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Acceleration of the PanIN Development in Mice Expressing Oncogenic K-Ras Due to a High Fat Diet

Bincy Philip

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Acceleration of PanIN Development in Mice Expressing Oncogenic K-Ras Due to a High Fat Diet

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A

THESIS

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The University of Texas
Health Science Center at Houston
and
The University of Texas
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Graduate School of Biomedical Sciences
In Partial Fulfillment
of the Requirements
for the Degree of

MASTER OF SCIENCE

by

Bincy Philip, B.S
Houston, Texas

May 2013
Dedicated to my parents for their constant love and never-ending support throughout my life. Thank you for working so hard for our family to give us the opportunity for a better future.
I would like to express my gratitude to my wonderful parents, Philipose Thomas and Marykutty Philipose, for their continued love throughout the last 23 years of my life. I give all my thanks to my brother Jibu Philip, my sister Jincy Philip, and my sister-in-law Sheril for their loving support from two thousand miles away. Though this past year has been a stressful and scary time for our family, we were able to persevere. I am amazed by the strength and fearlessness of each and every one of you. I am forever grateful for my uncle Jacob Mathew, my aunt Accamma Jacob, and my little sisters Lina and Anshu for being the constant support for our family throughout every hardship and happiness in our lives.

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Acceleration of the PanIN Development in Mice Expressing Oncogenic K-Ras Due to a High Fat Diet

Bincy Philip

Supervisory Professor: Craig D. Logsdon, Ph.D.

Obesity is postulated to be one of the major risk factors for pancreatic cancer, and recently it was indicated that an elevated body mass index (BMI correlates strongly with a decrease in patient survival. Despite the evident relationship, the molecular mechanisms involved are unclear. Oncogenic mutation of K-Ras is found early and is universal in pancreatic cancer. Extensive evidence indicates oncogenic K-Ras is not entirely active and it requires a triggering event to surpass the activity of Ras beyond the threshold necessary for a Ras-inflammation feed-forward loop. We hypothesize that high fat intake induces a persistent low level inflammatory response triggering increased K-Ras activity and that Cox-2 is essential for this inflammatory reaction. To determine this, LSL-K-Ras mice were crossed with Ela-CreER (Acinar-specific) or Pdx-1-Cre (Pancreas-specific) to “knock-in” oncogenic K-Ras. Additionally, these animals were crossed with Cox-2 conditional knockout mice to access the importance of Cox-2 in the inflammatory loop present. The mice were fed isocaloric diets containing 60% energy or 10% energy from fat. We found that a high fat diet increased K-Ras activity, PanIN formation, and fibrotic stroma significantly compared to a control diet. Genetic deletion of Cox-2 prevented high fat diet induced fibrosis and PanIN formation in oncogenic K-Ras expressing mice. Additionally, long term consumption of high fat diet, increased the progression of PanIN lesions leading to invasive cancer and decreased overall survival rate. These findings indicate that a high fat diet can
stimulate the activation of oncogenic K-Ras and initiate an inflammatory feed forward loop requiring Cox-2 leading to inflammation, fibrosis, and PanINs. This mechanism could explain the relationship between a high fat diet and elevated risk for pancreatic cancer.
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1.1 Introduction

Currently, pancreatic ductal adenocarcinoma (PDAC) is the fourth deadliest malignancy in the United States and every year over 43,000 Americans are diagnosed with this disease (77). Majority of these patients will die within a year of diagnosis and mortality rates have increased in the last decade (79). Only 5% of patients will survive up to five years after diagnosis (77). It is estimated that 19,480 men and 18,980 women will die from pancreatic cancer in 2013 alone (78). The severity of this disease stems from the lack of early detection and effective treatments with surgical resection as the only treatment presently. However, for most patients, by the time of diagnosis, the cancer has already metastasized and it is too late for surgical resection to be effectual.

1.2 Early Stages of Pancreatic Cancer

1.2.1 Bi-functional Roles of the Pancreas

The pancreas is an irregularly shaped, flat digestive gland that is located behind the stomach and runs along the duodenum and spleen (Figure 1.1). The location of the organ makes it a difficult for visualization using current methods of detection and increases the risk of cancer to go undetected. The pancreas consists of three types of cells with diverse functions that are either part of the exocrine or endocrine gland (40). The exocrine gland of the pancreas is comprised of acini that secrete digestive enzymes such as zymogens and ductal cells that allow for transport of fluid drainage from the acinar cell to the stomach as well as secretion of biocarbonate for neutralization of stomach acid (40). The pancreatic duct, running through the entire pancreas, is the main duct for the secretion of pancreatic fluid.
from the exocrine gland into the duodenum and merges with the bile duct (Figure 1.1). Islets of Langerhans make up the endocrine gland and are responsible for the body’s insulin production (40). Together these compartments are necessary for the normal function of the gastrointestinal tract. Cancer in the pancreas can interfere with proper digestion due to the destruction of bile ducts and reduction of pancreatic enzymes produced.

**Figure 1.1:** Diagram of the major properties of the pancreas. Animated representation of the major ducts and cells comprising the pancreas.
1.2.2 Pancreatic Intraepithelial Neoplasia

Currently, there is major controversy present between the origin of pancreatic ductal adenocarcinoma, whether it derives from duct cells or acinar cells transforming into pancreatic intraepithelial neoplasias (PanINs), noninvasive ductal lesions classified as precursors to pancreatic cancer (51). Recent evidence supports the initiation of PanIN formation to be of acinar cell origin in which acinar cells differentiate into ductal precursor lesions in a process called acinoductal metaplasia (29). Specifically in mice it has been recorded that mouse acinar cells can transdifferentiate into preneoplastic ductal lesions in the presence of oncogenic K-Ras (29).

Despite the controversy, the transformation from normal epithelial cells to PanINs requires morphological changes to the cell and nuclei (Figure 1.2). PanIN-1a lesions are classified as the first stage of transformation of the normal flat epithelial cells, acinar or ductal, into columnar cells with the visible presence of mucin production (30, 47, 50, 70). The cells are usually uniform, basally oriented, and the nucleus remains round. However, these cells can transform further and form papillary protrusions in which acinar atrophy becomes highly prevalent, becoming PanIN-1b lesions. PanIN-2 lesions are distinguishable from PanIN-1 in the development of cytologic atypia accompanied by loss of cellular polarity causing overcrowding (32). The nuclei become elongated and the cells appear to be pseudo-stratified. The progression to PanIN-3 lesions are characterized by extensive nuclear atypia, cellular crowding, and cells form papillai budding off into the ductal lumen (30, 32, 47, 50, 70). The presence of PanIN lesions is common in healthy individuals with the risk increasing with age, and they do not always lead to
pancreatic cancer. Progression to PDAC requires the further activation of oncogenic mutations such as K-Ras and tumor suppressors’ p16 and p53 to drive this reaction.

Figure 1.2: Progression of PanIN formation leading to invasive PDAC. Schematic of the different stages of PanIN development starting from transformation of normal epithelial cells to columnar cells leading to PanIN-1a lesions. This is followed by PanIN-1b lesions characterized by papillary protrusions of cells with changes in nuclear morphology. PanIN-2 lesions consist of nuclear atypia accompanied by pseudostratification and overcrowding of cells. These lesions develop into PanIN-3 lesions in which the nuclei are elongated and overcrowded with clusters of cells budding off into the lumen. This progression of PanIN lesions can eventually lead to invasive carcinoma. The initiation of PanIN formation is due to genetic alterations such as mutation in K-Ras leading to accumulation of other oncogenic mutations in p16 and p53. PanIN pictures were reprinted with permission from The American Journal of Surgical Pathology, Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions, Hruban, R.H., et al., 2001. 25(5): p.579-86, Copyright 2001.

1.2.3 Inflammation and Fibrosis

In the initiation of the metaplastic transformation of normal pancreas cells to ductal precursors, inflammation is highly required. Recent evidence from our lab has shown the importance of an inflammatory mediator to drive PanIN formation (14). The link between inflammation and cancer was noted back in 1863 by Rudolf Virchow who observed the presence of leukocytes in neoplastic tissues throughout
tumor areas and stroma (4). This area of neoplastic growth consists of immune cells, such as macrophages, dendritic cells, and lymphocytes, which are designed to migrate to sites of infection and activate unique mechanisms through release of pro-inflammatory cytokines to eliminate the pathogens and allow for tissue reformation (4). However, this network of inflammatory mediators, although necessary to eliminate infection, enhances survival, proliferation, and growth of these tumor cells through DNA damage, angiogenesis, invasion, metastasis, and evading immunity (4, 55). Therefore, chronic inflammation resulting in a “overhealing” as suggested by Sir Alexander Haddow, drives pathogenesis (24, 25).

The activation of inflammation in the pancreas leading to the recruitment of the immune system is initiated by damaged acinar cells (55, 66, 67). Environmental factors such as obesity and genetic mutations are major contributors to inflammation in the pancreas. Once inflammation is initiated by acinar cells and stromal elements undergoing cellular mutations, quiescent pancreatic stellate cells (PSCs), myofibroblast cells, are activated. Activated PSCs travel to sites of injury and develop extracellular matrix (ECM) proteins, predominately alpha smooth muscle actin, collagen I and III, fibronectin, and proteoglycans, leading to fibrosis in the pancreas to help repair the damage (18, 19, 55, 65). Pancreatic cancer is known as a fibrotic tumor with 90% of the tumor volume with pronounced desmoplastic reaction in the stroma lead by PSCs releasing increased levels of ECM deposition exceeding the rate of ECM degradation (46, 58, 65). Overproduction of ECM proteins by stellate cells correlates with poor prognosis of pancreatic cancer. Additionally, stromal cells are manipulated by inflammatory cells and tumor cells to promote tumor cell growth, invasion and migration, indicating the
importance of inflammation and fibrosis in the initiation of PanIN formation (2, 57, 65).

