invasion (Padua et al., 2008). Migration and metastasis of hepatocellular carcinoma was seen with increased levels of ANGPTL4 expression (Li et al., 2011). Differential expression of ANGPTL4 have been correlated to poor survival rates of individuals diagnosed with gastric and colorectal cancer. However, the underlying mechanisms of action and functions of the different ANGPTL4 isoforms are not known. In this chapter, we provided insight into the expression and secretion of these isoforms.

Expression and secretion of fANGPTL4, nANGPTL4, and cANGPTL4 in three pancreatic and three breast cancer cell lines was examined under normoxia and hypoxia. No protein secretion was detected for any cell line under normoxia. fANGPTL4 was secreted by two pancreatic (Panc-1, Capan-1) and all three breast cancer cells (MDA-
MB231, MCF7, and SKBR3) cultured under hypoxia. Two sets of pancreatic cancer lines secreted nANGPTL4 (MiaPaCa2 and Capan-1), but cANGPTL4 was not detected in any of the conditioned medium. Only the Capan-1 cells secreted both fANGPTL4 and nANGPTL4. Hypoxia induced expression of ANGPTL4 RNA was observed in the six cancer cell lines, but not in HEK293 cells used as a control. Panc-1 and MDA-MB231 cells exhibited the largest increase in expression of ANGPTL4. While expression levels of ANGPTL4 did significantly increase in all cancer cells, cellular proliferation rates did not increase for any of the cell lines.

ANGPTL4 belongs to a family of proteins involved in angiogenesis, the process in which proliferation of endothelial cells leads to the formation of new blood vessels (Tan et al., 2012). This is essential for tumor growth and metastasis. Expression levels of proangiogenic cytokines and enzymes, like cycloxygenase 2 (COX2), have been used in assessing the angiogenic potential of cancer cells (Sobolewski et al., 2010). COX2 expression has been detected in Capan-1 and Capan-2, but not detected in MiaPaCa2 or Panc-1 cell lines (Deer et al., 2010). Prostaglandin E2 is a product of COX2 that has been indicated to have a tumorigenic role. Capan-1 cells exhibited the third largest increase in ANGPTL4 expression under hypoxia, and they were the only cells to secrete both fANGPTL4 and nANGPTL4. It has been suggested that prostaglandin E2 and hypoxia enhance ANGPTL4 expression and cANGPTL4 secretion. Also, Capan-1 cells possess a mutation and do not express SMAD4/DPC4, a tumor suppressor and a member of the transforming growth factor family (Deer et al., 2010). The overall aggressiveness of PDAC cells has been linked to angiogenesis, and these results show that Capan-1 cells have high angiogenic potential (Deer et al., 2010).
ANGPTL4 full length protein, along with nANGPTL4 and cANGPTL4, can be found in blood plasms and many tissues (Zhang et al., 2017). Few studies have demonstrated a structure-function relationship for these isoforms. However, cANGPTL4 has been associated with anoikis resistance, the ability of cancer cells to escape apoptosis after disconnection from the extracellular matrix (Tan et al., 2012; Tjeerdema et al., 2014). This isoform has been shown to interact with multiple beta-integrins elevating cellular levels of ROS (Terada and Nwariaku, 2011). Increased cellular levels of ROS are also seen with chronic ER stress. Previous studies have demonstrated that sustained increases in ROS levels are involved in the initiation and progression of aggressive tumors (La Paglia et al., 2017). We show that cANGPTL4 does interact with integrin β1 on the surface of Panc-1 cell when cultured under hypoxic conditions. These results support the idea that cANGPTL4 and its interactions play a role in cancer cell progression.

The metastatic capacity of PDAC highlights the importance of understanding cell adhesion. This property influences tumor growth and influences metastasis. Cell adhesion is mediated by the interactions of surface molecules with the extracellular matrix (Chong et al., 2012). Panc-1 and Capan-1 cells have demonstrated tight adherence to a mixture of components (Deer et al., 2010). Panc-1 cells exhibited the strongest adherence to collagen I, II, IV, and fibronectin. Cellular localization of fANGPTL4, nANGPTL4, and cANGPTL4 was investigated using immunofluorescent labeling and confocal imaging in Panc-1 and MDA-MB231 cells since they exhibited the greatest increase in ANGPTL4 expression under hypoxia. cANGPTL4 was identified on the extracellular matrix of Panc-1 and MDA-MB231 cells, but a fluorescent signal was
not detected in any of the samples probed for fANGPTL4 or nANGPTL4. Furthermore, colocalization of cANGPTL4 and integrin-β1 was seen in Panc-1 cells. In a previous study, cANGPTL4 induced vascular disruption through a direct and sequential association with integrin α5β1, E-cadherin, and claudin-5 (Huang et al., 2011). We provide supportive evidence that demonstrated cANGPTL4 interacted with integrin β1. Using immunofluorescence and confocal imaging, colocalization of these proteins was seen on the surface of Panc-1 cells cultured under hypoxic conditions. These results highlight the significance of future structural studies examining which cANGPTL4 residues interact with integrin β1.
4.1 Introduction

ANGPTL4 has been identified as a secreted protein affecting cell-matrix communication. It interacts with extracellular vitronectin and fibronectin. ANGPTL4 has shown to play a novel role in wound healing. It binds to integrin β1 and β5 on keratinocytes aiding in wound closure by re-epithelialization. Natural wound healing shares similarities with tumor progression such as inflammation, matrix remodeling, and cell migration (Goh et al., 2010). Furthermore, studies have indicated that cANGPTL4 interacts with integrin α5β1, VE-cadherin and claudin-5 to disrupt endothelial continuity through integrin-mediated PAK/Rac signaling (Huang et al., 2011). However, a full list of this protein’s interacting partners is not available.

