STUDIES OF AXL, HER2, AND PDGFRβ RECEPTOR TYROSINE KINASES IN MUTANT *KRAS*-DRIVEN PANCREATIC CANCER *IN VITRO*

by

Fahad Alshahrani

B.S. (King Khalid University) 2013

MOCK GRANT PROPOSAL

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL SCIENCE

in the

GRADUATE SCHOOL

of

HOOD COLLEGE

December 2018

Accepted:

Ricky Hirschhorn, Ph.D. Committee Member	Ann Boyd, Ph.D. Director, Biomedical Science Program
Oney Smith, Ph.D. Committee Member	
Rachel Beyer, Ph.D. Project Adviser	April Boulton, Ph.D. Dean of the Graduate School

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DEDICATION

I dedicate my proposal to my family and friends. To my loving parents, Gamla Bint Mohamed and Ayid Alhansholi, I thank you for your prayers and words of encouragement and inspiration. I have a special feeling gratefulness for all your support emotionally and financially. I always knew that you want the best for me. To my beloved wife, Azza Bint Saud, I thank you with love for your unfailing emotional support. You has been my best friend, loved, encouraged, and helped me get me through this period with positive way. To my lovely children, Abdulaziz and Amir, you have made me stronger and kept me hopping. I hope I have been a good father during my study life. To my brothers and sisters, I thank you for your all supports and encouragement. I would like to thank you all.

ACKNOWLEDGEMENTS

I would like to express my appreciation and thanks to the professors at Hood College, my advisor Dr. Rachel Beyer, my committee members Dr. Ricky Hirschhorn and Dr. Oney Smith, and program director Dr. Ann Boyd for generously offering their expertise, guidance, and time. Your ideas, discussion and feedback have been definitely valuable. I am very grateful to all of you.

Mock Grant Application
Modeled after Department of Health and Human Services
Public Health Services
(based on Form PHS 398)

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Type	Activity	Version – HCBMS.011712
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and punctuation.)*

STUDIES OF AXL, HER2, AND PDGFRβ RECEPTOR TYROSINE KINASES IN MUTANT *KRAS*-DRIVEN PANCREATIC CANCER *IN VITRO*

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PROGRAM DIRECTOR/PRINCIPAL INVESTIGATOR — **New Investigator** ☐ **No** ☒ **Yes**

3a. NAME (Last, first, middle)	3b. DEGREE(S)	3h. eRA Commons User Name
Alshahrani, Fahad Ayidh	MS	N/A

3c. POSITION TITLE
Graduate Student

3d. MAILING ADDRESS *(Street, city, state, zip code)*
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3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT
Department of Biology

3f. MAJOR SUBDIVISION
Biomedical Science Program

3g. TELEPHONE AND FAX *(Area code, number, and extension)*
TEL: N/A FAX: N/A

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4. HUMAN SUBJECTS RESEARCH	4a. Research Exempt
☒ No ☐ Yes	N/A

4b. Federal-Wide Assurance No.	4c. Clinical Trial	4d. NIH-defined Phase III Clinical Trial
N/A	☒ No ☐ Yes	☒ No ☐ Yes

5. VERTEBRATE ANIMALS ☒ No ☐ Yes — 5a. Animal Welfare Assurance No. N/A

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)

9. APPLICANT ORGANIZATION
Name
Address
Department of Biology
Hood College
401 Rosemont Ave
Frederick, MD 21701

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☐ State ☐ Local
Private: → ☒ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
DUNS NO. N/A Cong. District N/A

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
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13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
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E-Mail: fa3@hood.edu

14. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*
N/A

DATE

PROJECT SUMMARY (See instructions):

This project proposes to demonstrate the overexpression of the selected tyrosine kinase receptor, RTK, (AXL, HER2, and PDGFRβ) and their participation in oncogenic *KRAS* driven pancreatic cancer *in vitro.* The experimental design is to analyze the mRNA expressions of using q-PCR and protein expression using flow cytometry and ELISA. The participation of these selected RTK in this type of cancer will be demonstrated after knockout of their genes separately using the CRISPR/Cas9 gene editing system followed by an MTT proliferation assay to monitor any change in the proliferation rate after inactivating these genes. The inhibition of the selected RTK using their inhibitors, which are currently available for pancreatic cancer treatment, will also be used to demonstrate the participation of these selected RTK and will be evaluated to determine if further future drug development is needed. Therefore, R428, Lapatinib, and Sunitinib inhibitors will be used to inhibit AXL, HER2, and PDGFRβ, respectively. The confirmation of this hypothesis depends on the reduction of the proliferation rate in cell lines and may lead to new therapeutic strategies for pancreatic cancer.

RELEVANCE (See instructions):

Pancreatic cancer is a rapidly spreading cancer with no symptoms in the early stages. It is the third most lethal cancer with only a 5% survival rate in the new patients within five years. The treatment of pancreatic cancer depends on the stage; this cancer can only be treated in the early stages.

PROJECT/PERFORMANCE SITE(S) (if additional space is needed, use Project/Performance Site Format Page)

Project/Performance Site Primary Location			
Organizational Name: Department of Biology, Hood College			
DUNS:			
Street 1: 401 Rosemont Ave		Street 2:	
City: Frederick	County: Frederick		State: MD
Province:	Country: United States		Zip/Postal Code: 21702
Project/Performance Site Congressional Districts: 6th Congressional District			
Additional Project/Performance Site Location			
Organizational Name:			
DUNS:			
Street 1:		Street 2:	
City:	County:		State:
Province:	Country:		Zip/Postal Code:
Project/Performance Site Congressional Districts:			

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2. Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Fahad Alshahrani	Full-time student
eRA COMMONS USER NAME (credential, e.g., agency login)	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
King Khalid University, Bisha, KSA	B.S.	2013	Medical Laboratory Science
Hood College, Frederick, MD	M.S.	2018 (anticipated)	Biomedical Science

A. Positions and Honors

Teaching-Assistant at University of Bisha

B. Selected peer-reviewed publications (in chronological order)

C. Research Support

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the project/performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. If research involving Select Agent(s) will occur at any performance site(s), the biocontainment resources available at each site should be described. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Hood College Laboratory

Clinical:

Animal:

Computer:

It will be applied by Hood College

Office:

Other:

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

The following major equipment is available on site:

- Centrifuges- For the collection of cell lysates
- PCR machines- DNA amplification and qRT-PCR
- Refrigerator and freezer
- Incubators
- Spectrophotometer- to determine the
- concentration and the integrity of nucleic acids
- Flow cytometer- to analyze the protein cell surface expression
- SpectraMax M2 microplate reader
- Hood
-

The following consumables will be purchased with grant funding:

- Pancreatic cancer cell lines
- Petri Dishes
- Flasks
- Tubes
- 96-well plates
- Phosphate Buffered Saline (PBS)
- Trypsin
- Dulbecco's modified Eagle's medium (DMEM)
- Pipette tips
- Tubes
- Oligo (dT) column
- Random hexamer primers
- Reverse transcriptase
- Transcriptase
- Specific primers and TaqMan probes
- StepOne Real-Time PCR System Software
- eBioscience flow cytometry staining buffer
- Specific conjugated antibodies for EGFR, AXL HER2, & PDGFRβ
- Attune NxT Flow Cytometer
- ELISA kits
- SpectraMax M2 microplate reader
- CRISPR non-homology knockout kits
- Fluorescence microscope
- MTT cell proliferation kits
- Antagonists (Erlotinib, Sunitinib, Lapatinib,& R428)

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- Transcriptase
- Specific primers and TaqMan probes
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- ELISA kits
- SpectraMax M2 microplate reader
- CRISPR non-homology knockout kits
- Fluorescence microscope
- MTT cell proliferation kits
- Antagonists (Erlotinib, Sunitinib, Lapatinib,& R428)

SPECIFIC AIMS

According to several reports, pancreatic cancer cells have overexpressed epidermal growth factor receptor (EGFR) implicated in tumorigenesis. Navas and colleagues (2012) demonstrated that signaling through EGFR is required for mutant *KRAS* to increase the growth rate of pancreatic tumor cells compared with $EGFR^{-/-}$ *in vitro*. To extend this study, other receptor tyrosine kinase (RTK), such as AXL receptor tyrosine kinase (AXL), human epidermal growth factor receptor (HER2), and platelet derived growth factor receptor beta (PDGFRβ), are highly expressed on the surface of oncogenic KRAS driven pancreatic cancer tissue (Pettazzoni *et al.* 2015). Thus, in this proposal, I would like to investigate which of these RTK (AXL, HER2, and PDGFRβ) is the most highly expressed besides EGFR on *KRAS* oncogene driven pancreatic cancer cell lines and determine the importance of its signaling for mutant *KRAS* driven pancreatic cancer *in vitro.*

Aim 1: The quantitation of mRNA expression of the selected RTK (EGFR, HER2, PDGFRβ, and AXL) in pancreatic cancer cell lines will be analyzed using q-PCR.

Cell lines that have mutant *KRAS* will be included, such as AsPc1, CFPAC, IMIMPC-2, MIAPaCa, PANC1, and SKPC. These cells have overexpression of EGFR (Navas et al, 2012). Additionally, cell lines that have wild-type *KRAS* will be included, such as BxPc3 and T3M4.

Aim 2: The quantitation of cell surface protein expression of the selected RTK will be analyzed using flow cytometry and the total protein expression using ELISA.

Aim 3: Evaluation the significance of the signaling through the selected RTK for *KRAS* oncogene on pancreatic cancer cell lines will be realized after disrupting their genes and inhibiting them separately on the selected cell lines.