1.2.4 Oncogenic K-Ras mutation

Oncogenic mutation in K-Ras is common in 15% of all cancers with the highest prevalence seen in pancreatic cancer with mutant K-Ras activated in more than 90% of cases (1, 17, 35, 52). The most common oncogenic mutation in K-Ras reported in PDAC is most predominately due to a point mutation in codon 12 (16). The earliest appearance of mutant K-Ras is at PanIN-1 development, the first stage of precursor lesions (31, 51). A meta-analysis of studies on K-Ras mutations showed an increase in K-Ras mutations starting with 36%, 46%, and 87% of lesions containing mutant K-Ras as PanIN lesions progressed from PanIN-1 to PanIN-3 (47). This increase of mutant K-Ras in the presence of PanIN lesions is not seen in PanINs that are present in chronic pancreatitis or normal pancreas tissue (47).

Since mutations in K-Ras are seen in the earliest genetic event occurring during transformation of normal epithelial cells to PanIN-1 lesions, it suggests that this mutation acts as a gatekeeper for this phenomenon. The activation of oncogenic mutation in K-Ras results in the accumulation of mutations in p53 and p16 which is required for the progression into higher grade PanIN lesions (56, 70) (Figure 1.2). A genetic loss of function mutation in the tumor suppressor, p16, is predominately present in PanIN-2 development and is due either by a homozygous deletion, intragenic mutation with loss of second allele, or promoter hypermethylation (39, 43, 70). Loss of p16 can eventually lead to oncogenic inactivation of p53 by the loss of one allele and mutation in the second allele.
allowing for uncontrolled cell growth and genetic instability (70). Activation of oncogenic K-Ras is the first of these genetic mutations and is essential for the further activation of mutations to drive carcinogenesis.

1.3 Endogenous K-Ras Activity and Signaling

Ras is a 21 kilo Dalton guanine nucleotide binding protein (G-protein) that regulates cellular functions involved in cell proliferation, survival, and differentiation. Ras genes were first identified as the Harvey and Kirsten sarcoma retroviruses and named Ras for rat sarcoma. There are three isoforms of Ras that are considered proto-oncogenes: H-Ras, N-Ras, and K-Ras (15). In a normal cell, Ras is bound to guanosine diphosphate (GDP) and remains in an inactive state (Figure 1.3). In response to growth factors, cytokines, and other exogenous signals, guanine nucleotide exchange factors (GEFs) will remove the GDP and bind guanosine triphosphate (GTP) switching the Ras protein to an active state by triggering a conformational change in the G domain allowing for high affinity for downstream targets (8, 15, 74, 86). Once Ras is bound to GTP and active, it can activate the downstream effector molecules, such as phosphoinositide 3-kinase (PI3-K), mitogen-activated protein kinase (MAPK), and Ral that are responsible for normal cellular function (39, 43, 70) (Figure 1.3). The molecular switch of Ras oscillates back to inactive, making the activity transient due to intrinsic GTP hydrolysis and is further enhanced by GTPase activating proteins (GAPs). GAPs are recognized as negative regulators of active Ras and aids in the hydrolysis of GTP to GDP conforming Ras back to an inactive state (8, 15).
Figure 1.3: Endogenous Ras vs. oncogenic K-Ras signaling. Diagram of signaling pathways of endogenous and oncogenic K-Ras and the difference in Ras activity. Endogenous K-Ras is bound to GDP in an inactive state and is activated by GEFs which remove GDP and bind GTP. GTP bound Ras can now activate its downstream effector molecules Raf, PI3K, and NF-κB leading to formation of phospho-ERK, phospho-AKT, and activation of Cox-2 leading to PGE2 which are necessary for cell growth, survival, and differentiation. Endogenous K-Ras due to intrinsic GTPase activity and interaction with GAPs return back to its inactive state through hydrolysis of GTP to GDP. In contrast, oncogenic K-Ras mutation blocks GAP interaction with Ras-GTP prolonging Ras activity. However, oncogenic K-Ras is still bound to GDP and must be stimulated by an inflammatory mediator for GEFs to move it to an active state. Once oncogenic K-Ras is active, it can activate downstream targets. Activation of Cox-2 leads to a positive feed forward loop enhancing K-Ras activity resulting in in prolonged K-Ras downstream signaling.
Ras binds and activates several different effector molecules and of those the most studied is the kinase Raf (15, 54, 69) (Figure 1.3). Ras-GTP binds to the N-terminal Ras binding domain (RBD) and the cysteine rich domain (CRD) of the Raf effector molecule. Normally Raf is in the cytoplasm but on activation by Ras-GTP, Raf is recruited to the plasma membrane (7, 15, 80, 82). Active Raf can now phosphorylate downstream effectors MEK1 and MEK2 which can go on to activate ERK1 and ERK2 through phosphorylation of threonine and tyrosine residues on their activation regions. ERK is involved in phosphorylation of transcription factors, kinases, and phosphatases (15). The Ras/Raf/MEK/ERK pathway was the first Ras effector signaling pathway determined.

Additionally, PI3-K is another effector molecule of Ras that is necessary for cellular function (Figure 1.3). PI3-K consists of heterodimers with a p85 regulatory subunit and a p110 catalytic subunit (15). Once PI3-K is activated, it recruits the complex to plasma membrane and phosphorylates phosphatidylinositol-(4, 5)-biphosphate (PIP2) to make phosphatidylinositol-(3, 4, 5)-triphosphate (PIP3). PIP3 is then involved in activating AKT resulting in the downstream signaling pathways.

1.4 Oncogenic Activity of Ras

Ras mutations are common in many cancers with varying mutation rates depending on the isoform of Ras. The most common mutation of Ras is seen in the K-Ras gene and accounts for more than 85% of cancer cases (15). In pancreatic cancer, an oncogenic mutation in K-Ras is due to a point mutation in codon 12 which limits the ability of GAPs to perform GTP hydrolysis (39, 43, 70). Point mutation in codon 12 blocks GAP interaction with Ras preventing it from removing
GTP and binding GDP so Ras remains active. Therefore, once oncogenic K-Ras is bound to GTP, it displays prolonged signal and is often said to be constitutively active (52). Recently, this idea has been questioned and shown to not be the case. Contrary to common belief, oncogenic K-Ras is inactive until stimulated by an exogenous signal (14) (Figure 1.3). Once it is activated, it is slow to return to basal levels. Activated oncogenic K-Ras can stimulate downstream targets and can generate inflammatory mediators through activation of NF-κB, Stat3, and others (14, 20, 33). Activation of oncogenic K-Ras beyond a threshold initiates a Ras-inflammation feed-forward loop involving Cox-2 where K-Ras activity is further increased and sustained (14, 33). Cox-2 activates prostaglandin E2 which can stimulate the activation of oncogenic K-Ras (Figure 1.3). Therefore, while the normal activity of Ras is short-lived, the activity of oncogenic K-Ras is constitutive and sustained after activation.

1.5 Risk Factors of Pancreatic Cancer

Genetic and environmental factors can lead to an elevated risk of developing pancreatic cancer in an individual's lifetime (Table 1.1). Genetic factors or the non-modifiable risk factors consist of aspects that the individual has no control over. These non-modifiable risk factors include age, gender, race, and heredity. (Table 1.1). The risk for developing pancreatic cancer increases as an individual gets older, with a significant increase in cancer incidence after age 50 (59, 79). Specifically, the incidence rate in men jumped from 1.2 for men 35-39 years of age to 100.5 in men that are 85 years of age and a similar trend was seen in women (79). Men are more susceptible to developing pancreatic cancer than women and the risk increases after 35 years of age (79).
Another major non-modifiable risk factor is race, with the highest pancreatic cancer incidence rates found in African Americans (59, 79). Asian Americans/Pacific Islanders appear to have the lowest mortality rates compared to all races (59, 79). Hereditary factors can also put individuals at risk for PDAC due to the high prevalence of the cancer in one’s family. About 5-10% of PDAC patients have a family history of pancreatic cancer (59, 79).

Although many risk factors are uncontrollable, there are several modifiable risk factors that can are preventable. These factors include smoking, alcohol, dietary factors, obesity, chronic pancreatitis, and diabetes (Table 1.1). Smokers have twice the risk of developing pancreatic cancer than non-smokers and 20% of PDAC cases are due to this habit. The risk of developing PDAC increases with prolonged use of cigarettes (9, 34, 49, 79). Risk due to alcohol use, on the other
hand, is not well determined but several studies show a positive association (21, 79). Of these modifiable risk factors, obesity has taken the spotlight recently and has become recognized as a major risk factor for pancreatic cancer. Several studies support a strong correlation between obesity and a 20% higher risk for PDAC development (3, 6, 79).

1.6 Obesity as a Risk Factor

1.6.1 Obesity and Inflammation

The growing trend of obesity has been increasingly prevalent in the United States and nearly two thirds of adults are obese or markedly overweight (12). This epidemic of obesity is prevalent in developing Westernized societies and could be especially related to consumption of diets rich in high levels of fat (76). In 2000, there were nearly 300 million obese adults worldwide and that number almost doubled in eight years (72). Along with adult obesity, childhood obesity is strikingly increasing with 40 million children under 5 years of age considered overweight in 2011 (61).