The human ANGPTL4 gene encodes a 45–65 kDa protein that is well conserved among various species (Yang et al., 2008). Reported molecular masses vary due to different isoforms and glycosylation. This protein undergoes posttranslational modifications resulting in an amino-terminal coiled-coil domain (nANGPTL4), and a carboxy-terminal fibrinogen-like domain (cANGPTL4). The cleavage site and
function are tissue dependent (Lei et al., 2011). In mouse melanoma cells, a protein fragment with an equivalent molecular mass as cANGPTL4 was revealed after expression of ANGPTL4 (Galaup et al., 2006). In a study of major epithelial tumors, like squamous cell carcinoma, only cANGPTL4 was found to be overexpressed (Tan et al., 2012).

The binding of nANGPTL4 to lipoprotein lipase (LPL) has been well studied and demonstrated repeatedly (Sukonina et al., 2006; Mysling et al., 2016; Wang and Eckel, 2009; Chi et al., 2015). Through the binding of its N-terminal coiled-coil domain to LPL, this protein is a regulator of plasma triglycerides. Additionally, it has been demonstrated that fatty acids act to reduce nANGPTL4’s effect of LPL activity (Chi et al., 2015). Fatty acids that produced this effect were oleic acid, palmitic acid, myristic acid, and linoleic acid. These fatty acids were found to have a high affinity and bind to nANGPTL4 as determined by surface plasmon resonance, isothermal titration calorimetry, and fluorescent studies (Robal et al., 2012). However, there was no mention of a study examining fatty acid binding to cANGPTL4.

Cancer cells have been shown to regulate cell signaling to stimulate the process of lipid biosynthesis to support continuous cellular proliferation (Phan et al., 2014). A major metabolic event in cancer progression is the activation of fatty acid synthesis, which results in the production of saturated fatty acids (SFA) as well as monounsaturated fatty acids (MUFA). This provides the cells with much needed energy and sustains the demand for phospholipids required for new cell membranes (Igal, 2010). A more comprehensive understanding is needed concerning this process as well as investigations studying the binding of biomolecules to SFAs.
There are many genetic alterations in cells that lead to disturbances in mechanisms that control cell division, cell death, and cell metabolism (Elmore, 2007). Malignant cells have a different metabolic activity than normal cells. They establish metabolic reprogramming in order to meet their substantial energy requirements. These cells show an increased uptake of glucose and fatty acid production. Research has implicated complex carbohydrates in cell adhesion processes, cell adhesion to the extracellular matrix, and cell-cell recognition. Furthermore, carbohydrates can hinder cell adhesion processes as well as cell-cell recognition (Nangia-Makker et al., 2002).

A more comprehensive understanding is needed on the various functions of each domain to understand which fragments actually play an active role in the progression of cancer cells (Tan et al., 2012). Also, information concerning posttranslational modifications and molecular mass vary for each fragment. These data are important when investigated protein binding. Studies have demonstrated that cANGPTL4 interacts with integrin α5β1, VE-cadherin and claudin-5 (Huang et al., 2011). Previous studies have shown that nANGPTL4 binds to fatty acids such as oleic acid, palmitic acid, myristic acid, and linoleic acid. However, it is cANGPTL4 that has been associated with angiogenesis, cancer cell invasiveness, increased cellular proliferation, cell migration, and cell adhesion (Tan et al., 2012; Zhu et al., 2011).

ANGPTL4 has been shown to play a role in the progression of numerous types of cancers, including PDAC (Tan et al., 2012; Kirbya et al., 2016; Galaup et al., 2006). The research described here is an examination of the structural properties of cANGPTL4. There is a discrepancy among previous studies concerning the reported molecular mass of ANGPTL4 (Tan et al., 2012). The most likely reason is variation in
cleavage forms and glycosylation that as a result of tissue dependence (Tan et al., 2012). Both glycosylated and non-glycosylated cANGPTL4 have been reported (Grootaert et al., 2012). Also, it is known that fatty acids bind to nANGPTL4, but it is unknown if fatty acid bind to cANGPTL4 (Robal et al., 2012). In fact, a repertoire of this protein’s interacting partners remains to be determined. We use X-ray crystallography and binding studies to obtain insight into the structural properties of cANGPTL4.