- CRISPR/Cas9 system will be used to nullify the selected RTK genes and observe the largest influential RTK leading to candidates for future drug development.
- Antagonist molecules specific to each RTK will be studied. Antagonists (or inhibitors) that are currently available for pancreatic cancer treatment will be used to determine if further drug development is needed for these potential targets in pancreatic cancer
- MTT assay will be used to evaluate the proliferation rate after knockout and inhibiting the selected RTK genes.

BACKGROUND AND SIGNIFICANCE

Any cell in our bodies differentiates to a specific cell state dividing in a controlled manner and die when damaged or exhausted. However, when cells infinitely grow out of control, they become cancer cells. Cancer is a genetic disorder caused by mutations in genes that are responsible for the control of cell proliferation. Thus, it could be inherited from the parents, but it can be also acquired within a person's lifetime due to mutations in cell division or environmental DNA damage. Cancer can originate in any organ and spread throughout the body by the process of metastasis and leads to deterioration of organ and organ systems resulting in death. According to the Cancer Research UK, in 2012, 8.2 million people died from cancer and 14.1 million new cases of cancer worldwide. According to the National Cancer Institute (NCI), in the U.S., the cancer mortality rate is 163.5/100,000 males and females every year from 2011 until 2015. Thus, researchers have worked hard to learn and understand the biology of cancer, ways of prevention and treatment hopefully minimizing serious side effects. The NCI spent more than $5.6 billion on cancer research in 2017 alone. Some types of cancer have plenty of dedicated studies and funds than others due to their prevalence worldwide. Therefore, a researcher's focus on particular cancers depends on the rate of morbidity and mortality of each cancer.

Overview of Pancreatic Cancer

According to NCI (2018), pancreatic cancer is the third type of cancer leading to death in both American men and women with 7.3 % of the cancer death after lung cancer and colon cancer. Furthermore, the NCI estimates 1.7 million new cases in all type of cancers; 55,440 (3.2%) of them will be pancreatic cancer. The five-year survival rate is

only 8.5%. Rahib and colleagues (2014) believe pancreatic cancer will be second cancer leading to death by 2030.

There are two types of pancreatic cancer depending on where it arises, exocrine tumor, which is the most common, and endocrine tumor. According to Ryan and colleagues (2014), exocrine tumors represent 85% of pancreatic cancer especially pancreatic ductal adenocarcinoma (PDAC), which is the deadliest common cancer due to the often late diagnosis. The endocrine tumor is much less common, and it is also called pancreatic neuroendocrine tumors (PanNETs) or islet cell tumors (Klimstra *et al.*2010). Pancreatic cancer has a high mortality rate in newly diagnosed patients, as its early stage is silent (asymptomatic) until the tumors surround the entire tissue (advanced stage), or it has metastasized to another organ (Vincent *et al.*2011). Caldas and colleagues (1995) state that since *KRAS* oncogene is found mutated in 90 to 95% of pancreatic cancer patients, and it is the most common genetic abnormality in pancreatic cancer. Mutations in the *KRAS* oncogene are often accompanied with mutations in other tumor suppressors, such as TP53 (tumor protein p53), CDKN2A (cyclin-dependent kinases inhibitor 2A), and SMAD4 (SMAD family member 4) to be constitutively active (Rozenblum *et al.*1997). Tumor suppressor gene normally slows down the cell division and monitor any damage in DNA for repairing or leading to cell death. Thus, any mutation leading to loss-of-function of these genes may allow for any an uncontrolled division. To reveal the genetic abnormalities in pancreatic cancer, Jones and colleagues (2008) analyzed 24 protein-coding exomes of PDAC using genomic analysis to characterize the mutational spectrum pancreatic cancer, with mutant *KRAS* as the predominant oncogene.

KRAS Oncogene in Pancreatic Cancer

Ras proteins are implicated in more than 30% of all types of cancer including 95% of pancreatic cancer. As result, the NCI (2013) establishes the RAS initiative program to study and explore more approaches to attack the oncogene *KRAS*. Ryan and colleagues (2014) suggest that the mutant *KRAS* is considered as the marker of poor prognosis in patients; in contrast, the depletion of *KRAS* leads to cell death and arrest in proliferation meaning it is critical to sustained the cell proliferation. The *KRAS* gene encodes for a small GTPase protein, KRAS protein, which is a member of RAS family proteins (K, H, and N). Ras proteins in general play a critical role in cell proliferation, survival, differentiation, migration, metabolism, and apoptosis (Figure 1).

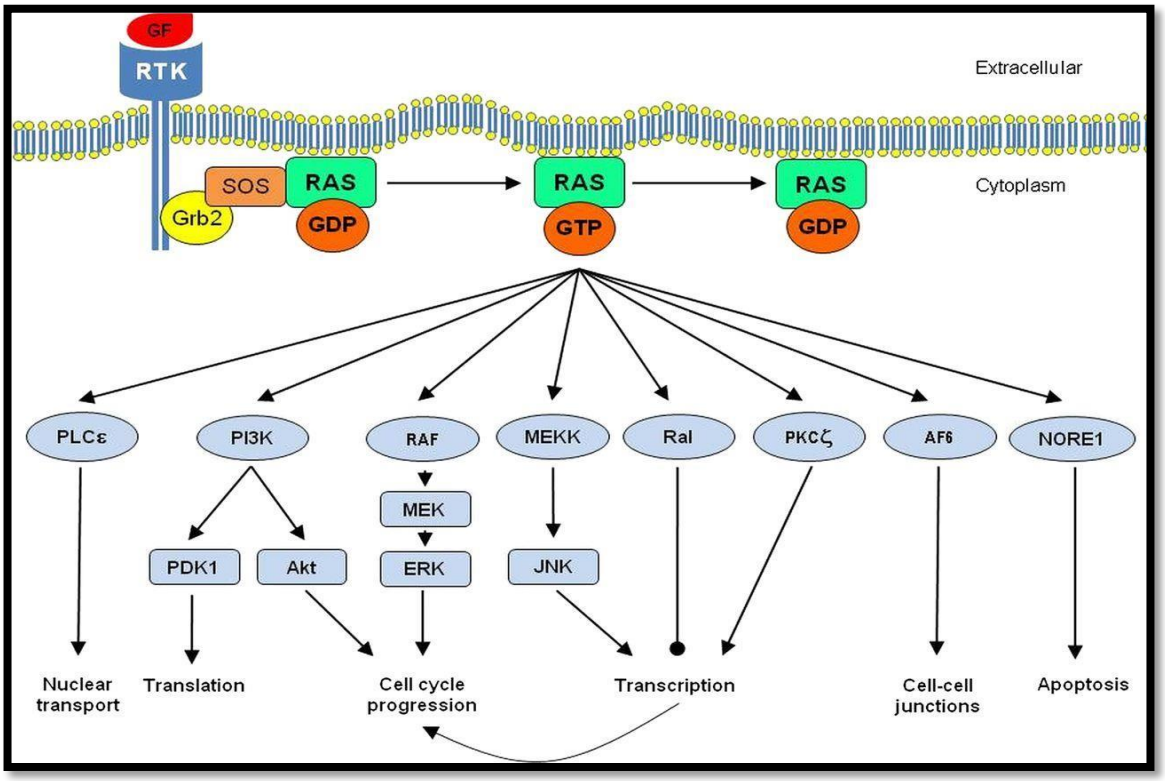

Figure 1. The activation of Ras protein normally happens after the extra signals interact and activate the RTK. Then, the active form of Ras protein activates many downstream pathways to control different biological processes within the cell (Chetty and Goverder 2013).

The activation of the Ras protein is normally regulated by an incoming external signal. When the external signals, such as epidermal growth factor (EGF), binds to a putative receptor tyrosine kinases (RTK), such as epidermal growth factor receptor (EGFR); this leads to activation of the on Ras protein, which subsequently turns on other proteins that ultimately turn on genes involving cell proliferation, survival, differentiation, migration, metabolism, and apoptosis. As result, any mutation that leads to permanent activation for Ras protein will cause unintended and superactive internal signals even in the absence of the external signals. The normal scenario is that the external signal interacts with its putative RTK creating dimerization and conformational change, which will

stimulate autophosphorylation for the cytosolic domain tyrosine residues. The phosphorylated domain will recruit adaptor protein GRB2 (growth factor receptor-bound protein 2), which then bind to Sos (Son of Sevenless) that encodes the guanine exchange factor (GEF). The complex of GRB2 and Sos will bind to inactive Ras, which is covalently attached to lipids in the plasma membrane. The binding between Sos and inactive Ras creates conformational change, which promotes the dissociation of GDP (guanine diphosphate) and allows GTP (guanine triphosphate) to bind and active Ras dissociates from Sos. Ras starts signaling (switch on) when bound to GTP and switches off when the hydrolysis of GTP to GDP by GTPase activating protein (GAP) occurs to terminate the signal. As long as the Ras protein is activated, it will interact with downstream effectors in different pathways (Chetty and Goverder 2013). In fact, only one single amino acid substitution is enough to activate Ras leading to uninterrupted signaling. The substitution of glycine to aspartic acid or valine at codon 12, 13, or 61 are the most common mutations (Caldas *et al.*1995). Moreover, the common mutations in *KRAS* are nonsynonymous mutations and occur in codons 12,13, and 61 leading to permanent activation in *KRAS*, oncogenic form (Stephen *et al.* 2014). In pancreatic cancer, the mutation in codon 12 of *KRAS* is the most frequent mutation with the additional presence of loss-of-function mutations in tumor suppressors (Zeitouni *et al.*2016).