Obesity is a disorder where there is an imbalance between energy intake and energy expenditure resulting in an accumulation of excess adipose tissue, and energy storing organ. Adipocytes increase in size to store fatty acids but these cells have limited storing capacity. For this reason, obesity is associated strongly with a chronic low grade inflammatory condition leading to metabolic dysfunction due to fatty acid deposition in other organs such as the liver (28, 63, 75). Systemic inflammation caused by obesity is primarily thought to derive from excess adipose tissue releasing high levels of pro-inflammatory molecules called adipokines and
cytokines such as TNF-α (28, 63). Most adipokines such as leptin, an adipose tissue specific protein regulating food intake, are seen to be elevated in obesity (63). In contrast, adiponectin, a protein which regulates fatty acid breakdown and glucose levels, is decreased. Leptin mainly binds to transmembrane receptor (OBR) or interleukin-6 (IL-6) allowing for downstream signaling through ERK and JAK-STAT3 (41). In cancer, leptin stimulates cell proliferation through activation of the MAPK pathway (41). TNF-α and IL-6 is produced by inflammatory cells in adipose tissue. Production of IL-6 promotes cell proliferation and metastasis through activation of JAK-STAT (41). Further, TNF-α is upregulated in a state of obesity and is known to bind to TNF receptors that lead to activation of NF-κB (41). Activation of NF-κB can lead to activation of Cox-2 promoting an inflammatory state. Increased systemic inflammation in obese individuals is linked with the infiltration of macrophages in adipose tissue (48, 63, 85). Additionally, excess adipose tissue is comprised of fibroblasts which release extracellular matrix proteins aiding in the development of fibrosis and contributes to metabolic dysfunction (63). Obesity induced systemic inflammation and fibrosis can lead to cardiovascular disease as well as an elevated risk for cancer (5).

1.6.2 Obesity and Cancer Risk

Obese and overweight individuals are classified based on one’s body mass index (BMI), a simple index measuring adiposity based on a person’s height and weight in which an elevated BMI greater than 25 kg/m² indicates the individual is overweight or obese if BMI is above 30 kg/m² (61, 83). Increased BMI is predominately correlated to morbidity and mortality due to cardiovascular diseases and the incidence of type 2 diabetes (62, 63, 72). However, several studies have
also linked elevated BMI to a greater risk of developing certain types of cancer (11, 12, 62, 68, 72, 76, 83) (Figure 1.4). It is estimated that in 2013, 580,350 Americans will die of some type of cancer (78, 79). Currently, between 7-41% of cancer deaths are attributable to excess body weight and that risk increases with obesity at a young age (61). Interestingly, the reduction of body weight, as seen in patients undergoing bariatric surgery can reduce cancer incidence, indicating that weight loss can prevent cancer risk and could potentially decrease the 1,600 deaths due to cancer each day (71, 79). Most consistently significant positive linear trends in death rates were seen in colorectal, endometrial, breast, uterine and kidney cancers with the highest observed relative risk seen in uterine cancer (11, 12).

1.6.3 Obesity and Pancreatic Cancer

Although these are the most common types of cancers affected by excess body weight, epidemiological studies also indicate there is a relationship between adiposity and cancers of esophagus, gastric cardia, pancreas, gallbladder, and liver (11, 12). Specifically, recent studies have shown a two-fold increased risk for pancreatic cancer in obese individuals (12). There has been conflicting results regarding the association between high BMI and pancreatic cancer. However, majority of epidemiology and cohort studies have found a strong linear relationship between excess body weight and increased risk for pancreatic cancer (3, 44, 45) (Figure 1.4).

Excess body weight has been recognized as a major contributor to promoting pancreatic cancer and increases the risk by 20% (3, 6, 10, 12, 44, 45, 53, 55, 68, 79). Individuals who are obese at a young age, ages 20-49 has an increased risk
for developing pancreatic cancer and an earlier age of disease onset (45, 79). Further, there was also a strong correlation between obesity at an older age and decreased survival in PDAC patients (45). Since accumulating evidence shows a direct linear relationship between excess body weight and pancreatic cancer risk, it is even more important to determine the molecular mechanisms involved.

**Relative Cancer Risk Assessment**

![Relative Cancer Risk Assessment Diagram](image)

**Figure 1.4: Relative cancer risk assessment through BMI.** Relative risk assessment of overweight and obese individuals according to body mass index. This data was taken from Calle, E. E., and R. Kaaks. 2004. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nature Reviews Cancer 4:579-591. BMI, body mass index, ND, not determined.
1.7 High Dietary Fat Increases Cancer Risk

With the growing epidemic of obesity, it is important to determine the dietary factors that could put individuals at risk for developing obesity mediated disorders such as cancer. A recommended healthy balanced diet should consist of 65% of one’s caloric intake made up of carbohydrates, 20-35% fat, and 10-35% protein (36). Since obesity is an imbalance between energy intake (amount of calories consumed) and energy expenditure (amount of calories lost), it is important to consider the quality and quantity of food consumption. It is evidenced that dietary fat should be consumed sparingly and that fat metabolism is complex compared to the breakdown of protein or carbohydrates. Several studies have shown that high fat consumption can enhance the growth of certain types of cancer, specifically pancreatic cancer (42, 73). Specifically, it was demonstrated that high fat diet induced obesity leading to low grade inflammation and accelerated PanIN development in a less specific K-Ras mouse model (42). Orthotopic implantation of Mia PaCa2 tumor cells into mice fed high fat diet showed dramatic increase in pancreatic tumor burden (84). Although the association of high fat and cancer development is known, the mechanisms of how high fat diet induces obesity and how obesity induces cancer initiation is not well understood.

1.8 Mouse Models of Pancreatic Cancer

To prove further that high fat diet induced obesity is a potential major contributor to the development of pancreatic cancer; different mouse models can be used. The most commonly used K-Ras mouse model is known as Pdx-Cre-1-K-Ras$^{G12D}$ and it is unique in that it utilizes a developmental promoter, Pdx-1, also
known as insulin promoting factor (IPF), to drive expression of Cre recombinase, a tyrosine recombinase enzyme made from P1 bacteriophage that is used to drive site specific recombination events (23, 27, 64). Since Pdx-1 is a transcription factor that is present early in the development of the pancreas and is known to lead to the differentiation of progenitor cells that give rise to acinar, ductal, and islet cells, using this promoter to drive Cre expression results in Cre expression in every cell of the pancreas. Pdx-1-Cre-K-Ras\textsuperscript{G12D} is a mouse made from a recombination of Pdx-1-Cre with a mouse using an endogenous promoter to knock-in mutant K-Ras expression. Mutation in K-Ras in this model is due to a point mutation that changes the glycine to aspartic acid at the 12\textsuperscript{th} residue, as is commonly seen in pancreatic cancer. The expression of mutant K-Ras is blocked by loxP-stop-loxP cassette which requires Cre activation for cleavage of this site and to drive oncogenic K-Ras expression in the entire pancreas at conception. Additionally, another widely used pancreatic cancer mouse model is known as p48\textsuperscript{Cre}-K-Ras\textsuperscript{LSL-G12D/+}, which similar to the Pdx-1-Cre-K-Ras\textsuperscript{G12D}, employs a developmental promoter p48 that is expressed later in development than Pdx-1 (26). This promoter is used to drive Cre expression and recombination leading to mutant K-Ras in all adult pancreatic cells (23).

Since Pdx-1-Cre-K-Ras\textsuperscript{G12D} and p48\textsuperscript{Cre}-K-Ras\textsuperscript{LSL-G12D/+} results in nonspecific expression of K-Ras during early embryonic development of the pancreas, cell type specific promoters were also used to drive Cre expression. As mentioned previously, the origin of pancreatic cancer is known to be ductal cells. However, due to the growing evidence that acinar cells can transform into ductal precursor lesions, an acinar specific promoter, elastase, is currently used. Short elastase promoters
have been used but these results in low efficiency of expression (37). Recently, full length elastase promoter was used to drive Cre expression that was tamoxifen regulated with 100% efficiency for acinar cells (37). Although there are various models using the elastase promoter, our lab specifically used a full length elastase promoter to drive Cre that is bound to an estrogen receptor (ER) and crossed with Tuveson K-Ras model to result in mouse model called LSL/BAC (37). When Cre is activated by tamoxifen binding to its estrogen receptor, Cre recombinase removes loxP sites and knock-ins expression of endogenous levels of mutant K-Ras. Recombination results in mutant K-Ras expression specifically to the acinar cells of the pancreas (37, 38). This model gives greater control of the expression of K-Ras and presents a more accurate representation of what could occur in the human condition.

All these mouse models develop PanINs that are histologically similar to human pathology. Furthermore, they are also known to express mucins, cytokeratin-19, Cox-2, and other factors that are seen to be highly present in human PanINs (23, 27).