4.2 Material and Methods

4.2.1 Expression and Purification of Recombinant Human cANGPTL4

The amino acid sequence of cANGPTL4 from various species was obtained from UniProt, aligned using Clustal Omega and evaluated for conserved residues. An expression plasmid was assembled from oligos using DNA Works at Helix System for human cANGPTL4 (185-406) with an N-terminal His6-tag. The plasmid was cloned into the NdeI and BamHI sites of pET3a (Novagen, USA) through homologous recombination. The plasmid was propagated in Escherichia coli strain DH5α cells (Genlantis, Inc. San Diego, CA), error-free clones were further selected by DNA sequencing (Eurofin, AL, USA), and were transformed into E. coli SHuffle cells (Genlantis, Inc., CA, USA) for protein expression. E. coli cells harboring an expression construct were grown in 1 liter of Luria-Bertani (LB) medium containing 500 µl of 100 mg/ml carbenicillin (50 µg/ml final concentration) at 30°C overnight with constant shaking at 250 rpm. The cell starter culture was centrifuged at 6000 rpm for six minutes, and the cell pellets were re-suspended in 50 mL of LB. Re-suspended cells were added to a 10 L terrific broth medium containing carbenicillin in a bioreactor with continuous
aeration. Cells were cultured until an OD$_{600}$ of 0.6 was reached, and then induced by adding isopropyl β-D-galactopyranoside (IPTG) to a final concentration of 0.6 mM to express the recombinant protein. Induction was maintained for 12 hours at 18°C with continuous aeration. Bacterial cells were harvested by centrifugation at 6000 rpm at 4°C for six minutes and stored at -80°C.

A 25 g cell pellet was re-suspended in lysis buffer consists of 50 mM Bis-Tris-propane pH 9.2, 20 mM imidazole, and 400 mM NaCl for protein isolation purification. After lysis and centrifugation at 17,000 g for 45 minutes, the protein was purified using a nickel affinity column equilibrated with the same buffer solution. The column was washed with lysis buffer, and protein was eluted from the column using lysis buffer containing an increased concentration of imidazole (375 mM). Protein purity was evaluated on a 12% Bis-Tris gel and Coomassie stain. Concentrated protein was further purified using size exclusion chromatography (S200) with 50 mM Bis-Tris-propane and 100 mM NaCl, pH 9.2. An Amicon Ultra 10K spin column was used to concentrate the protein to 12 mg/mL and it was stored at 4°C. Protein purity and sequence were confirmed by mass spectrometry.

4.2.2 Proliferation Assay with Recombinant Human cANGPTL4

The biological activity of recombinant cANGPTL4 was measured with a cell proliferation assay. Panc-1, MiaPaCa2, and BxPC3 grown in cell culture were transfected in duplicate samples with 1 ng/µL and 10 ng/µL of recombinant cANGPTL4 using Xfect Protein Transfection Reagent (Clontech). The assay was repeated with the addition of 20uM Diphenyleneciodonium (an inhibitor of NADPH oxidase). Cells from
each line were also transfected with GFP control at 1 ng/µL and 10 ng/µL. The number of viable cells in culture 12 hours post-transfection were determined with CellTiter-Glo Luminescent Cell Viability Assay (Promega).

4.2.3 Crystallization Screening

Approximately 2 mL of cANGPTL4 at 11 mg/mL was trace fluorescently labeled with carboxyrhodamine-succinimidyl ester for visualization of protein crystals in crystallization plates (Invitrogen) (Forsythe et al., 2006). Sitting-drop vapor diffusion screening was used by adding 50 µl of solution to reservoirs and protein to precipitate volumes of 2:1, 1:1 and 1:2 into 96-well plates (Hampton Research). In order to maximize the likelihood of obtaining crystals, this process was completed using three commercial screens: HRHT (Hampton Research), JCSG+ (Molecular Dimensions), and MCSG-3 (Anatrace). A fourth screen was setup using an in-house devised 96 condition crystallization screen with more instances of precipitants that appear only a few times in commercial screens. Plates were scanned daily using fluorescence with a Crystal X2 imaging device to look for the formation of protein crystals. Crystals were identified by their having a high fluorescence intensity relative to precipitated protein. Starting crystallization conditions were identified as 0.1 M HEPES, 1.6 M ammonium sulfate, 0.1 M sodium chloride, pH 7.5. Screening around these conditions was carried out by varying the ammonium sulfate concentration and the buffer pH. Improved conditions, used for ligand binding studies, were found to be over the pH range of 7.5 to 7.7, with ammonium sulfate reservoir concentrations ranging from 0.9 M to 1.2 M.
Ligand binding studies were set up in 4 x 4 blocks of wells in the 96 well sitting drop plates, with the pH varying from 7.5 to 7.7 in the X-axis and ammonium sulfate from 1.2 to 0.9 M in the Y-axis. Six blocks were set up for each 96 well plate. Ligand binding crystallization plates were set up by hand. Fatty acid ligands tested included butyric, myristic, palmitic, stearic palmitoleic, oleic, linoleic, and arachidonic acids. Carbohydrate ligands tested included glucose, fructose, sucrose, lactose, arabinose, mannose, and ribose. One 4x4 well block of each plate was used for growing ligand-free crystals for comparison. The fatty acid ligands were taken up in ethanol, then each was added to the protein solution in an equimolar ratio. The carbohydrates were taken up in dH$_2$O and added to the protein solution at a 10:1 (ligand:protein) ratio. The starting protein solution concentration was 11 mg/mL in the final gel filtration buffer (0.025 M Bis-Tris Propane, 0.2 M NaCl, pH 9.5). The crystals obtained at the lower ammonium sulfate concentrations were typically best for collecting diffraction data.

Crystals were harvested using appropriate loop sizes (MiTeGen), then briefly soaked in a cryoprotectant solution containing reservoir solution. Glycerol was initially used, at 20%, but it was discontinued once it was found in the ligand binding pocket due to concerns that it could displace the fatty acid ligands. Ethylene glycol was subsequently employed at 20% concentration as the cryoprotectant.