Receptor Tyrosine Kinase (RTK)

Ardito and colleagues (2012) reveal the contribution of RTK especially EGFR in *KRAS* mediated pancreatic cancer suggesting another synergistic approach between the two genes. RTK is cell surface receptor with high affinity to many signals (growth factor, hormones, and cytokines). As such, they play a crucial role in many cellular processes,

such as cell cycle and metabolism. There are 58 known RTK in humans classified into 20 subfamilies (Lemmo and Schlessinger 2010). The activation of RTK occurs after the growth factor triggers dimerization between the external oligomer domains leading to a conformational change in the cytosolic domains. This conformational change results autophosphorylation at tyrosine kinase residues leading to the recruitment of adaptor proteins mentioned above. RTK dose not only regulate the normal cellular processes but also have a crucial role in many cancerous cellular processes (Zwick *et al.*2001). Baer and colleagues (2015) suggested that some RTK are implicated in pancreatic tumorigenesis. HER2 (or *ERBB-2*) has been found frequently overexpressed in human pancreatic carcinoma samples (Yamanaka *et al.*1993). PDGFRβ is also found commonly highly expressed in pancreatic cancer tissues (Hwang *et al.*2003). Additionally, Koorstra and colleagues (2009) demonstrate that the AXL receptor is overexpressed in pancreatic cancer. EGFR, which is overexpressed, induces the activation of KRAS in order to induce the tumorigenesis in pancreatic tissue (Ardito *et al.* 2012). Proteomic analysis shows the high proportion expression of AXL, PDGFR, EGFR, and HER2 RTK in human pancreatic cancer samples (Pettazzoni *et al.*2015). Thus, the purpose of this project is to investigate the participation of these additional three overexpressed RTK (AXL, HER2, and PDGFRβ) on the proliferation of pancreatic cancer cell *in vitro*.

EGFR Receptor Tyrosine Kinase

EGFR is cell surface protein encoded by *EGFR* (or *ERBB1*) gene, which is a member of ERBB family (including ERBB1, ERBB2, ERBB3, and ERBB4) (Troinani *et al.* 2012). *EGFR* gene is located in chromosome 7p11.2 with 31 exons. EGF, the ligand, has a high and potent affinity to EGFR inducing the receptor dimerization after binding.

EGFR has implicated in many types of cancer including pancreatic cancer. EGFR is frequently found overexpressed in human pancreatic cancer (Lemoine *et al.* 1992). Northern blot and immunostaining show the overexpression of EGFR in pancreatic cancer (Friess *et al.* 1999). The EGFR plays role in metastasis and recurrence of pancreatic cancer (Topita *et al.* 2003). Troiani and colleagues (2012) state that overexpressed EGFR is detected in 90% of pancreatic cancer tissues. *In vitro*, Navas and colleagues (2012) demonstrated that the signals through EGFR are essential for oncogenic *KRAS*-driven pancreatic cancer.

AXL Receptor Tyrosine Kinase

The AXL protein is encoded by *AXL* gene, which is a member of TAM group (Tyro3-Axl-Mer) of RTK subfamily. O'Bryan and colleagues originally isolated AXL from chronic myelogenous leukemia cells and its name was derived from Greek word "anexelekto", or uncontrolled (1991). The *AXL* gene is located on chromosome 19q13.2 with 20 exons (Verma *et al.* 2011). Growth arrest-specific gene 6 (Gas6) is AXL's ligand, which induces the dimerization leading to the autophosphorylation tyrosine residue in the cytosolic domain of AXL; Gas6 is a member of vitamin K-dependent protein family (Wu *et al.* 2014). AXL receptor is found high frequency expressed in pancreatic cancer patients. Koorstra and colleagues (2009) demonstrate that AXL receptor is commonly overexpressed in pancreatic cancer tissues and associated with distant metastasis. Knockdown of *AXL* gene in pancreatic adenocarcinoma cells decreased the invasion and increased apoptosis (Song *et al.* 2011). Using a monoclonal antibody against AXL receptor reduces pancreatic cancer cell proliferation *in vitro* (Leconet *et al.* 2014).

HER2 Receptor Tyrosine Kinase

The HER2 (human epidermal growth factor receptor 2) protein is encoded by *ERBB2* gene, which is a member of ERBB family. *ERBB2* is located on chromosome 17q2 at the long-term with 32 exons (Ghaneh *et al.* 2007). No ligand for HER2 has yet been identified, but it can heterodimerize with other members of the ERBB receptor family to initiate different signaling pathways (Rusnak *et al.* 2001). Yamanka and colleagues (1993) reveal that most pancreatic cancer patients have a high expression of HER2. Furthermore, immunohistochemistry analysis reveals the vast difference in expression of HER2 in normal and cancerous pancreatic cells; HER2 was rarely expressed in normal pancreatic cells while it was overexpressed in 86% of pancreatic cancer cases (Day *et al.* 1996). Kelber and colleagues (2012) demonstrated that HER2 involves in the PDAC progression, so inhibition HER2 could be a potential therapy in pancreatic cancer. The overexpressed HER2 in pancreatic cancer is associated with poor prognosis (Shibata *et al.* 2018).

PDGFRβ Receptor Tyrosine Kinase

PDGFRβ (platelet-derived growth factor receptor beta) is a transmembrane protein encoded by *PDGFRB* gene, which is a member of PDGFR RTK family. *PDGFRB* gene is located in 5q32 with 24 exons. PDGFRβ's ligand is PDGF-BB (platelet-derived growth factor-BB), which was originally identified in whole blood but not in cell-free plasma (Heldin *et al.*1999). Immunohistochemical analysis reveals the overexpression of PDGFRβ in metastasis pancreatic carcinoma; inhibition of PDGFRβ decreases the proliferation and metastasis in vivo (Hwang *et al.* 2003). Furthermore, Chen and colleagues (2006) demonstrate the high expression of PDGFRβ in most pancreatic cancer samples using

Immunohistochemical analysis. *In vitro*, inhibition of PDGFR in pancreatic cancer significantly diminishes the tumor growth and survival rate (Taeger *et al.* 2011).

Tyrosine Kinase Inhibitors (TKIs)

TKIs are medicinal drugs to inhibit tyrosine kinases, which are enzymes activating the signals transduction pathway. In 1988, Yaish and colleagues describe that TKIs work by competing with ATP at the catalytic binding site of tyrosine kinase to inhibit the processes of adding phosphate, so TKIs is also called Tyrphostins, which is a short name for tyrosine phosphorylation inhibitors. The TKIs are effective cancer therapy as anti-cancer, and Imatinib was the first TKI used in oncology clinics in 2001 and followed by other drugs (Hartmann *et al.* 2009). In 2005, the U.S Food and Drug Administration (FDA) approved the combination of erlotinib, EGFR TKI, and gemcitabine, which are chemotherapy drugs, to treat metastatic pancreatic cancer (Takimoto *et al.* 2008). The activation and overexpression of HER2 are implicated in the resistance of EGFR-targeted drugs, so inhibition HER2 could restore the sensitivity to EGFR TKIs (Yonesaka *et al.* 2011). Inhibition HER2 by using Lapatinib could be a potential therapy to treat overexpressed HER2 pancreatic cancer (Shibata *et al.* 2018). Sunitinib is an inhibitor to PDGFRβ reducing angiogenesis of tumor and triggering apoptosis (Strawn *et al.* 1996). Sunitinib is approved drug against PDGFR and other kinases to treat pancreatic neuroendocrine tumors (Raymond *et al.* 2017). Ludwig and colleagues (2017) found a small molecule, called R428 (or BGB324), can promote the chemotherapy and impair the tumor immune invasion in pancreatic cancer by inhibiting AXL receptor. These TKIs will be used in this project to see their effects in these selected RTK.

CRISPR\Cas9

The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)\ CRISPR associated protein 9 (Cas9) system is revolutionary gene-editing technology that can precisely modify, knockout, or delete individual genes much faster than before (Munshi 2016). CRISPR\Cas9 is an adaptive immune system in bacteria and archaea to protect them from the viral infection (ex. phage) and foreign genetic elements using RNA guided DNA cleavage by Cas9 protein (Cong *et al.* 2013). CRISPR is found in about 50% of the sequence of the bacterial genome and 90% in archaea genome (Hille *et al.* 2018). The first CRISPR locus was detected and discovered accidentally by Japanese researchers in 1987 in Escherichia coli (Isoino *et al.* 1987). Barrangou and colleagues (2007) demonstrated that bacteria are resistant to phage infection because the bacteria integrated small DNA segments of the phage into the CRISPR region, called spacers, after the phage infection. This CRISPR\Cas9 immunity occurs in three steps, which are spacer acquisition, crRNA processing, and interference. In the spacer acquisition, after the first phage's infection and its DNA is inserted into the cell, and the foreign DNA will be recognized and cleaved by two Cas proteins (Cas1 and Cas2) into small segments, called protospacers, which will integrate into the CRISPR locus (new repeat spacers) in the bacterial DNA. In the crRNA processing, CRISPR region will be transcribed in mRNA, called precursor CRISPR-RNA (pre-crRNA), which then are cleaved by Cas6 to be short crRNAs. Moreover, there is another RNA called transactivating RNA (tracrRNA). In the interference phase, the tracrRNA will hybridize with crRNA and then will join Cas9 protein creating a complex to monitor, recognize, and cleave the exogenous DNA from the second infection (Figure 2).