1.9 Study Perspective

In the current study, we investigated the role of obesity in animals with an oncogenic mutation in K-Ras in the pancreas by treating animals with a high fat diet to induce obesity. Previously, a high fat diet was shown to prompt inflammation and accelerate development of PanIN lesions in mice harboring a developmental expression of oncogenic K-Ras (42). This model, however, had spontaneous formation of PanINs. We hypothesized that a consumption of high fat diet has the
potential to act as an inflammatory stimulus to activate oncogenic K-Ras signaling and initiate the development of PanIN lesions. To test this hypothesis, we used an elastase-BAC mouse model that expresses oncogenic K-Ras specifically in the adult acinar cells (LSL/BAC) and has low levels of spontaneous PanIN development (37). Since Cox-2 is known to be critical in inflammatory mechanisms, we tested whether the expression of Cox-2 in acinar cells would be critical for high fat diet induced pathogenesis in animals with oncogenic K-Ras mutation. Determining the risk of a high fat diet in the presence of mutant K-Ras is essential because of the increasing numbers of obese Americans every year. The number of pancreatic cancer cases could be decreased by simple prevention tactics.
CHAPTER 2. MATERIALS AND METHODS
2.1 Genetically constructed transgenic animals.

All genetically engineered transgenic animals were acquired from the Mouse Models of Human Cancer Consortium Repository. To determine the combinatorial effects of high fat diet with oncogenic K-Ras LSL-K-Ras\textsuperscript{G12D}/Ela-CreERT (LSL/BAC) were constructed using the LSL-K-Ras\textsuperscript{G12D} mice expressing conditional knock-in mutant K-Ras\textsuperscript{G12D} and mice with a full length elastase gene promoter (Ela-CreERT) was used to drive the expression of tamoxifen-regulated CreERT specific to adult pancreatic acinar cells as previously described. LSL-K-Ras\textsuperscript{G12D} and Ela-CreERT mice were bred to generate LSL-K-Ras/Ela-CreERT (LSL/BAC) double transgenic mice resulting in targeted expression of K-ras\textsuperscript{G12D} in adult mice pancreatic acinar cells. Furthermore, to determine the role of Cox-2 in K-Ras induced PanIN formation, COXKO/LSL-K-Ras\textsuperscript{G12D}/Ela-CreERT (COXKO/LSL/BAC) were made by crossing Cox-2 conditional knockout mice (Cox-2 KO) and LSL/BAC mice to generate a triple transgenic mice with additional targeted deletion of Cox-2 and expression of K-Ras\textsuperscript{G12D} in the pancreas acinar cell after Cre activation with tamoxifen.

Additionally to further determine the survival rate and PanIN progression; LSL/Pdx-1 (LSL/IPF) mice were also generated by crossing LSL-K-Ras\textsuperscript{G12D} with pancreatic specific Cre (Pdx-1-Cre) mice that have targeted expression of K-Ras\textsuperscript{G12D} in all embryonic cells of the pancreas during development. These animals do not require the activation of Cre with tamoxifen for activation of oncogenic K-Ras.
2.2 Treatment of animals.

2.2a Short term diet treatment of animals.

The treatment of all animals was reviewed and approved by the University of Texas M.D. Anderson Institutional Animal Care and Use Committee. LSL/BAC animals, COXKO/LSL/BAC, and littermate control animals (BAC and COXKO/BAC) were induced with tamoxifen through oral gavage for three consecutive days to initiate oncogenic K-Ras expression starting at age 40 days. Animals were then placed on either a high fat diet (HFD; Test Diet 58Y1- Van Heek DIO, Lab Supply, Fort Worth) which contains 60% energy derived from fat (LSL/BAC; n=10 and BAC; n=9), (LSL/Pdx-1; n=6 and Pdx-1; n=6) and (COXKO/LSL/BAC; n=9; COXKO/BAC; n=5) or control diet (CD; Test Diet DIO 58Y2 Rodent Purified Diet, Lab Supply, Fort Worth) which provides 10% energy from fat (LSL/BAC; n=8 and BAC; n=5), (LSL/Pdx-1; n=5 and Pdx-1; n=5) and (COXKO/LSL/BAC; n=5 and COXKO/BAC; n=5) on day three of tamoxifen induction. All animals were fed on diet for 30 days. The body weight of each animal was measured weekly and after treatment, the groups were sacrificed and pancreata were harvested for histology and protein analysis.

2.2b Long term survival diet treatment of animals.

LSL/Pdx-1 and Pdx-1 animals were placed on their respective diets either HFD or CD right after they were weaned and left on diet till they needed to be sacrificed (70-100 days). LSL/BAC and BAC groups of animals were induced with tamoxifen at 40 days and placed on either diet accordingly on the third day of induction. They were left on diet till they had to be euthanized (150- days). The body
weight of each animal was measured monthly and after treatment, the groups were sacrificed and pancreata were harvested for histology and protein analysis. Survival of animals was plotted using the survival curves on GraphPad Prism. The percent change in body weight from day 1 to day 30 was calculated and graphed along with the percent total pancreas weight/total body weight.

2.3 Ras activity assay.

Levels of GTP bound Ras was measured using a Raf pull-down assay kit (Millipore, Bellerica). Snap-frozen pancreas samples were homogenized and sonicated briefly on ice in lysis buffer containing 25 mM 4-(2-hydroxyl-ethyl)-1-piperazineethanesulfonic acid (pH 7.5), 1% IGEPAL CA-630, 150mM NaCl, 0.25% sodium deoxycholate, 10% glycerol, 25mM NaF, 10mM MgCl₂, 1mM ethylendediaminetetraacetic acid, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1mM sodium orthovanadate. The samples were centrifuged at 15,000 g for 20 minutes at 4°C to remove cellular debris and the lysates were collected. Equal concentrations of total 700 μg were incubated for 45 minutes at 4°C with agarose beads coated with the Raf-Ras binding domain. 500ul of Mg²⁺ lysis buffer was used to wash the beads three times, 30μl of 4x loading buffer was added, lysates were vortexed, centrifuged at 15,000g for 30 seconds at 4°C, then heated for 5 minutes at 95°C. Protein levels of active and total Ras were measured by immunoblotting through a western blot protocol. Quantification of bands was performed using an Odyssey V3.0 instrument (LI-COR, Nebraska) and the densities of each sample for active Ras/total Ras were plotted using Graphpad Prism 5.
2.4 Western blot analysis.

Pancreas mouse tissue samples from each group were homogenized using RIPA buffer. Lysates were prepared and either a 15% or 12% SDS-PAGE gel was used to separate the proteins. Proteins were transferred from a SDS-PAGE gel to a nitrocellulose membrane and blocked for 30 minutes at room temperature with 5% non-fat dry milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20. Membranes were incubated at 4°C overnight with primary antibodies: Rabbit Anti-GAPDH G9545 (1:1000; Sigma Aldrich), Mouse Anti-Ras 05-516 Clone 10 (1:1000; Millipore), p-p44/42 MAPK (T202/Y204) p-ERK 9106S (1:1000; Cell Signaling), and p44/42 MAPK (total ERK) 9102 (1:1000; Cell Signaling). Immunodetection of proteins was performed using Alexa-Fluor goat anti-mouse 800 or goat anti-rabbit 680 conjugated secondary antibodies for detection with Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln). Images were converted to grayscale, analyzed using Odyssey V3.0 and graphed.

2.5 Histology.

Pancreas tissue from mice were removed, washed with PBS, and incubated in formalin overnight. The following day, formalin-fixed samples were embedded in paraffin and sectioned onto slides at 5μm thick. Slides were then stained with hematoxylin and eosin (H&E) and histological assessment was performed by a pathologist. Slides were scored from 1-3 on the level of inflammation (1: scattered immune cells, non-aggregating; 2: intermediate level of immune cells present; 3: aggregates of immune cells within the pancreatic parenchyma forming a follicle-like formation), fibrosis (1: occasional periductular or scant perilobular fibrosis; 2:
intermediate level; 3: definite extensive fibrosis with resultant parenchymal (acinar) atrophy, and frequency of PanIN lesions in each single lobule containing PanIN lesions, irrespective of the number of cross sections of ducts present, was counted. Each lesion was also scored on the grade of PanIN (PanIN-1, PanIN-2, or PanIN-3).

2.6 Immunohistochemistry.

Unstained paraffin embedded mouse pancreata were stained for: Rabbit anti-alpha smooth muscle actin ab5694 (1:1000; Abcam), and Rabbit p-ERK 4370 (1:100; Cell Signaling). Slides were deparaffinized using the standard procedure of two washes of xylene, 100% Ethanol, 95% Ethanol, and one wash of 80% Ethanol. Antigen retrieval was performed using DAKO target retrieval solution (10x concentrate) with a steamer for 20 minutes at 98°C. The slides were allowed to cool for 15 minutes and followed with endogenous blocking with H₂O₂ for another 15 minutes. After washes with PBS slides were blocked with 1:12 fish gelatin for 1 hour and incubated with primary antibody overnight at 4°C. Washes were done with PBS and PBS containing 0.05% Tween 20. Sections were incubated with rabbit on rodent horseradish peroxidase-labeled polymer RMR622H (Biocare) and positive labeling was detected with DakoCytomation Liquid DAB+ Substrate System. Gill no.3 hematoxylin solution was used to counterstain each section. Immunohistochemical staining for macrophages, Anti-F4/80 Rat (1:20; eBioscience), was performed after deparaffinization and antigen retrieval as mentioned previously. Nonspecific binding was removed by endogenous blocking with H₂O₂ and protein blocking with fish gelatin 1:12 from a 40% stock. After washing, biotin goat anti-rat secondary was applied to sections followed by incubation with streptavidin.
2.7 Immunofluorescence.