4.2.4 Diffraction Data and Structure Elucidation

Diffraction data were collected at Argonne National Lab on beamline SBC-CAT on 19BM or ID. Diffraction images were integrated and scaled using HKL 2000 and 3000. Phenix (version 1.16.1) was used to obtain phasing. Molecular replacement was performed in Phenix. Then, molecular replacement was repeated using a different
structure from the PDB for two sets of diffraction data as a comparison. Protein structure model building and refinement was completed using both Phenix (Adams et al., 2002) and COOT (Crystallographic Object-Oriented Toolkit) crystallographic software.

X-ray diffraction data were collected, images were indexed, and integration was completed using DENZO. Once integrated, diffraction data were scaled with the SCALEPACK program in HKL 2000 or 3000. Crystallographic resolution was cutoff at an I/σ value greater than 2. Scaled intensities were converted to structure factors. Files were transferred to Phenix for phasing and elucidation of structures.

Phasing was complete in Phenix using molecular replacement with PHASER. Molecular replacement was performed in Phenix using PDB 1Z3S as a model for the first crystallographic structure. Then, PDB 6EUB was used as a molecular replacement model for two sets of diffraction data as a comparison. Each structure underwent refinement using Phenix: refine. COOT crystallographic software was used to visualize the Fo-Fc and 2Fo-Fc electron density maps and manually fit residues or ligands. Three high resolution crystallographic derived structures were determined and deposited in the protein data bank.

4.2.5 Palmitic Acid and cANGPTL4 Binding Assay

Capan-1 cells expressing cANGPTL4 were grown on hydrophilic, tissue culture-treated ibidi 8-well microchamber plates. Cells were grown to approximately 80% confluency. Capan-1 cells in 2 of the 8 wells were incubated with BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) tagged palmitic acid, cells in 2 wells were incubated with fluorescently-labeled recombinant cANGPTL4 combined with BODIPY
(4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) tagged palmitic acid, cells in 2 wells were incubated with fluorescently-labeled recombinant cANGPTL4 without palmitic acid, and serum free media only was placed in the final 2 wells. Cells were incubated at 37°C for 3 hours. Following incubation, unbound palmitic acid and recombinant cANGPTL4 were removed by washing extensively with PBS. Cells were fixed with 4% paraformaldehyde for 10 minutes. They were washed three times in PBS, briefly washed with 0.1M ammonium chloride, washed again with PBS, and blocked with 3% normal goat serum for 1 h at room temperature. Following three washes in PBS, cells in the 2 wells that were incubated with fluorescently labeled palmitic acid were incubated overnight at 4 °C with anti-human cANGPTL4. Cells in the rest of the wells were left in 3% normal goat serum during this time. After three washes with PBS at room temperature, cells were incubated with Alexa Fluor 647 secondary antibodies for 2 hours. Actin-red 555 and Nuc-blue were added for actin and nuclear staining. Imaging was performed without primary antibodies served as negative controls. Images were acquired using a Zeiss LSM710 confocal microscope.

4.2.6 Thermal Shift Assay to Determine Kd for Palmitic Acid Binding

Thermal shift assays were performed to measure the binding of palmitic acid and cANGPTL4 using a Tycho NT.6 from Nanotemper (Adams et al., 2016). Use of this instrument was kindly provided by PMI BioPharma Solutions (Nashville, TN). Briefly, the assay was carried out by mixing cANGPT4 in a buffer consisting of 0.05M HEPES, 0.2M NaCl, pH 7.5 with palmitic acid solution in (solvent), such that the final
concentration was fixed at 25% while the palmitic acid varied between 0.2 µM and 25 µM. Assays were carried out at cANGPTL4 concentrations of 0.25 and 1.0 µM.

4.3 Results

4.3.1 Protein Analysis

To examine the purity of recombinant human cANGPTL4, the protein was analyzed by SDS-PAGE with Coomassie blue staining (Supplemental 2A). A protein band was obtained at the expected molecular weight at approximately 26,600 kDa. To confirm the protein sequence, a sample was sent to the University of Alabama in Birmingham for mass spectrometry analysis. Results identified the sequence of residues 185 - 400 of ANGPTL4’s C-terminal domain (Supplemental 2B).

4.3.2 Biological activity

To determine if the recombinant protein was active, a cellular proliferation assay was performed. Low concentrations (1 ng/µL and 10 ng/µL) of protein were transfected into Panc-1, MiaPaCa2, and BxPC3 grown in cell culture. A two-tailed T-test was performed to determine statistical significance. Cellular proliferation rates were not significantly increased in Panc-1 cells transfected with 1 ng/µL of protein but were increased in cells transfected with 10 ng/µL (p = 0.05) (Figure 4.1). Cellular proliferation rates were significantly increased in MiaPaCa2 cells transfected with 1 ng/µL (p = 0.023) and 10 ng/µL (p = 0.0081). No significant increase in cellular proliferation was seen in BxPC3 cells.
Figure 4.1: Box plots depicting cellular proliferation rates of Panc-1, MiaPaCa2, and BxPC3 cells post-transfection with recombinant human cANGPTL4. Recombinant human cANGPTL4 protein was transfected into cells at 1 ng/µL and 10 ng/µL protein. Number of viable cells were determined 12 hours post-transfection with CellTiter-Glo Luminescent Cell Viability Assay.