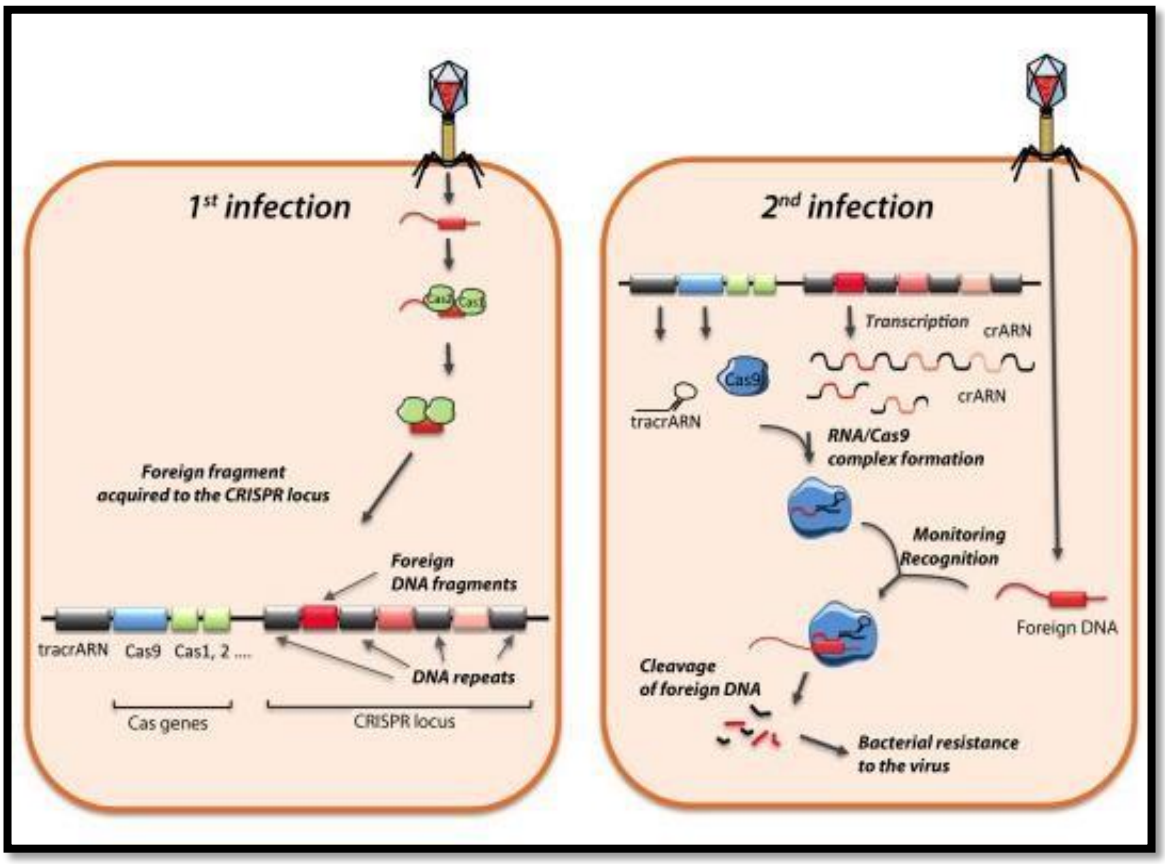

Figure 2. The three phases (adaption, expression, and interference) of the CRISPR/Cas9 system is to create the adaptive immunity in prokaryotic cells. The first phase will occur within the first infection after two enzymes (Cas1 and 2) cleave the foreign nucleic acid to small segments that integrated in the CRISPR locus creating genetic memory. The other two phases will be during the second infection to protect from the reinvading foreign nucleic acid (Duroux-Richard *et al.* 2017).

Cas9 is RNA-guided DNA endonuclease that binds to the middle chain of DNA and cut it. In the bacteria cell, Cas9 cuts in a targeted manner that is guided by crRNAs (called guide RNAs). In 2013, Cong and colleagues engineered the type II prokaryotic CRISPR system to be a genome-editing tool in eukaryotic cells. After they took the CRISPR region from two different bacteria (Streptococcus thermophilus and Streptococcus pyogenes), they designed and inserted spacers and crRNA for a specific sequence in human DNA into this CRISPR region. They followed this by inserting this

modified CRISPR region and Cas9 protein into human cell line using plasmids. As a result, they demonstrated that the gene editing using CRISPR technology could be used in human cells. The modified CRISPR consists of Cas9 that is derived from Streptococcus pyogenes (SpCas9) and a guide RNA (gRNA), fusion crRNA and fixed tracrRNA. The gRNA is ~20 bases complementary to the target sequence in the gene of interest to guide the endonuclease (SpCas9) (Ran *et al.* 2013). The crRNA will locate this sequence while tracrRNA will bind to crRNA to form the active complex. The SpCas9 is able to modify the DNA sequence, such as a double-strand break (DSB). The specificity of CRISPR\Cas9 depends on two factors: the target sequence and Protospacer Adjacent Motif (PAM). The target sequence is ~20 bases long while PAM sequence is 3 bases located in the host sequence and recognized by Cas9; the SpCas9 PAM sequence starts with 5`-NGG-3`, which occur every 8-12 base pairs in the human genome (Ran *et al.* 2013). As result, the gRNA will be modified to 20 bases plus NGG (5`-N20-NGG-3`) to help the Cas9 making its activity in any DNA sequence with this form (Sander and Joung 2014).

The end result is a DSB in the DNA sequence triggering the cell to repair using one of two repair pathways, either non-homologous end joining (NHEJ) pathway or homology-directed repair (HDR) pathway (Figure 3).

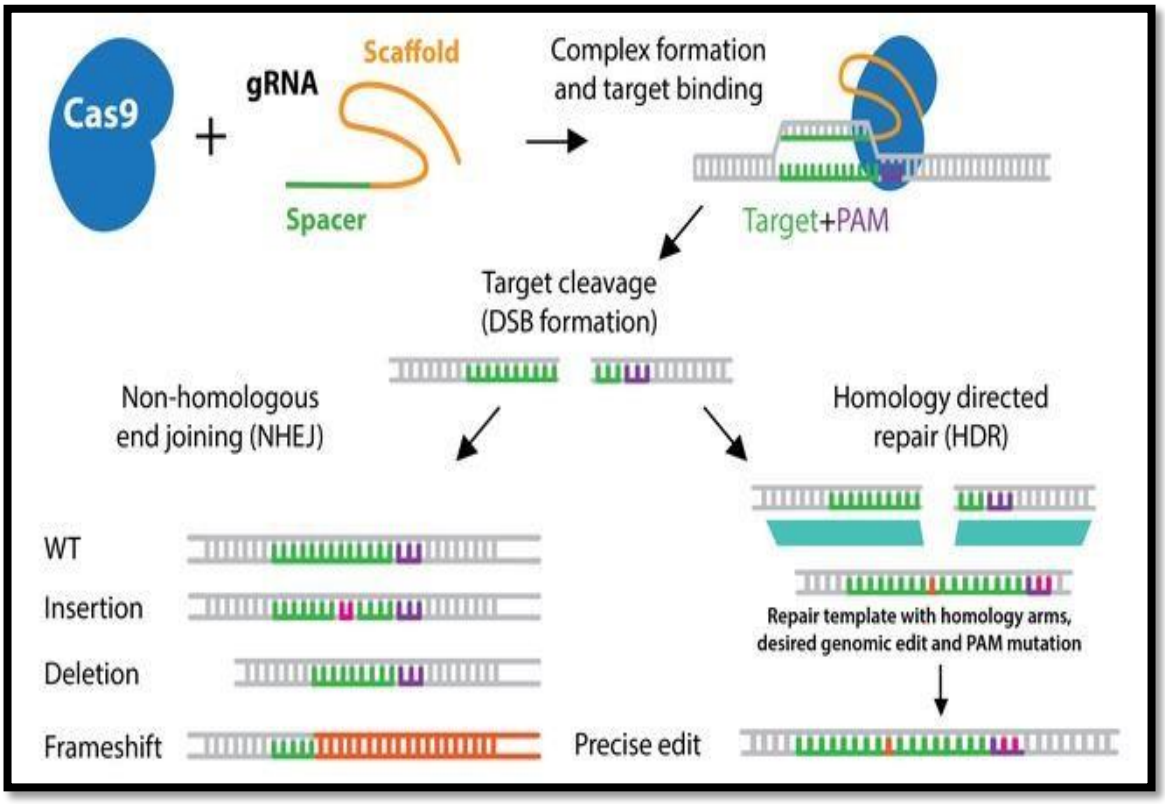

Figure 3. After Cas9 and gRNA complex bind to PAM (purple) and the target sequence (green), DSB repair method either NHEJ or HDR occurs. NHEJ often causes insertion (pink) or deletion (indels) creating frameshift (orange) leading to loss of function. HDR requires a donor complementary template to the DSB site leading to precise and accurate editing, and it is usually used to introduce a specific mutation (red) (Addgene 2018).

The NHEJ pathway works during G0 and G1 phases of the cell cycle (Bolderson *et al.* 2009). It often causes insertion, deletion, or frameshift causing a premature stop codon at the DSB site leading to loss of function of the gene of interest. In contrast, HDR is a repair mechanism during S and G2 phases of the cell cycle, and it requires a donor complementary template to the DSB site (Bolderson *et al.* 2009). According to Krejci and colleagues, there are three steps to repair DSB by HDR pathway, trimming the 5`end at DSB site (referred as resection), strand invasion, annealing followed by synthesis, and ligation. In contrast, NHEJ is much simpler; the ends of the cleaved DNA molecules are

rejoined together without any homologues donor DNA sequence in three steps (synapsis, end processing, and ligation) (Figure 4) (2003).