Unstained OCT embedded frozen pancreas sections were fixed with cold acetone for 10 minutes and then washed with PBS. Slides were then treated with protein blocking solution (8% fish gelatin) for 20 minutes at room temperature. Primary antibodies Cox-2 (1:300; Cayman Chemicals), iNOS (1: 100; Thermo Scientific), F4/80 (1:50; eBioscience) was diluted in protein blocking solution and applied overnight at 4°C. The next day, slides were washed in PBS, incubated in protein blocking solution for 10 minutes and secondary antibody Alexa 594 Goat Anti-rabbit (1:800; Life Technologies) diluted in protein blocking solution were applied for 1 hour at room temperature. The samples were then washed and counterstained with nuclear stain Hoechst 33342 (1:10000) for 10 minutes. After nuclear staining the slides were washed, and coverslips were placed using glycerol/PBS containing propyl gallate as mounting media. Slides were then visualized under an epifluorescence microscope (Zeiss) and the total area and intensity of fluorescence were measured using Simple PCI. The sum of the total Cox-2 area for each tissue sample was divided by the sum of the total nuclear area. Each sample was normalized to the average of all the LSL/BAC animals on CD.

2.8 Collagen Detection.

Collagen I fibers were observed by using paraffin embedded pancreatic tissue and staining with Mason-Trichome stain (Sigma Aldrich). Deparaffinization was performed as described above in the immunohistochemistry section. The tissue samples were incubated in preheated Bouin’s solution, washed with water, and stained with Weigert’s Iron Hematoxylin solution. This was followed by staining in Biebrich Scarlet-Acid Fuchsin, Phosphophotungstic/Phosphomolybdic Acid
Solution, and Aniline Blue Solution. The slides were then washed with 1% acetic acid, washed, and rehydrated.

2.9 Statistics.

Data was analyzed using GraphPad software (Graphpad Prism Version 4.0 for Windows; GraphPad Software, San Diego, CA, www.graphpad.com). Results are expressed as mean ± SEM. Data was analyzed by t-test or One-way ANOVA and results are considered significant at p<0.05.
CHAPTER 3: RESULTS
3.1 Introduction

Obesity is known as a major risk factor for pancreatic cancer, the fourth deadliest malignancy in the United States (78). Several studies have stated a correlation between elevated BMI and excess body weight with an increased risk of developing pancreatic cancer (3, 12, 44, 45, 61, 68). There is also an established link between childhood obesity and significant risk for PDAC. In the past two decades, the rate of obesity has doubled to 59 million Americans because of the adoption of a diet high in fat content. The increasing concern as obesity rates rise, makes it even more important to determine the mechanistic molecular effects of a high fat diet on PDAC development.

More than 90% of pancreatic cancer cases display an oncogenic mutation in K-Ras (26, 35, 39, 43, 70). Ras is a guanine nucleotide binding protein (G-protein) that is highly necessary for the function of cellular signaling pathways. In the normal state, Ras is bound to GDP and remains in an inactive state until guanine exchange factors (GEFs) that are controlled by growth factor receptors, cytokines, and other signaling molecules remove the GDP and load GTP to Ras. This action exchanges Ras from an inactive state to an active state to stimulate activation of phosphoinositide 3-kinase (PI3-K), mitogen-activated protein kinase (MAPK), Ral, and Cox-2 which are all necessary for cell growth, differentiation, and survival (39, 43, 70). Due to intrinsic GTP hydrolysis and interaction with GTPase activating proteins (GAPs), hydrolysis of GTP to GDP returns Ras back to its inactive state making the normal activity of Ras very transient. When an oncogenic mutation of K-Ras is present at codon 12, it prevents the ability of GAPs to help in the hydrolysis of GTP to GDP, therefore prolonging the activity of Ras and making Ras
constitutively active (39, 43, 52, 70). However, oncogenic K-Ras requires an inflammatory stimulus to become activated. Once it is stimulated, it is slow to return to basal levels and can go on to generate inflammatory mediators through activation of NF-kB, Cox-2, and others. High Ras activity beyond the threshold can initiate the inflammatory feed-forward loop in which these mediators can activate and prolong K-Ras activity (14, 33).

An oncogenic mutation in K-Ras is present in the early stages of pancreatic cancer at the formation of precancerous lesions known as pancreatic intraepithelial neoplasia (PanINs), non-invasive ductal precursor lesions to invasive cancer (51). Eventually, oncogenic K-Ras can lead to the accumulation of mutations in tumor suppressors’ p53 and p16 which can advance the PanINs to higher grade PanINs progressing towards PDAC. Inflammation and desmoplasia accompanies PanIN formation in the initiation of this process (51). Therefore, we hypothesized that high dietary fat could act as an inflammatory stimulus activating and prolonging oncogenic K-Ras activity to initiate PDAC development. In addition, we predicted high fat diet consumption starting at an early age would increase the progression of PanIN development. To prove this we analyzed how diets with varying fat content affected the pancreas of animals harboring an oncogenic K-Ras mutation and examined it for an increase in inflammation, fibrosis, and PanIN formation. Along with K-Ras, we investigated the important mediators that were crucial for this reaction to occur by testing the expression of Cox-2 in pancreas acinar cells expressing oncogenic K-Ras to determine if it was essential for high fat diet induced inflammation, fibrosis, and PanIN development.
We found that high fat diet consumption in the presence of oncogenic K-Ras, accelerated and progressed PanIN development, fibrosis, and inflammation using a mouse model that recapitulates the potential human dietary fat risk. These animals had elevated Ras-GTP levels and increased downstream signaling. We also saw survival rates were decreased in animals on high fat diet. In addition, we were also able to show a direct link between Cox-2 and K-Ras indicating it is a key regulator of these events. Therefore, these results show K-Ras to be a key protein involved in cancer initiation and shows Cox-2 to be critical for these events, suggesting potential intervention possibilities for prevention.

3.2 Combinatorial Effects of High Fat Diet and Oncogenic K-Ras

3.2.1 High fat diet increases the total body weight and pancreas weight

Animals were induced with tamoxifen for three days to activate expression of oncogenic K-Ras and placed on either a control or high fat diet for thirty days (Figure 3.1). Cre-mediated conditional oncogenic K-Ras knock-in mice (LSL/BAC) expressing endogenous levels of oncogenic K-Ras specifically in the pancreatic acinar cells were compared to the littermate controls (BAC). The group of animals treated with diet containing 60% energy from fat for thirty days, gained weight rapidly compared to animals on the control diet containing 10% energy from fat (Figure 3.1). After thirty days of dietary treatment, BAC and LSL/BAC on high fat diet doubled their body weight and had excess fatty deposits throughout the peritoneal cavity in comparison to mice fed a control diet (Figure 3.1). Additionally, only animals expressing oncogenic K-Ras fed a high fat diet had increased pancreas weight compared to other groups.
Figure 3.1 High fat diet increased total body in all mice and pancreas weight in mice expressing oncogenic K-Ras. BAC and LSL/BAC mice were induced with tamoxifen for three days and on day three was placed on respective diets for 30 days (A). All groups were weighed once every week and the percent change from week 1 to week 5 of treatment was calculated. BAC and LSL/BAC mice fed a HFD (60% kcal of fat) had a greater change in weight (**p= 0.0047 LSL/BAC HFD vs. LSL/BAC CD and #p=0.0295 LSL/BAC HFD vs. BAC mice on CD; ***p= 0.0003 BAC HFD vs. LSL/BAC mice on CD, and ##p=0.0031 BAC HFD vs. BAC CD) compared to the mice fed a CD (10% kcal of fat) (B). On day 30 the mice were sacrificed and the pancreas was removed, weighed and the percent weight of the pancreas/the total body weight was graphed for all groups. Mice expressing oncogenic K-Ras and fed the HFD had a significantly greater pancreas weight compared to BAC animals on HFD and CD, as well as LSL/BAC animals on CD (**p=0.0030 LSL/BAC HFD vs. LSL/BAC CD; **p=0.0031 LSL/BAC HFD vs. BAC CD; **p=0.0012 LSL/BAC HFD vs. BAC HFD) (C).
3.2.2 High fat diet initiates inflammation, fibrosis, and the formation of PanIN lesions

BAC control mice and LSL/BAC mice fed either a control or high fat diet were histologically accessed for morphologically changes within the pancreas (Figure 3.2). BAC animals had normal pancreata and were indistinguishable from each other after thirty days of treatment with respective diets. LSL/BAC mice expressing endogenous levels of oncogenic K-Ras fed a control diet infrequently developed small areas of fibrosis or PanINs. In contrast, LSL/BAC mice on high fat diet developed extensive areas of fibrosis accompanied by multiple PanIN-1 lesions. Each pancreas sample was also scored on the level of inflammation, fibrosis, and the number of PanIN lesions present throughout the specimen (Figure 3.2). When LSL/BAC animals fed a control diet were compared to those on high fat diet, the level of inflammation and fibrosis was significantly higher. Similarly, those on high fat diet had six-fold the number of lobules containing PanIN-1 lesions. The consumption of high fat diet in the presence of oncogenic K-Ras mutation in the pancreas could initiate and accelerate the formation of PanIN lesions, which is an established early step in the initiation of pancreatic cancer.