4.3.3 Protein Crystals

In order to screen for crystallization conditions, cANGPTL4 at 11 mg/mL was trace fluorescently labeled with carboxyrhodamine-succinimidyl ester and used in multiple crystallization screens. Thin plate-shaped protein crystals formed in a well containing 0.1 M HEPES pH 7.5, 0.1 M sodium chloride, and 1.6 M ammonium sulfate (Figure 4.2). Once crystallization conditions were identified, additional sitting-drop vapor diffusion plates were created with the addition of various unsaturated fatty acids, saturated fatty acids, and carbohydrates. Crystals were found in wells containing protein plus myristic, palmitic, oleic, linoleic, or stearic acid.
Figure 4.2: Protein crystals of recombinant human cANGPTL4. A) Thin plate-shaped crystals of recombinant human cANGPTL4 formed in a well containing 0.1 M HEPES pH 7.5, 0.1 M sodium chloride, and 1.6 M ammonium sulfate. B) Crystals of recombinant human cANGPTL4 formed in a well containing 0.1 M HEPES pH 7.5, 0.1 M sodium chloride, 1.6 M ammonium sulfate, and palmitic or myristic acid. Scale is microns per smallest division.

4.3.4 Protein Structure of Recombinant Human cANGPTL4 Monomer

To analyze the amino acid sequence of cANGPTL4 (180-400), Clustal Omega was used to align the sequences from various species. The analysis showed similarities and several well conserved residues between them (Figure 4.3). There are 4 well conserved cysteine residues at C188, C216, C341, and C354. The monomeric structure of cANGPTL4 has a topology of α1-β1-β2-β3-α2-β4-β5-β6-β7-η1-β8-α4-β9-α5-β10-
η2-β11-β12 (Figure 4.4). It is stabilized by two disulfide bonds formed by C188 - C216 and C341 - C354 (Figure 4.5). The recombinant cANGPTL4 protein was designed to begin with P185 and end with M400.

Figure 4.3: Analysis of the amino acid sequence of cANGPTL4 (180-400) shows similarities and conserved residues between various species. There are 4 well conserved cysteine residues at C188, C216, C341, and C354.
Figure 4.4: Monomeric structure of recombinant human cANGPTL4 showing $\alpha_1$-$\beta_1$-$\beta_2$-$\beta_3$-$\alpha_2$-$\beta_4$-$\beta_5$-$\beta_6$-$\beta_7$-$\eta_1$-$\beta_8$-$\alpha_4$-$\beta_9$-$\alpha_5$-$\beta_10$-$\eta_2$-$\beta_11$-$\beta_12$ topology. Each secondary structure element is colored as cyan for $\alpha$-helices, fuchsia for $\beta$ strands, and pink for loops.

Figure 4.5: Atomic resolution image of 4 conserved cysteine residues in cANGPTL4. A) Disulfide bond between C188 and C216 B) Disulfide bond between C341 and C354. The 2Fo-Fc electron density maps were contoured at 1$\sigma$. 

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4.3.5 Glycerol Binding to cANGPTL4

cANGPTL4 protein crystallized with one molecule in the asymmetric unit (Table 4.1). The electron density map contained peaks near Trp349, Trp350, His356, and Ser357. The crystal structure of cANGPTL4 in complex with glycerol was solved to a crystallographic resolution of 2.11 Å in a C222₁ space group. Molecular replacement was used to solve the structure, and it was refined to $R/R_{\text{free}}$ values of 21.3/23.6. A glycerol molecule forms a hydrogen bond with Trp349, Trp350, His356, Ser357 (Figure 4.6). Coordinates were deposited in the PDB with the accession ID 6U0A.

4.3.6 Palmitic Acid Binding to cANGPTL4

Crystals of the cANGPTL4/palmitic acid complex grew in the same C222₁ space group. The monomeric structure was solved to a 1.75 Å resolution and refined to $R/R_{\text{free}}$ values of 19.4/21.1 (Table 4.2). The carboxylic head of palmitic acid forms a hydrogen bond with Thr353 while hydrophobic side chains of amino acids in the binding pocket stabilize the alkyl tail by van der Waals interactions. Previous experiments of fatty acid binding protein 5 (FABP5) have resulted in an enlargement of the ligand pocket volume as a fatty acid would bind to Cys127. The electron density of the cANGPTL4/palmitic acid complex reveals the unequivocal presence of disulfide bonds between Cys341 and Cys354 (Figure 4.7). Coordinates were deposited in the PDB with the accession ID 6U1U.
Table 4.1: Refinement statistics for crystal structure of recombinant human cANGPTL4 with glycerol, PDB ID 6U0A.
Figure 4.6: Structural analysis of cANGPTL4 binding pocket with glycerol. Glycerol forms a hydrogen bond with Trp349, Trp350, His356, Ser357. The 2Fo-Fc electron density maps were contoured at 1σ.
Table 4.2: Refinement statistics for crystal structure of recombinant human cANGPTL4 with palmitic acid, PDB ID 6U1U.

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Figure 4.7: Structural analysis of cANGPTL4 binding pocket with palmitic acid. The carboxylic head of palmitic acid forms a hydrogen bond with Thr353 while hydrophobic side chains of amino acids in the binding pocket stabilize the alkyl tail by van der Waals interactions. B) Electron density map showing a disulfide bond between Cys341 and Cys354. The 2Fo-Fc electron density maps were contoured at 1σ.