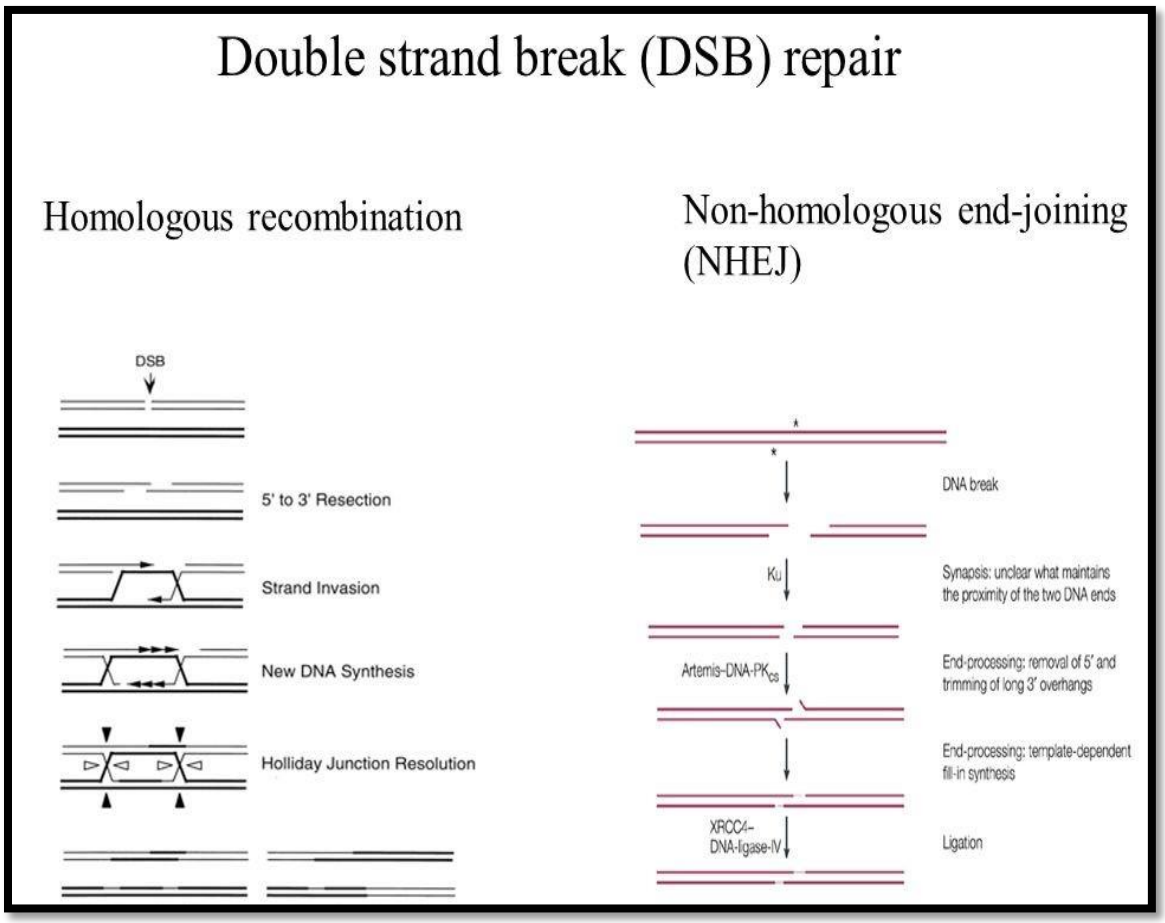

Figure 4. DSB repair occurs in two different pathways: NHEJ or HDR. In NHEJ pathway, after DSB occurred, a protein called Ku interacts with break ends (synapsis) and recruits other protein (Artemis-DNA-PK_{cs}), which process the ends to be compatible for ligation. In the HDR pathway, the DSB site will be resected; then, the donor identical sequence will invade and anneal followed by synthesis and ligation (Krejci *et al.* 2013).

The DSB repair methods of DNA use three different enzymes to accomplish these steps, nuclease to resect the DNA damage, polymerase to insert the new DNA strand, and ligase to restore the integrity of DNA strand. CRISPR/Cas9 system will be used in this project to knockout the selected RTK individually and then evaluate the cell proliferation

in different pancreatic cell lines. When any cell line has major consequences in reducing proliferation rate or increasing apoptosis rate after the knockout, this indicates that the signaling through the nullified RTK gene is very critical to sustain the cell proliferation on the selected pancreatic cancer cell lines.

Given the observations made by Navas and colleagues (2012) demonstrating the importance signaling through EGFR in oncogenic *KRAS* pancreatic cancer cell lines, I propose extending this to three additional RTKs (AXL, HER2, and PDGFRβ). The purpose of the project will be to evaluate the expression of the selected RTK including EGFR as a control and investigating their roles in oncogenic *KRAS* driven pancreatic cancer using CRISPR and TKIs to modulate expression. CRISPR will be used to ablate the selected RTK and see the largest significant effect leading to candidates for future drug development. Additionally, TKIs that currently available in the market evaluated to determine if further future drug development is needed for these potential targets in pancreatic cancer.

PRELIMINARY REPORT / PROGRESS REPORT

Previous work in this field demonstrates that the selected RTK (EGFR, AXL, PDGFRβ, and HER2) are frequently overexpressed in pancreatic cancer tissue. Separately, Troita in 2003 and 2012 demonstrated that EGFR is highly expressed in most pancreatic cancer patients and implicated in metastasis and the reappearance of pancreatic carcinoma (Torita *et al.* 2003; Troiani *et al*. 2012). The AXL RTK is also overexpressed and implicated in metastasis in pancreatic carcinoma specimens (Koortra et al. 2009). In 2011, Song and colleagues demonstrate the critical role of AXL RTK for pancreatic tumor proliferation by nullifying its genes, leading to an increase the apoptosis processes and diminish the invasion. HER2 is overexpressed in 89% of pancreatic carcinoma specimens (Day *et al*. 1996), and it is implicated in pancreatic cancer development, so inhibition of HER2 could be a potential therapy (Kelber *et al*. 2012). Immunohistochemical analysis reveals the high-frequency expression of PDGFRβ in pancreatic cancer patients (Chen *et al*. 2006). According to Taeger and colleagues (2011), targeting PDGFR using TKI *in vitro* reduces the pancreatic cancer cell proliferation, so it could be a therapeutic drug to treat pancreatic cancer. Together, Pettazzoni and colleagues (2015) demonstrate the high expression of the selected RTK in pancreatic cancer specimens using proteomic analysis. On the other hand, many studies show that mutant *KRAS* is the major driving molecular event in pancreatic cancer. More than 90% of pancreatic cancer cases are caused by mutations in KRAS protein (Waddell *et al*. 2015). Martinko and colleagues (2018) demonstrate that oncogenic *KRAS* upregulates the expression of some cell surface proteins in pancreatic cancer in order to accomplish migration and adhesion. Oncogenic *KRAS* upregulates the expression of EGFR in the pancreatic cancer mouse model to form the

tumorigenesis (Ardito *et al*. 2012). Navas and colleagues (2012) demonstrate that oncogenic *KRAS* completely depends on signals through EFGR in the absence of mutations in the other tumor suppressors. They also show that EGFR signals are required to form a tumor in oncogenic *KRAS* driven pancreatic cancer in the absence of some tumor suppressors. These studies show the crucial role of overexpressed EGFR for oncogenic *KRAS* to form tumorigenesis in pancreatic cancer providing an approach to treat pancreatic cancer by inhibiting the EGFR. This evidence supports the idea that AXL, PDGFRβ, and HER2 may be overexpressed and have critical targetable participation for oncogenic *KRAS* driven pancreatic cancer *in vitro*. Thus, they could be potential targets for therapeutic approaches to treat oncogenic *KRAS* driven pancreatic cancer.

RESEARCH DESIGN / METHODS

Pancreatic cancer is one of the most fatal cancers in the world with a mortality rate of 95% (Ilic &Ilic, 2016). More than 90% of pancreatic cancer cases are caused by mutations in the KRAS protein (Waddell *et al.* 2015). In 2012, Navas and colleagues demonstrated that signaling through EGFR, which is overexpressed, is essential for oncogenic *KRAS* driven pancreatic cancer. To extend this study, other RTK such as AXL, HER2, and PDGFRβ are also overexpressed and implicated in pancreatic cancer (Pettazzoni *et al.* 2015). In this project, I propose to investigate the expression of these three selected RTKs and the significance of their signaling for oncogenic *KRAS* on pancreatic cancer cell lines.