3.2.3 High fat diet increases infiltration of inflammatory mediators into the pancreas

Animals treated with both control and high fat diet was analyzed for signs of inflammation specifically for the presence of immune cells such as macrophages (Figure 3.3). Macrophages are the first responders of the body’s innate response when a pathogen invades the body and releases pro-inflammatory cytokines that can recruit lymphocytes and other immune cells to help fight the infection.
Figure 3.2 Consumption of a high fat diet led to large areas of fibrosis, inflammation, and PanIN lesions throughout the pancreas. Representative H&E stain of BAC animals on CD (A), and HFD (B), oncogenic K-Ras LSL/BAC animals on CD (C) and HFD (D) showed high fibrosis and areas of precancerous lesions in HFD fed oncogenic K-Ras expressing mice. Littermate controls had a healthy pancreas after 30 days of treatment regardless of diet. Each tissue sample was scored on the level of inflammation, fibrosis, and precancerous lesions (average of number of PanIN-1 lesions) seen in the entire tissue. LSL/BAC mice on HFD had a significantly higher level of inflammation (**p=0.009 LSL/BAC HFD vs. LSL/BAC CD; ###p=0.0008 LSL/BAC HFD vs. BAC CD; and *p=0.0433 LSL/BAC CD vs. BAC CD) (E) and fibrosis than mice on CD (*p=0.0108 LSL/BAC HFD vs. LSL/BAC CD; ###p<0.001 LSL/BAC HFD vs. BAC CD; and ###p=0.0006 LSL/BAC HFD vs. BAC HFD) (F). Each sample was graded based on the number of PanIN lesions in the entire tissue. Only PanIN-1 lesions were found in all the tissues. HFD fed LSL/BAC mice had significantly higher number of precancerous lesions (**p=0.0162 LSL/BAC HFD vs. LSL/BAC CD; ###p<0.001 LSL/BAC HFD vs. BAC HFD and CD) (G).
We predicted that animals consuming a high fat diet when an oncogenic mutation of K-Ras was present within the acinar cells of the pancreas would have a greater recruitment of macrophages, as well as other inflammatory mediators. Immunohistochemical staining of F4/80+, a marker for most mature macrophages, of BAC animals on control and high fat diet showed no signs of macrophages in the tissue. Additionally, LSL/BAC animals on control diet had low levels of inflammation. In contrast, LSL/BAC animals on high fat diet had an increased infiltration of F4/80+ macrophages around the areas of PanIN lesions (Figure 3.3).

**Figure 3.3 Consumption of High Fat Recruits Macrophages into the Pancreas.** Staining for F4/80+ macrophages on BAC animals on CD (A), HFD (B) LSL/BAC animals on CD (C), and HFD (D). LSL/BAC animals on HFD demonstrate strong macrophage infiltration.
Another inflammatory marker that is used is inducible nitric oxide synthase (iNOS), which is commonly released by macrophages, endothelial cells, and stromal cells. In the presence of inflammation, these cells rapidly express iNOS producing large amounts of nitric oxide, a free radical gas important for infectious and anti-tumor mechanism of the innate immunity.

Figure 3.4 Inflammatory iNOS production is increased by a high fat diet. Immunofluorescence tissue staining for iNOS of BAC animals on CD (A), HFD (B) LSL/BAC animals on CD (C), and HFD (D) indicated higher iNOS expression in mutant K-Ras animals on HFD than on mice on CD. Quantification of intensity and area of fluorescence of iNOS in the pancreas showed a significant increase in iNOS expression in LSL/BAC animals on HFD (**p=0.0083 LSL/BAC high fat vs. LSL/BAC CD) (E).
Overproduction of nitric oxide is present in many cancers leading to oxidative stress and tissue damage. BAC animals on control and high fat diet had low levels of iNOS as predicted because the pancreases were completely normal (Figure 3.4). LSL/BAC animals on high fat diet had increased levels of iNOS production in the fibrotic areas of the tissue compared to LSL/BAC animals on control diet (Figure 3.4).

Similarly, levels of Cox-2, an enzyme activated by Ras and involved in formation of biological mediators involved in inflammation like prostaglandin E2 (PGE2), was analyzed by immunofluorescence (Figure 3.5). Levels of Cox-2 were significantly elevated in LSL/BAC animals fed a high fat diet. LSL/BAC animals on control diet and the littermate control animals on either diet had low levels of Cox-2 expression in the pancreas. Immunohistochemistry staining for co-localization of Cox-2 and F4/80+ macrophages indicates that Cox-2 is highly present in the PanIN lesions and is also expressed by macrophages within the tissue.

3.2.4 High fat diet induces excessive fibrotic stroma formation

PDAC consists predominately of fibrotic stroma and only 10-20% of the tumor mass is made up of cancer cells. Fibrosis is a major early initiating step in pancreatic cancer and is activated by a pancreas specific myofibroblast cell called pancreatic stellate cells (PSCs). Normally, in the presence of inflammation in the pancreas, quiescent PSCs in the stroma will become activated and migrate to areas of injury. PSCs will then produce extracellular matrix proteins such as collagen I and alpha smooth muscle actin (αSMA), known as markers for these cells. We
hypothesized that when animals expressing oncogenic K-Ras are fed a high fat diet, quiescent PSCs will become activated and produce excess stromal formation.

**Figure 3.5** High fat diet acts an inflammatory stimulus in the pancreas, increasing the level of Cox-2. Immunofluorescence tissue staining for Cox-2 on BAC animals on CD (A), HFD (B) LSL/BAC animals on CD (C), and HFD (D) indicated higher Cox-2 expression in mutant K-Ras animals on HFD than on mice on CD. Quantification of intensity and area of fluorescence of Cox-2 in the pancreas showed a significant increase in Cox-2 expression in LSL/BAC animals on HFD (***p<0.0001 LSL/BAC high fat vs. LSL/BAC CD) (E).
Trichome Mason Staining of pancreata from BAC mice on either diet and LSL/BAC mice on control diet showed normal collagen production around the blood vessels (Figure 3.6). However, LSL/BAC mice on high fat diet had increased levels of stromal collagen I throughout the pancreas. Likewise, immunohistochemical staining to detect presence of αSMA, an indicator of activated stellate cells, indicated elevated expression levels in LSL/BAC mice on high fat diet compared to LSL/BAC mice on control diet and littermate controls on either diet (Figure 3.6). Both stains support the potential of high fat diet in activating stellate cells, increasing stromal activity resulting in definite fibrosis.

3.2.5 High fat diet can stimulate oncogenic K-Ras activation and its downstream targets

To determine if a high fat diet could increase Ras activity in mice expressing endogenous levels of oncogenic K-Ras, we measured levels of GTP-bound Ras in the pancreas of all mice through a Raf-pull down assay (Figure 3.7). LSL/BAC animals on high fat diet had significantly amplified activated Ras levels compared to LSL/BAC animals fed with control diet. Further, western blot and immunohistochemistry analysis indicated that levels of phospho-ERK, a downstream target of activated K-Ras, were elevated in LSL/BAC animals on high fat diet (Figure 3.7). This supports the potential of high fat diet to stimulate oncogenic K-Ras activation and increase its downstream activity. Phospho-ERK elevation was not seen in LSL/BAC mice fed a control diet.
Figure 3.6 High fat diet increases stromal activity in mice with mutant K-Ras. Collagen staining of BAC animals on CD (A), HFD (B) LSL/BAC animals on CD (C), and HFD (D) showed high levels of collagen production in LSL/BAC animals fed a HFD. Immunohistochemical staining for alpha-smooth muscle actin was also performed as another marker for stromal activity for BAC animals on CD (E), HFD (F) LSL/BAC animals on CD (G), and HFD (H). It further supported high stromal activity in HFD fed LSL/BAC mice by increased expression of alpha-SMA.
Figure 3.7 Activation of Ras downstream pathways were increased significantly by HFD. Ras activity was also measured through Raf-pull down assay and correlated with Phospho-ERK expression levels. LSL/BAC animals on HFD had a significantly higher Ras activity compared to BAC and LSL/BAC animals on CD (**p=0.0087 LSL/BAC HFD vs. LSL/BAC CD; **p=0.0095 LSL/BAC HFD vs. BAC CD; #p=0.0381 LSL/BAC HFD vs. BAC HFD) (A). Western blot analysis for phospho-ERK supported this observation by demonstrating a significantly higher Phospho-ERK expression in HFD fed LSL/BAC mice compared to BAC mice and LSL/BAC mice on CD (**p=0.0043 LSL/BAC HFD vs. LSL/BAC CD; **p=0.0095 LSL/BAC HFD vs. BAC CD; #p=0.0476 LSL/BAC HFD vs. BAC HFD) (B). Immunohistochemical staining of phospho-ERK for BAC animals on CD (C), HFD (D) LSL/BAC animals on CD (E), and HFD (F) showed substantial Phospho-ERK activity concentrated in mice expressing oncogenic K-Ras on HFD.
3.2.6 Cox-2 is essential for high fat diet induced fibrosis in mice with oncogenic K-Ras

Normally, activation of K-Ras leads to downstream activation of Cox-2 and PGE2 needed for inflammation in our bodies. We predicted that Cox-2 would be elevated in animals expressing oncogenic K-Ras fed a high fat diet. To determine the mechanistic effects of high fat diet specifically in the pancreas, we examined pancreatic Cox-2 levels and were able to identify that high levels of Cox-2 was present in LSL/BAC animals treated with high fat diet (Figure 3.5). To further analyze the role of Cox-2, LSL/BAC mice were crossed with Cox-2 conditional knockout mice to produce triple transgenic mice that have oncogenic K-Ras expression but lack Cox-2 expression in the acinar cells. COXKO/LSL/BAC and the littermate control animals (COXKO/BAC) were treated with either control diet or high fat diet for thirty days. No signs of inflammation, fibrosis, or PanIN lesions were evident in any of the groups regardless of treatment with high fat diet (Figure 3.8). Further, samples were assessed for the level of inflammation, fibrosis, and number of PanIN foci present within the tissue and COXKO/LSL/BAC animals on high fat diet were compared to BAC and LSL/BAC animals on control or high fat diet (Figure 3.8). The levels of inflammation, fibrosis, and PanIN lesions were markedly decreased in COXKO/LSL/BAC animals on high fat diet (Figure 3.8). Additionally, Ras activity was significantly decreased in animals with the expression of oncogenic K-Ras but lacking Cox-2 in the acinar cells (Figure 3.9). This was true for COXKO/LSL/BAC animals regardless of the diet. This data supports the hypothesis and indicates a critical importance of Cox-2 in initiating inflammation and acinar cell metaplasia into PanIN lesions caused by high fat diet consumption. Furthermore it
specifies the necessity for Cox-2 to increase Ras activity above the threshold to induce these reactions.