### 4.3.7 Myristic Acid Binding to cANGPTL4

Myristic acid is another long chain saturated fatty acid that was added to cANGPTL4 in crystallization trials. A space group of C222₁ was once again obtained, and the monomeric structure was solved to a resolution of 2.37 Å. The structure was refined to R/R\text{free} values of 19.4/23.8 (Table 4.3). Once again, the carboxylic head of palmitic acid forms a hydrogen bond with Thr353 while the alkyl tail is stabilized by van der Waals interactions (Figure 4.8). Coordinates were deposited in the PDB with the accession ID 6U73.
Table 4.3: Refinement statistics for crystal structure of recombinant human cANGPTL4 with myristic acid, PDB ID 6U73.
Figure 4.8: Structural analysis of cANGPTL4 binding pocket with myristic acid. The carboxylic head of palmitic acid forms a hydrogen bond with Thr353 while hydrophobic side chains of amino acids in the binding pocket stabilize the alkyl tail by van der Waals interactions. Electron density map showing a disulfide bond between Cys341 and Cys354. The 2Fo-Fc electron density maps were contoured at 1σ.

4.3.8 Binding of cANGPTL4 to Palmitic Acid Cell Culture Assay

In order to examine cANGPTL4 binding to palmitic acid and cellular location of the complex, Capan-1 cells were incubated with BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) tagged palmitic acid and lucifer yellow tagged recombinant cANGPTL4 in serum free media. Images obtained with confocal microscopy showed cells with cANGPTL4 (yellow), cells with palmitic acid (green), and cells with both molecules. A merged image reveals both cANGPTL4 and palmitic acid colocalized inside Capan-1 cells (Figure 4.9, top panel). No autofluorescence was seen in DIC imaging of these cells.

In order to investigate the binding of palmitic acid to endogenous cANGPTL4, Capan-1 cells were incubated with only BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-
indacene) tagged palmitic acid for 3 hours. Then the cells were incubated with Actin-red 555 and Nuc-blue. Images obtained with confocal microscopy showed the nuclei (blue), actin filaments (red), and palmitic acid (green) located inside Capan-1 cells. Fatty acids, like palmitic acid, would remain outside cells without being bought into the cells by a transporter, such as cANGPTL4 (Figure 4.9, bottom panel).

Figure 4.9: Examining the binding of palmitic acid and cANGPTL4 in Capan-1 cells. Top panel: Capan-1 cells incubated with BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) tagged palmitic acid and lucifer yellow tagged recombinant cANGPTL4 in serum free medium. DIC image shows no autofluorescence in the first image. The second image shows cells with cANGPTL4 (yellow), third image shows cells with palmitic acid (green), and fourth image shows cells with cANGPTL4 and palmitic acid inside the cells. Bottom panel: Capan-1 cells incubated with only BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) tagged palmitic acid, Actin-red 555, and Nuc-blue. The first image shows nuclei only, second image shows cells with cANGPTL4 (yellow), third image shows cells with palmitic acid (green), and the fourth image shows cells with cANGPTL4 and palmitic acid inside the cells.
4.3.9 Binding of cANGPTL4 to Palmitic Acid Thermal Shift Assay

Having found palmitic acid bound to the cANGPTL4, the binding affinity of the fatty acid to the protein was determined using a thermal shift assay. This assay was performed using a Tycho NT.6 from Nanotemper courtesy of PMI BioPharma Solutions (Nashville, TN) (Adams et al., 2016). The principle of the assay is that the protein, in the presence of varying concentrations of ligand, is heated from 35 to 90 °C and the intrinsic fluorescence emission at 330 and 350 nm measured as a function of temperature. The 350/330 ratio changes as the protein unfolds, and the midpoint of the ratio curve vs. temperature is plotted as a function of the ligand concentration. Bound ligand that stabilizes the protein structure shifts the midpoint to a higher concentration, and a plot of the midpoint vs. ligand concentration can be used to estimate the binding constant of the ligand for the protein. The binding of palmitic acid to cANGPTL4 was evaluated at two protein concentrations, 0.25 and 1.0 µM, and over the range of palmitic acid concentrations from 0.2 to 25 µM. The experiments were carried out in duplicate. The results indicated a Kd ranging from 0.77 to 1.3 µM at a protein concentration of 1.0 µM (Figure 4.10 and Table 4.4). This value is well within the range of the plasma free palmitic acid concentration, which ranges from 0.3 to 4.1 mmol/L in healthy adults.
Figure 4.10: Thermal shift assay measuring the binding of palmitic acid and cANGPTL4 at 0.25 μM and 1.0 μM using a Tycho NT.6 from Nanotemper.