Aim1: The quantification the mRNA expressions of the selected RTK (EGFR, HER2, PDGFRβ, and AXL) in pancreatic cancer cell lines will be analyzed using q-PCR

Cell Culture

The pancreatic cell lines (Table1) are carrying hotspot mutations in *KRAS* accompanied with mutations in other tumor suppressors genes (*CDKN2A*, *TP53*, and *SMAD4*) to be constitutively active. Tumor suppressor genes normally slow down the cell division and monitor any damage in DNA for repairing or leading to cell death; therefore, any mutations leading to loss-of-function may allow the uncontrolled division. These cell lines (Table1) will be purchased from American Type Culture Collection (ATCC; Manassas, VA). They will be cultivated *in vitro* in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), 2mM L-glutamine, 50 U/ml penicillin, and 50ug/ml streptomycin (ATCC; Manassas, VA). These cell lines will be checked

microscopically, and the subcultures will be made as necessary. Navas and colleagues (2012) demonstrate that these cell lines have overexpression of EGFR leading to increasing the growth rate of the mutant *KRAS* pancreatic cancer cell lines compared to the wild type *KRAS* cell lines. Therefore, the expression level of HER2, AXL, and PDGFRβ will be tested and evaluated in these cell lines. Since of Navas and colleagues (2012) demonstrate the overexpression of EGFR on these selected cell lines, the quantification overexpression of EGFR will be evaluated again for confirmation. There are two cell lines (BxPc3 and T3M4) selected that have wild-type *KRAS*.

Table 1. Summary of pancreatic cancer cell lines.

Cell line	*KRAS*	*CDKN2A*	*TP53*	*SMAD4*
AsPc1	G12D	Frameshift	Frameshift	WT
CFPAC-1	G12V	Methylated	Mutation	Deletion
IMIMPC-2	G12D	Deletion	Mutation	WT
MIAPaCa-2	G12C	Deletion	Mutation	WT
PANC-1	G12D	Deletion	Mutation	WT
SKPC	G12V	Methylated	Mutation	Deletion
BxPc3	WT	Mutation	Mutation	Mutated
T3M4	WT	Methylated	Mutation	WT

Quantitative Polymerase Chain Reaction (q-PCR)

This technique measures the expression levels of the selected RTK mRNAs in the cell lines listed in Table 1. The total RNA of the selected RTK will be isolated using TRIzol reagent method followed by spectrophotometry with the ratio absorbance 260 x 40 ng/uL to measure the concentration of total RNA and the ratio absorbance 260/280 to assess the integrity of RNAs. A two-step qRT-PCR approach will be used to quantify the mRNA expression of the selected RTK. First, cDNA of each RTK will be generated using random hexamer primers and reverse transcriptase-PCR (RT-PCR). Second, q-PCR will be used to amplify the synthesized cDNA of the selected RTK using specific primer sequences and TaqMan probes that will be designed using IDT PrimerQuest software (Table 2).

Table 2. The forward (F) and reverse (R) primers and probe (P) sequences of RTK for RT-qRT-PCR analysis.

RTK		Sequence (5'->3')	Start	Stop	Length	Tm
EGFR	F	5`GCCTCCAGAGGATGTTCAATAA3`	406	428	22	62
	P	5`TGAGGTGGTCCTTGGGAATTTGGA3`	431	455	24	68
	R	5`GTTATGTCCTCATTGCCCTCA3`	517	538	21	62
HER2	F	5`ACGCCTGATGGGTTAATGAG3`	130	150	20	62
	P	5`AGAACCTTTGCTGTCCTGTTCACCA3`	233	258	25	68
	R	5`CTCTACCTCCAGCACAGAATTT3`	285	280	22	62
PDGFRβ	F	5`GCGGAGACCATTAGGAAGTTT3`	522	543	21	62
	P	5`GCGTTCCTGGTCTTAGGCTGTCTT3`	570	594	24	68
	R	5`CTAATCCTCTGCCAGCTTTCA3`	609	630	21	62
AXL	F	5`AACCTTCAACTCCTGCCTTC3`	1513	1533	20	62
	P	5`CTTGGCTCTCTTCCTTGTCCACCG3`	1592	1616	24	68
	R	5`GAAAGAAGGAGACCCGTTATGG3`	1618	1640	22	62

After amplification using QuantStudio 3 Real-Time PCR System (Thermo Fisher; Ballenger Creek, MD), the data will be analyzed using StepOne Real-Time PCR System Software (Thermo Fisher; Ballenger Creek, MD). The fluorescence signal will be emitted due to the hydrolysis of the probe sequence by the 5` to 3` via the exonuclease activity of *Taq* DNA polymerase. This hydrolysis will break up the fluorescent dye on the 5` end from non-fluorescent quenching dye on the 3` end of the probe and will result in an increased fluorescein signal (Figure 5). To analyze the data, the comparative threshold cycle (Ct) or 2-$\Delta\Delta C(T)$ method will be used for relative quantification of gene expressions. The relative quantification is to analyze the change in the target gene expression relative to the reference sample, such as untreated control sample or the expression of the same mRNA in another organ (Livak and Thomas 2001). To normalize the PCR for the amount of RNA, endogenous control gene will be used. The housekeeping gene whose expression level does not change across sample and treatment acts as the endogenous (internal) control gene. There are some appropriate housekeeping genes acting as internal control genes for qPCR experiment, such as GAPDH, β-actin, β_2-microglobulin, and rRNA (Livak and Thomas 2001).. The internal control gene is usually used to determine that the gene expression is not affected by the treatment during the experiment (Livak and Thomas 2001). The Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene will be used as the internal control gene in this q-PCR analysis. Serial dilutions of cDNA for the target genes and internal control gene are important step to assess the efficiency between the amplicons during the PCR experiment (Livak and Thomas 2001). The 2-$\Delta\Delta C(T)$ method presumes that the efficiencies between the cDNA amplifications of the target genes and the internal control gene are close 100%. The Ct values (ΔCt) of the target genes and the internal

control gene will be calculated in every experimental sample. After that, the differences of ΔCt values between them are calculated ($\Delta\Delta C(T)$). Lastly, the fold-change in the target gene expressions between two samples is equal to 2-$\Delta\Delta C(T)$. The Ct value is the required number of cycles to reach the Ct threshold reflecting the amount of the gene expression in the cell. Thus, the lower Ct value indicates the higher levels of RNA expressions while the higher Ct value indicates lower levels of RNA expressions. All required reagents, primers, TaqMan probes, and enzymes will be purchased from Thermo Fisher (Ballenger Creek, MD).

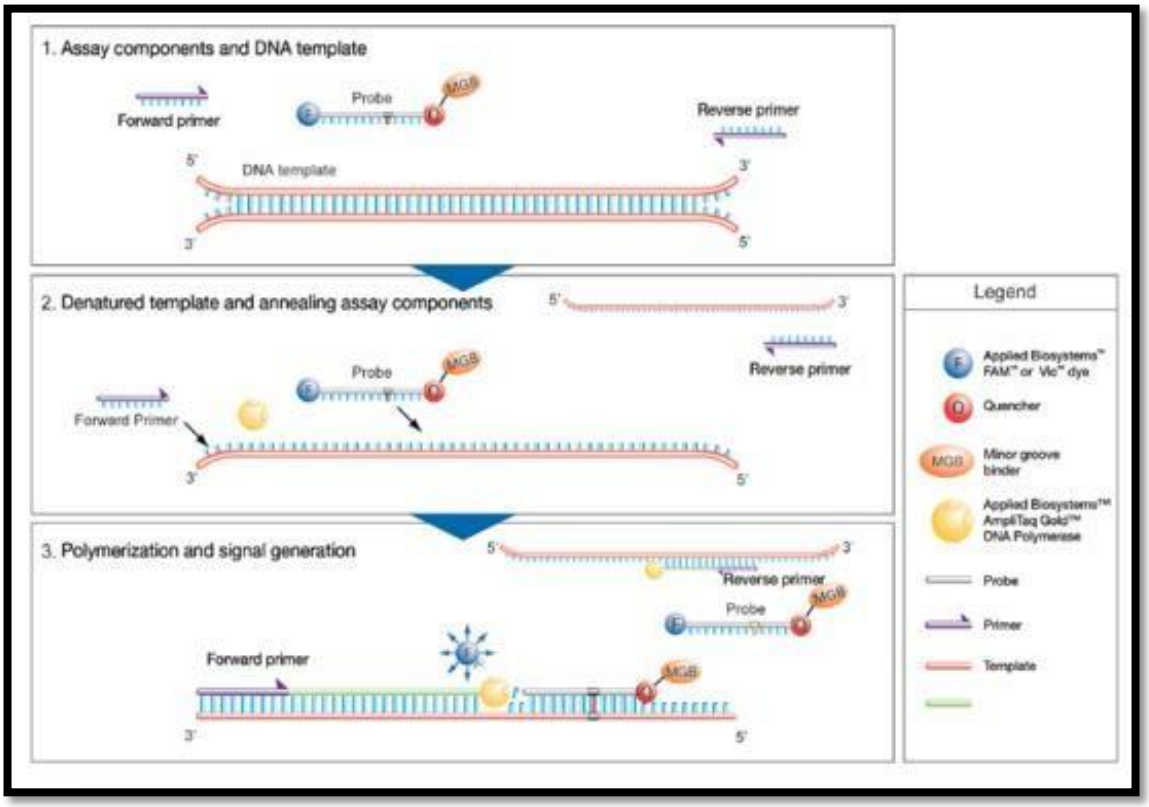

Figure 5. TaqMan mRNA expression assay principle. 1) the fluorescent dye on the 5` end quenched to MGB-nonfluorescent dye quencher on 3`end of the probe sequence. 2) after increasing the temperature to denture the sequences, the temperature will be reduced to allow the annealing 3) *Taq* DNA polymerase will synthesis the new daughter sequences using the unlabeled primers (forward and reverse primers) and template. When *Taq* DNA polymerase reach the probe, it will cleave probe and separate the fluorescent dye (Thermo Fisher 2018).