Figure 3.8 Cox-2 deletion in the pancreas blocks K-Ras induced effects of high fat diet. Histology of conditional Cox-2 knockout mice without oncogenic K-Ras expression treated with CD (A), HFD (B), and Cox-2 knockout mice with oncogenic K-Ras expression fed CD (C) and HFD (D) displayed a healthy, normal pancreas with no signs of inflammation, fibrosis, or PanIN lesions.
Figure 3.9 Cox-2 deletion prevents the activation of oncogenic K-Ras. Quantification of fibrosis, inflammation and PanINs, for COXKO/LSL/BAC animals treated with CD and HFD compared to BAC and LSL/BAC animals data shown in Figure 3.2. Tissue samples were scored and LSL/BAC animals on HFD had substantially higher levels of inflammation (**p=0.0002 LSL/BAC HFD vs. COXKO/LSL/BAC HFD) (E), fibrosis (**p=0.0001 LSL/BAC HFD vs. COXKO/LSL/BAC HFD) (F), and number of PanIN-1 lesions (**p=0.0006 LSL/BAC HFD vs. COXKO/LSL/BAC HFD) compared to COXKO/LSL/BAC animals (G). Ras activity was also compared between LSL/BAC and COXKO/LSL/BAC animals on CD and HFD (H). There was a significant reduction in Ras activity in COXKO/LSL/BAC animals on either diet compared to LSL/BAC animals (**p=0.005 LSL/BAC HFD vs. LSL/BAC CD and COXKO/LSL/BAC HFD).
3.3 Influence of High Fat Diet on Tumor Progression and Survival Rate

3.3.1 High fat diet decreases survival rate in animals expressing oncogenic K-Ras

To determine how long the animals would survive if left on the respective diets longer than thirty days, BAC and LSL/BAC animals were fed with control or high fat diet starting at thirty days of age and left till they needed to be sacrificed. LSL/BAC animals on high fat diet had significant decrease in survival rate and survived up to only 250 days compared to those animals on control diet which survived much longer (Figure 3.10).

![Figure 3.10](image)

**Figure 3.10 High fat diet decreased overall survival rate in animals with oncogenic K-Ras.** LSL/BAC animals on CD and HFD were started on diet after induction with tamoxifen and monitored daily for survival. Animals on HFD had a significant reduction in survival rate compared to control animals which remain alive (*p=0.0214) (A). Similarly, LSL/Pdx-1 mice were monitored for survival after treatment with either diet and animals on high fat diet had a decrease in overall survival (*p=0.0233) (B).
Additionally, LSL/Pdx-1 mice, expressing oncogenic K-Ras in all the cells of the pancreas at development, were also placed on control or high fat diet to determine if high fat diet could decrease survival. Similar to LSL/BAC animals, LSL/Pdx mice on high fat diet survived up to 145 days on the diet while animals on control diet survived about 220 days (Figure 3.10). This data supports the ability of high fat diet to decrease survival in animals expressing an oncogenic mutation in K-Ras.

3.3.2 High fat diet accelerates progression of PanIN-2 and PanIN-3 formation

Corresponding with overall survival, animals on high fat diet had an acceleration of PanIN formation which progressed at a rapid rate. LSL/Pdx-1 and LSL/BAC animals fed either a control or high fat diet had drastic histological differences in their pancreata (Figure 3.10). LSL/Pdx-1 animals sacrificed after 90 days of high fat diet consumption had extensive fibrosis and inflammation accompanied by foci containing PanIN-2 and PanIN-3. This varied significantly from LSL/Pdx-1 animals sacrificed forty days later which only contained PanIN-1 foci throughout the pancreata. Scoring of level of inflammation, fibrosis, and PanIN level showed significantly elevated levels compared to LSL/Pdx-1 animals on control diet (Figure 3.11). LSL/Pdx-1 animals on high fat diet had increased number of PanIN foci with progression to PanIN-2 and PanIN-3 (Figure 3.11). Similarly, LSL/BAC animals fed either diet had a linear trend and animals sacrificed at 160 days displayed prominent accelerated PanIN formation and cystic lesions in animals on high fat diet (Figure 3.12). This phenomenon was not present in LSL/BAC animals on control diet which exhibited small areas of low grade PanIN formation. Further treatment of the respective diets induced a severe desmoplastic reaction with
progression to invasive pancreatic cancer after 205 days of high fat diet (Figure 3.12).

Figure 3.11 HFD accelerates cancer development in Pdx-Cre-K-RasG12D. Histological assessment of the pancreas of Pdx-Cre mice days on CD (A), HFD (B), Pdx-Cre-K-RasG12D mice on CD (C) and HFD (D) treated 70-100 shows a drastic progression of PanIN lesion formation in Pdx-Cre-K-RasG12D animals on HFD. Each sample was scored on the level of inflammation, fibrosis, and the number of each grade of PanINs present in the entire tissue. Pdx-Cre-K-RasG12D mice on HFD had significantly higher levels of inflammation (**p=0.0056), fibrosis (**p=0.0056), and number of PanIN-1 lesions (*p=0.00115) than Pdx-Cre-K-RasG12D on CD. Pdx-Cre-K-RasG12D mice on HFD had large areas of reactive stroma accompanied with the presence of PanIN-2 and PanIN-3 lesions that were not seen in animals on CD.
The tumor burden in LSL/BAC animals on high fat diet was significant. These LSL/BAC animals developed metastasis to the lungs and liver unlike the control diet fed animals which did not demonstrate this phenotype.

Figure 3.12 High fat diet accelerated PDAC and metastasis in LSL/BAC animals. Pancreata of LSL/BAC animals on CD or HFD was compared and LSL/BAC animals on HFD obtained a large tumor in the pancreas while the LSL/BAC animals on CD signs of fibrosis (A) Pancreata of HFD had large cysts compared to animals on CD and lung metastasis was present (B). Histological assessment of the pancreas of mice fed CD sacrificed at 160 days (C) compared to an animal sacrificed at 205 days (D) shows an increase in fibrosis. In contrast, shows extensive cystic regions and accelerated PanIN formation in animals was observed in LSL/BAC animals on HFD at 160 days (E). Comparison to LSL/BAC animals at 205 days indicates a rapid progression to PDAC (F).
CHAPTER 4: DISCUSSION
4.1 Introduction

Recent evidence has shown a strong link between high fat diet induced obesity and pancreatic cancer but very little is known about the mechanism involved. Since K-Ras is highly mutated in pancreatic cancer, our study specifically investigated if oncogenic K-Ras increases the risk of developing pancreatic cancer when a high fat diet is consumed. Our data supports the ability of a high fat diet in the presence of physiologically relevant levels of oncogenic K-Ras in adult pancreatic acinar cells to initiate pancreatic cancer development (Figure 4.1). Further, it demonstrated the rapid progression of PanIN lesions and decreased overall survival when the high fat diet consumption was prolonged. To address this question, we used for the first time, an acinar specific K-Ras model. Previous work has indicated a high fat diet can accelerate PanIN development in p48$^{Cre}$-K-Ras$^{LSL-G12D/+}$, a less specific acinar-Cre mouse model (42). It was also evidenced that tumor promotion is associated with inflammation and TNFR1 signaling showing a role of TNF-α in enhancing PanIN formation (42). Additionally, this study demonstrated that high fat diet does cause changes in metabolic function seen by insufficiency in the exocrine gland (42). This model, unlike the acinar-Ras mice, develops spontaneous PanINs early in development because it utilizes an embryonic promoter to drive the expression of oncogenic K-Ras in all the cells of the pancreas. Therefore, although it demonstrates that high fat diet does increase PanIN formation and result in metabolic dysfunction in the pancreas, it is not an accurate physiological representation of the true effects of high fat diet. Pancreatic cancer is not disease of infancy and tumors arise in adult individuals. Therefore, in contrast, our model more closely mimics human disease due to the lack of
spontaneous pathologies in mice expressing endogenous levels of K-Ras only in the adult acinar cells (14, 22, 33).

4.2 Mechanistic Link Between High Fat Diet and Pancreatic Cancer

The association between obesity and growth of pancreatic cancer was observed in xenograft tumors. Orthotopic implantation of human pancreatic cancer cells (Mia PaCa2) into mice fed a high fat diet drastically increased tumor burden (84). Although there was strong evidence suggesting a connection between cancer promotion and a high fat diet, a mechanistic conclusion was lacking. Additionally, although it is important to note that cancer growth can be increased, the mechanisms involved in growth of cancer are different than those seen in cancer initiation, and the mechanisms of cancer initiation could provide ideas for prevention of pancreatic cancer risk caused by a high fat diet.