Table 4.4: Thermal shift assay measuring the binding of palmitic acid and cANGPTL4 at 0.25 μM and 1.0 μM using a Tycho NT.6 from Nanotemper.
4.4 Discussion

ANGPTL4 is a secreted protein known to play a role in the progression of multiple carcinomas (Tan et al., 2012; Zhu et al., 2011; Huang et al., 2011; Kirbya et al., 2016; Kim et al., 2000). This protein has been implicated in the metabolic reprogramming of cancer cells during EMT (Teo et al., 2017). A decline in cellular energy resulting in decreased metastasis was seen in cancer cells lacking ANGPTL4 during EMT. Additionally, it was shown that ANGPTL4 controlled the expression of certain 14-3-3 proteins to meet cellular energy requirements for EMT. Previous investigations revealed that ANGPTL4 promoted anoikis resistance and tumor growth by triggering a 14-3-3 adapter protein, which isolated apoptotic proteins (Teo et al., 2017). However, how ANGPTL4 controls the expression of 14-3-3 and triggers the adapter protein remains unknown. Our crystallographic derived models reveal a common binding site for multiple ligands (deposited in the PDB as IDs 6U0A, 6U1U, and 6U73). While we do not show ANGPTL4 in complex with a 14-3-3 protein, we do show cANGPTL4 in complex with glycerol and fatty acids. This provides considerable insight into how cANGPTL4 can provide cancer cells with energy necessary for proliferation and EMT.

The regulation of lipid and glucose metabolism is a well-established function of ANGPTL4 (Xu et al., 2005; Sukonina et al. 2006). The protein has also been associated with diabetes, coronary artery disease, and obesity (Tan et al., 2012; Zhu et al., 2011). Cancer cells are known to undergo metabolic reprogramming to meet energy requirements, and ANGPTL4 has been recognized as a controller of this reprogramming (Teo et al., 2017). This study investigated the fragment cANGPTL4 in PDA. Our
findings provide novel insight into cANGPTL4’s metabolic role by showing for the first time how this protein may transport palmitic acid into pancreatic cancer cells.

Palmitic acid binding by cANGPTL4 was found to be in the low µM range, at a level consistent with free plasma concentrations. The binding site was also occupied by glycerol in another crystallographic derived structure (Deposited as PDB ID 6U0A). There is a possibility that this site could accommodate a mono- or a di-glyceride. Crystals have been obtained for cANGPTL4 grown in the presence of mono-myristin, but diffraction data has not been collected or processed for them at this time.

cANGPTL4 has been reported as both glycosylated and non-glycosylated, depending on the expressing tissue (Tan et al., 2012; Zhu et al., 2011). While we were not able to verify this due to corrupted data, we were able to show that the non-glycosylated recombinant protein behaved similarly to the endogenous protein in terms of binding to integrin-β1, thus indicating that glycosylation is presumably not required for this function. Additionally, we found that the recombinant protein binds to fatty acids and glycerol, and that it then transports the fatty acids into the cell. Again, immunofluorescence and confocal microscopy studies show that the endogenous protein also binds to and transports fatty acids into the cell. The fatty acid binding function of cANGPTL4 is a novel finding of this research, revealing a hitherto unknown function and role for the protein. Further, while studies with the un-liganded protein show that it is located on the extracellular matrix, the fatty acid liganded protein is not located there, but rather inside the cell. Thus, the binding of fatty acids serves to redirect the protein to a new role, the potential of which remains to be explored. This may well indicate a therapeutic function that can be exploited through the design of a pharmaceutical that
binds to the fatty acid binding of cANGPTL4 to deliver it to cancer tumors. While non-trivial, this could potentially bypass the problem of a general targeting of cANGPTL4 which could result in the disruption of other needed cellular functions in which it participates.

Since cANGPTL4 is a multifunctional protein, and it is not unreasonable to hypothesize that the protein-fatty acid complex also has a signaling function. What that signaling function would be is not known and was not revealed in this research. This is an important area for understanding any potential side effects associated with pharmaceuticals developed to bind to the protein. Also, the signaling may not be directly related to cancer progression, but rather may be linked to one of cANGPYL4’s other functions. For example, the nANGPTL4 is known to affect lipoprotein lipase activity. As the cANGPTL4 is binding a fatty acid, it is not unreasonable to suggest that the signaling function is connected to regulation of fatty acid metabolism, and not directly to cancer progression.

If a signaling function were present, then a starting point to explore the nature of that function would be a pull-down assay. The work presented herein shows that glycosylation is apparently not relevant to the fatty acid binding and transport therefore this assay is simplified and can be carried out using non-glycosylated recombinant protein. On the basis of the immunofluorescence in this study, the protein-fatty acid complex is pulled into the cytoplasm. A pull-down assay would use cell cytoplasm isolates with added either bare or fatty acid bound cANGPYL4. After pull-down the proteins bound to the cANGPTL4 would be identified by 2D electrophoresis followed by sequencing. The sequence information would reveal protein(s) that the cANGPTL4 is
binding to, and thus what internal pathways are being impacted. There may be no
difference between liganded and unliganded cANGPYL4 in the pulled down binding
partners. This would not be surprising, as the unbound protein stays on the cell surface
and is not expected to be internalized.

In this study, we provide insight into cANGPTL4’s structure and function. These
results reveal a novel function of this protein and suggest how it may aid in cancer cell
progression. As discussed above, additional investigations are needed. Protein structures
are now available for use in future studies of cANGPTL4.
Chapter 5

5.1 Summary

PDAC has a relative five-year survival rate of 34% with early stage diagnoses that drops to 3% with metastasis (Noone et al., 2017). ANGPTL4 studied in detail here has been indicated in the progression of this cancer and various others (Tan et al., 2012; Zhu et al., 2012; Huang et al., 2011; Kirby et al., 2016). There were three specific aims for this research. First, to understand how cANGPTL4 expression drives malignant phenotype. Second, to extend the existing structural-function studies from bacterially produced to human cancer cells. Third to determine the specific residues of cANGPTL4 that interact with binding partners. For the first aim, we examined differential expression of ANGPTL4 in pancreatic and breast cancer cell lines with varying phenotypical characteristics. For the second aim, we investigated the secretion and cellular location of full-length ANGPTL4 and both isoforms. We explored the interaction of endogenous cANGPTL4 with integrin β1 and palmitic acid. Finally, we determined structures of cANGPTL4 protein complexed to glycerol, palmitic acid, and myristic acid. This allowed us to determine specific residues of cANGPTL4 that interacted with each.