Expected Results

All selected RTK will be above the Ct threshold if the gene is expressed (shown in Figure 6). I expect that the gene expression of the selected RTK may be as higher as EGFR in the most mutant *KRAS* cell lines compared to the wild-type *KRAS* cell lines.

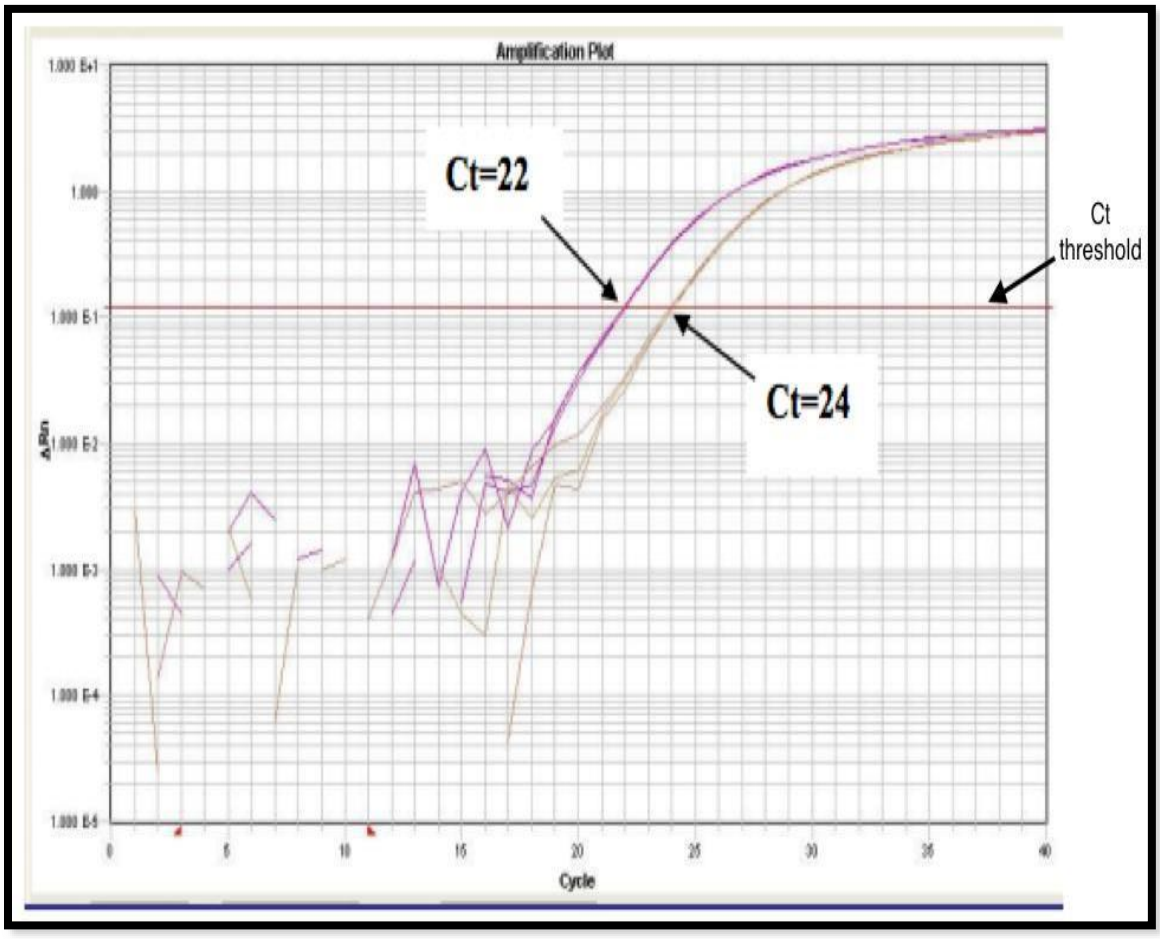

Figure 6. An example data of RT-qRT-PCR shows that the gene with 22 Ct value (purple line) has a higher expression than the other one with Ct=24 (brown line). The horizontal red line indicates the Ct threshold (University of Montreal 2018).

Aim 2: The quantification the cell surface protein and the total expression levels of the selected RTK.

Flow cytometry

This technique is used to quantify the cell surface protein expression of the selected RTK. To prepare the cells for flow cytometry analysis, the cells will be resuspended in an eBioscience flow cytometry staining buffer purchased from Thermo Fisher (Ballenger Creek, MD). Fluorescence-conjugated monoclonal antibodies for the selected RTK will be added to the buffer and incubated for 30 minutes at room temperature (or at 40^{o}C for 1 hour). Each antibody is conjugated with a different fluorochrome (Table 3) (Thermo Fisher; Ballenger Creek, MD). The cells will be washed and resuspended with the eBioscience flow cytometry staining buffer and analyzed by Flow Cytometry on an Attune NxT Flow Cytometer purchased from Thermo Fisher (Ballenger Creek, MD).

Table 3. The primary conjugated antibodies for RTK.

RTK	Primary Antibodies
EGFR	EGFR Monoclonal Antibody (ICR10), PE
HER2	ErbB2 (HER-2) Monoclonal Antibody (2G11), FITC, eBioscience™
PDGFRβ	CD140b (PDGFRB) Monoclonal Antibody (APB5), APC, eBioscience™
AXL	Axl Monoclonal Antibody (MAXL8DS), PE-Cyanine7, eBioscience™

ELISA for Analysis of Protein Expression

Enzyme-linked immunosorbent assay (ELISA) is a technique for detecting and quantifying the total protein expression of the selected RTK. ELISA kits will be purchased from Thermo Fisher (Ballenger, MD) with Catalog numbers: AXL (#EHAXL), EGFR (#KHR9061), PDGFRβ (#EHPDGFRB) and HER2 (#BMS207). Each ELISA kit includes a pre-coated 96-well plate with target-specific antibodies, secondary (conjugated) antibodies, chromogen, and all required solutions and reagents. After the cell suspension and lysate, the antigens (RTK) will be added to the pre-coated plates followed by the addition of secondary antibodies forming a "sandwich." Addition of chromogen is used to visualize the expression producing a colored product, reflecting quantity of protein in the initial lysate. Finally, the ELISA plate will be measured using a SpectraMax M2 microplate reader purchased from MTX Lab System (Bradenton, FL).

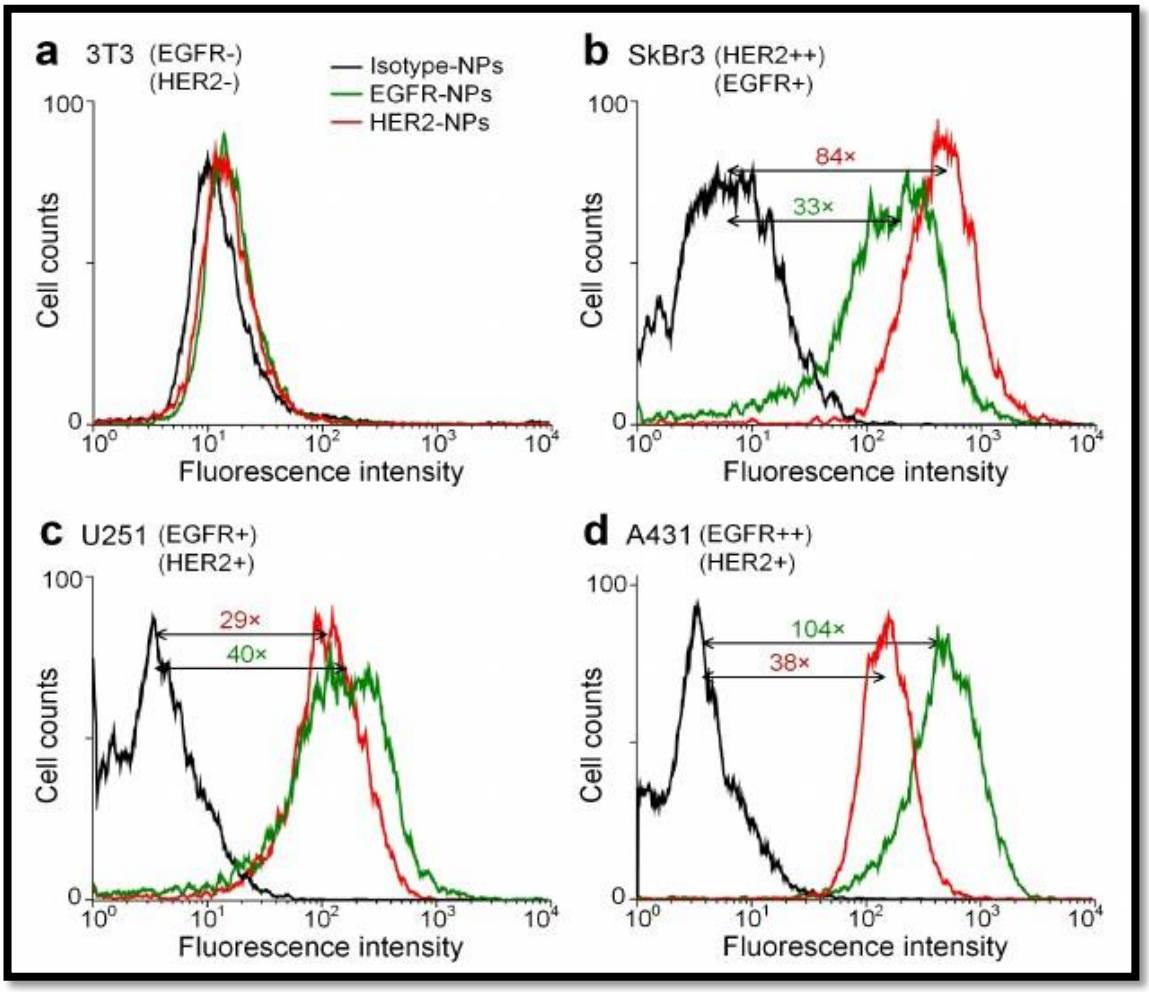

Figure 7. An example data of cell surface expression levels of EGFR and HER2 in different cell lines using flow cytometry. A. 3T3 cell line does not express both RTKs, the RTK expressions are similar to the negative control (Isotype). B.SkBr3 cell line express the EGFR and overexpress HER2. C. U251 cell line result showing the expression of both RTKs. D. A431 cell line result showing the overexpression of EGFR and the expression of HER2. The colored numbers represent the ratio of the fluorescence intensity between the RTK and the negative control (Wang *et al.* 2015).