Oncogenic point mutation in K-Ras is the earliest recorded mutation occurring during the normal epithelial cell transformation to PanIN lesions (16, 17, 31, 35, 47, 56). Although mutations in K-Ras are mostly associated with cancer, large subsets of healthy individuals who never develop PDAC also harbor K-Ras mutations in their pancreas (35, 81). In fact, oncogenic K-Ras requires an inflammatory stimulus for activation and stimulation beyond the pathological threshold, increase K-Ras activity eventually leading to pancreatic cancer. This concept was recently demonstrated when mice carrying an oncogenic K-Ras mutation and treated with caerulein, LPS or elevated cholecystokinin (CCK) levels to induce inflammation, resulted in a Cox-2 mediated feed-forward loop driving sustained K-Ras activity (14). Our study indicated the ability of high fat diet to also
act as an inflammatory agent to trigger the activation of oncogenic K-Ras and its downstream targets, specifically phospho-ERK in LSL/BAC animals (Figure 4.1). Ras activity and phospho-ERK was highly increased in these animals supporting the ability of a high fat diet to sustain high K-Ras activity. This data is crucial in understanding the possible mechanistic link between oncogenic K-Ras and obesity-induced acceleration of normal acinar cell transformation into ductal lesions.

High fat diet also increased the stimulation of quiescent pancreatic stellate cells inducing increased stromal formation (Figure 4.1). The role of PSCs and the stromal microenvironment in pancreatic cancer is currently not understood. Despite this, active PSCs are universally present in the early stages of PDAC and predicted to promote pancreatic cancer progression (60). Stellate cell activation is primarily due to inflammatory mediators and not a response to acinar cell damage or death, since acinar cell apoptosis does not activate PSCs (20). Our study demonstrated the impact of high fat diet on promoting high levels of inflammation seen by increased infiltration of macrophages, as well as the elevated levels of Cox-2 and iNOS (Figure 4.1). Levels of iNOS are elevated in many cancers and thought to promote cancer growth (85). The induction of iNOS by macrophages and epithelial cells could aid in fibrosis and PanIN formation.

Moreover, acinar cells containing high Ras activity can release mediators that can aid in this process, initiating fibrosis. Similarly, animals expressing oncogenic K-Ras had increased fibrosis by showing high levels of ATC2 and collagen I that are normally produced by PSCs. Mediators released by mutated K-Ras influence acinar cells could have been involved in the recruitment of immune cells, activating PSCs and instigating stromal formation. Recently, we established
PGE2 as a mediator involved in stellate cell activation (13). Therefore, Cox-2 activity induced by oncogenic K-Ras is a possible mechanistic explanation for development of fibrotic stroma.

Along with the increased levels of inflammation and fibrosis observed in this study, we were also able to validate that high fat diet can stimulate oncogenic K-Ras activity to drive the initiation of PanIN lesion formation. All animals on high fat diet had pronounced PanIN-1 formation compared to our animals on control diet in only thirty days of treatment with either diet. This study proves the importance of an oncogenic mutation in K-Ras in instigating the early development of PDAC. Additionally, it emphasizes the elevated risk of developing PDAC in the presence of oncogenic K-Ras and consumption of high dietary fat.

As mentioned previously, Cox-2 is highly important in the inflammatory positive feed forward loop enhancing oncogenic K-Ras activity (14). To support this finding, our data illustrates Cox-2 was essential for the high fat diet induced effects on oncogenic K-Ras activity, as well as stromal and PanIN lesion development (Figure 4.1). Interestingly, although our data shows K-Ras is the primary driving mechanism in the promotion of PDAC development, it requires the presence of Cox-2 to activate and mediate the Ras feed forward loop sustaining high oncogenic K-Ras activity. A recent paper illustrating the ability of high fat diet to accelerate PanIN formation, also demonstrated a link between TNF-α and tumor promotion in high fat diet induced obesity (42). Since it is known that in obesity, levels of TNF-α are significantly increased and that TNF-α is an activator of NF-κB, this finding correlates with our data. This observation could be a key mechanistic explanation for the protective effect of Cox-2 inhibitors, such as aspirin, on reducing pancreatic
cancer risk and could present a possible method for prevention for obesity related syndrome causing PDAC.

Figure 4.1: Mechanistic link between high fat diet and oncogenic K-Ras. HFD causes inflammation in the pancreas which leads to oncogenic K-Ras activation. Activation of oncogenic K-Ras leads to the downstream activation of Cox-2, Phospho-ERK, and infiltration of macrophages into the stroma, as well as activation of quiescent stellate cells producing extracellular matrix proteins; alpha SMA and collagen I increasing stromal activity. Cox-2 stimulates a positive feed forward loop increasing K-Ras activity and further enhancing inflammation, fibrosis, and the recruitment of inflammatory mediators to the pancreas. Therefore, the presence of Cox-2 is essential in the promotion of oncogenic K-Ras activity to initiate PanIN formation.
4.3 Progression to PDAC with High Fat Diet

Since a mechanistic link was established between oncogenic K-Ras and high fat diet, it was essential to determine how continuous consumption could enhance this reaction. Previously, we observed PanIN-1 development accompanied by extensive fibrosis and inflammation after only thirty days of treatment with high fat diet. A recent study had shown that treatment of LSL/Pdx-1 mice, expressing oncogenic K-Ras in all the cell of the pancreas, with a high fat diet progressed PanIN-2 and PanIN-3 development (42). In this current study, we validated the results from this model and also used an acinar specific oncogenic K-Ras model (LSL/BAC). Both studies indicated the ability of high fat diet to accelerate PanIN development. LSL/Pdx-1 mice advanced PanIN formation with widespread fibrosis and inflammatory cell invasion as was noted in the previous studies. LSL/BAC animals on high fat diet had a rapid progression of PanIN development and after six months animals had developed pancreatic cancer. Furthermore, not only did the animals have localized tumor in the pancreas, the cancer had metastasized to the liver and lungs. These results were not seen in the animals fed the control diet.

In addition, we were able to record the overall survival of these animals through the course of dietary treatment. It was previously recorded in several cohort and epidemiology studies that obesity decreased overall survival in individuals with pancreatic cancer (45). We confirmed this observation by showing continuous treatment with high fat diet significantly decreased survival compared to the control diet. In fact, LSL/Pdx-1 animals on high fat diet survived up to 150 days compared to our control animals that survived about 250 days. Similarly, LSL/BAC animals on high fat diet became sick at a rapid rate while majority of the control animals remain
alive. Overall, these studies accurately demonstrated the elevated risk of developing cancer early associated with high fat diet consumption by the rapid progression of PanIN development leading to invasive cancer, as well as the reduction in overall survival. This observation provides clear evidence suggestive for prevention of pancreatic cancer by reduction of dietary fat.

4.4 Future Directions

Although this work has accurately associated an oncogenic K-Ras mutation with initiating the development of PDAC when animals were treated with a high fat diet, there still remains many unanswered questions. It is important to determine the actual mechanism of how high fat diet activates oncogenic K-Ras and if inflammation is a result of oncogenic K-Ras activation or a cause. Furthermore, it would be essential to investigate further the role of Cox-2 in this reaction to understand how deletion of Cox-2 eliminates the high fat diet induced fibrosis and PanIN formation. Since COXKO/LSL/BAC animals were only treated with the control and high fat diet for thirty days, we can conclude that deletion of Cox-2 in acinar cells delay K-Ras mediated high fat diet induced PanIN formation. However, it would be critical to determine the long term effects of this treatment on these animals.

It would also be important to determine the other inflammatory mediators involved and which are upregulated or downregulated in this process. Protein analysis of tissues could also provide information on the other proteins affected by high fat diet consumption and how K-Ras and Cox-2 might enhance or decrease their activity. Obesity related markers such as leptin, TNF-α, adiponectin, and
others should also be evaluated to determine how adipose tissue secreted molecules could enhance K-Ras mediated PanIN development. This will give a complete understanding of the relationship between obesity, systemic inflammation, and how they may work together to potentially induce oncogenic K-Ras in the pancreas leading to fibrosis and PanIN development.

Along with the added mechanistic knowledge that is necessary to understand this pathology, knowing that continuous high fat diet consumption accelerates PanIN progression to PDAC in only six months in mice expressing oncogenic K-Ras, demonstrates the necessity for prevention tactics. Potential Cox-2 inhibitors could be studied to block inflammatory Cox-2 feed forward loop increasing Ras activity resulting in early PanIN formation. Additionally, antioxidants have also been previously shown to reduce cancer risk and could be tested in this mouse model as a potential preventative treatment. With the rise in obesity, it is even more necessary to fight this epidemic and decrease cancer risk.
CHAPTER 5: REFERENCES


of v-raf, a unique oncogene transduced by a retrovirus. Proc Natl Acad Sci U S A 80:4218-4222.


VITA:

Bincy Philip was born in Kerala, India on August 11, 1989 to Philipose C. Thomas and Marykutty Philipose. She came to the United States when she was four years old and lived in Des Plaines, Illinois for her childhood. After finishing her high school career at Maine East High School, Bincy attended the University of Illinois in Urbana-Champaign to obtain her Bachelor's degree in Biochemistry and graduated in May 2011. The summer of 2011, she entered the University of Texas Health Science Center Graduate School of Biomedical Sciences to pursue a degree in Cancer Biology. Her passion for pancreatic cancer research brought her to work under Dr. Craig Logsdon where she was able to gain considerable scientific knowledge and drive for moving products from the lab to help patients in the clinic. Bincy plans on pursuing a career in clinical research and she is enthusiastic to continue her interest in oncology.