Through a combination of these complimentary biochemical and biophysical techniques, this work shows for the first time that cANGPTL4 binds fatty acids, a previously unknown property of this protein. Further, the results show that the protein
then carries the bound fatty acid back into the cell. This may be a means of supplying energy to the cell, needed for the rapid growth and proliferation of cancer cells but also may have a signaling function.

5.2 Future Studies

The research addressed much of the specific aims but leaves some details unknown. For example, we do not know the role or roles of the cANGPTL4-fatty acid complex. Experimentally, this could be explored adding the recombinant cANGPTL4-fatty acid complex to a cell culture, briefly incubating them, washing the cells, then lysing them, and thereby recovering the cANGPTL4 with any bound proteins using a pull-down assay. This would provide information on any complex formed together with a sample of that complex. Further work would be needed to determine the function, but this would provide a suitable starting point for that direction. Alternatively, as the protein in our case is already Hexa-His tagged it may be sufficient to pass the lysed cells supernatant over a Ni\(^{++}\) affinity column. A similar analysis could then be used to determine any complexes found. This was not done with the present work as they exceeded our original goals but are a question for the future.

cANGPTL4 binds to a range of fatty acids but we have limited structural knowledge on how this occurs. This should also be further explored. Crystals have been obtained in the presence of the monoglyceride monomyristin, stearic acid, dodecanoic acid, palmitoleic acid, oleic acid, and linoleic acid, but the structures have not been determined or refined at this point. To obtain a complete picture of binding, these structural approaches require binding affinity studies, as well as determination of whether the different acids all behave the same as the cANGPTL4-palmitic acid complex with
respect to fatty acid transport into the cell. This provides a self-contained project for a future student. Additionally, crystals have been obtained of cANGPTL4 with added fructose, glucose, and arabinose to investigate this protein’s ability to bind to other fuel sources. The diffraction data has not yet been processed.

There is evidence to suggest that the binding of fatty acids may have other effects as well, such as preventing the binding of other proteins. Previous studies have shown that recombinant cANGPTL4 binds to integrin, claudin, and cadherin, serving to disrupt cell-cell contacts and promoting metastasis. However, these studies were carried out with the apoprotein, not liganded with fatty acids. Adding recombinant apoprotein to the cell culture shows that it is bound to the cell surface, as would be expected on the basis of results from a previous study (Huang et al., 2011). However, adding the holoprotein, a cANGPTL4-fatty acid complex, to the cell culture does not show protein bound to the cell surface but rather protein inside the cell, with the fatty acid still apparently bound. To resolve these observations, the binding studies of Huang et al. would need to be repeated using the cANGPTL4-fatty acid complex and compared to previous results to determine if the bound fatty acid affects the proteins ability to bind to the three cell surface proteins.

One concern was the role of glycosylation on cANGPTL4’s biological activity. We carried out cell culture studies using immunofluorescence and confocal imaging. These studies showed the same responses from endogenous protein as found when we added recombinant, non-glycosylated, protein to the culture media. These are positive results indicating that, for the scope of the questions probed in this dissertation,
conclusions drawn on the basis of the recombinant protein are also valid for the endogenously produced protein.

We were unable to express sufficient endogenous protein from cell culture to attempt structural studies. The significant resources necessary to increase this production were simply not available. Further, the three known binding partners are all integral membrane proteins that are known to be glycosylated, considerably raising the difficulty of carrying out classical crystallization and X-ray diffraction structural studies. An approach using cryo-electron microscopy is potentially better suited to the complexes with low abundance glycosylated integral membrane proteins and their low probability of crystallization.

5.3 Conclusion

This research significantly expands our knowledge on the functional range of the protein cANGPTL4 addressing the initial research goals. It has also opened up more research questions that have been guided by the results produced. A total of three structures were determined and deposited in the PDB consisting of glycerol, palmitic acid, and myristic acid with cANGPTL4. This will enable others to explore these protein complexes for the first time.
Works Cited


El Sayed, SA. & Mukherjee, S. 2019. "The pancreas is a composite organ, which has exocrine and endocrine functions." In *Pancreas Physiology.* StatPearls Publishing LLC.


Supplemental Figure 1: Autofluorescence was accessed by DIC imaging of Panc-1, MiaPaCa2, Capan-1, MDA-MB231, MCF7, and SKBR3 cells. No autofluorescence was detected in any of the cell lines. Scale bar = 100µm.
Supplemental Figure 2: Protein analysis of recombinant human cANGPTL4. A) SDS-PAGE with cANGPTL4. The strong band indicates successful expression and purification of cANGPTL4. This protein is largely impurity free and well suited for crystallization trials. B) Mass spectrometry analysis identified the amino acid sequence of residues 185-400 of recombinant human ANGPTL4’s C-terminal domain.