Expected Results

The expression of the cell surface proteins of the selected RTK will be highly expressed in all cell lines that have oncogenic *KRAS* compared to the wild-type *KRAS* cell lines. The data shown in Figure 7 shows the different expression levels of EGFR and HER2 in four different cell lines, which are 3T3 (a normal mouse fibroblast does not

express EGFR and HER2 expression), SkBr3 (a human breast adenocarcinoma cell line highly expresses of HER2 and moderately expression of EGFR), U251 (a human glioblastoma cell line moderately expresses EGFR and HER2), and A431 (a human epidermoid carcinoma cell line highly expresses of EGFR and moderately expression of HER2) (Wang *et al.* 2015). This figure is used as an example of output result from flow cytometer. I expect the expression of the selected RTK on the cell membrane will be higher in the mutant *KRAS* cell lines compared to wild type *KRAS* cell lines. In the ELISA, the result will show the expression of the total protein of the selected RTK in mutant *KRAS* cell lines higher than the wild type *KRAS* cell lines.

Aim 3: Evaluation the significance of the signaling through the selected RTK for *KRAS* oncogene on pancreatic cancer cell lines will be realized after disrupting their genes and inhibiting them separately on the selected cell lines.

CRISPR/Cas9 System

This technique will be used to knockout the genes of the selected RTK in the selected cell lines to demonstrate the largest potential for a therapeutic effect in pancreatic cancer cell lines. CRISPR/Cas9 method requires two components: endonuclease (Cas9) and a guide RNA (gRNA). The gRNA, a 20 base pair target specific sequence, will guide Cas9 to the target sequence in the gene of interest (GOI). CRISPR works through the creation of DNA double-strand break (DSB). The creation of DSB stimulates one of two repair pathways, either non-homologous end-joining (NHEJ) or homology-directed repair (HDR). NHEJ is more sufficient to disrupt the gene by introducing insertion or deletion in

the translation sequence (Sander and Joung 2014). Therefore, The CRISPR non-homology mediated method will be used in this knockout. The CRISPR non-homology mediated knockout kits will be purchased from ORIGENE website (Rockville, MD). Each kit contains two gRNA vectors and linear donor DNA containing EF1a-GFP-P2A-Puro as remarkable genes for selection. One gRNA is enough to make the knockout, but to ensure the efficiency of the knockout, two gRNA vectors are provided. The gRNA vector, named pCas9-Guide vector, is an all-in-one vector meaning it expresses human codon-optimized Cas9 and gRNA containing the inserted target sequence and gRNA scaffold. These gRNAs (Table 4) are selected according to their efficiency and specificity score using bioinformatics tools, such as the CHOPCHOP web tool (Labun et al. 2016).

Table 4. The sequence of gRNA with PAM (red color) of the selected RTK.

RTK	gRNA 1	gRNA2
EGFR	5`TCCTCCAGAGCCCGACTCGCCGG3`	5`GCTGCCCCGGCCGTCCCGGATGG3`
HER2	5`TGTGGCTTCCAGGACCCCCGCGG3`	5`GATGTGTTGTGTTTACCTTGAGG3`
PDGFRβ	5`GACTTCCCATCCGGCGTTCCTGG3`	5`GTCTTAGGCTGTCTTCTCACAGG3`
AXL	5`AGACGATGGGATGGGCATCCAGG3`	5`CGATGGGATGGGCATCCAGGCGG3`

The linear donor DNA will be integrated into the cutting site after the gRNA vector cut the target sequence. The gRNA vector and donor DNA will be cotransfected in the selected cell lines (Figure 8).

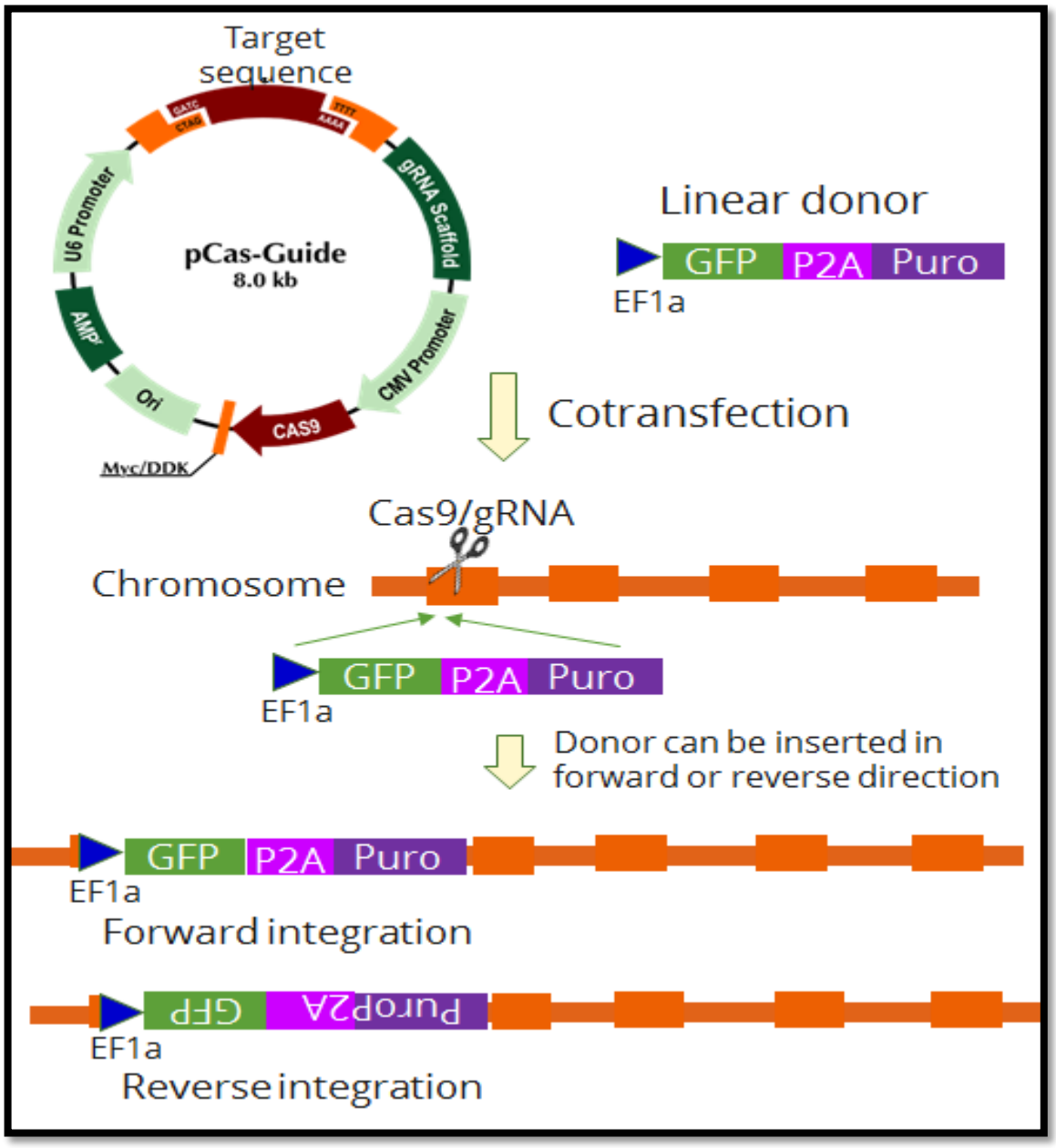

Figure 8. This figure shows the CRISPR non-homology mediated knockout process. pCas-Guide plasmid will be cotransfected with the linear donor sequence that encodes for the report proteins. Then, the Cas9/gRNA complex will cleave the target sequence creating DSB while the linear donor will integrate either forward or reverse integration with cleavage site leading to loss of function of the target gene (ORIGENE 2018).

After this cotransfection, the gRNA will guide Cas9 to the target sequence by annealing to the complementary sequence. Cas9 will then bind to PAM (protospacer adjacent motif) and uncoil the double strand followed by DSB. The linear donor DNA will be inserted in the cleavage site followed by re-ligation. PCR will be used to verify the integration of the donor DNA. After twenty days post-transfection, including cell passaging, the cells will be sorted based on GFP positivity. The single cell will be sorted into the wells of a 96 well plate. Cells that proliferate will be confirmed to be GFP positive and an aliquot assayed by ELISA to confirm the lack of protein expression meaning the disruptions of RTK genes. In addition, DNA sequencing will be conducted verifies the disruption of the RTK gene. Sanger sequencing (DNA sequencing) is a technique to determine the order of nucleotides in the DNA sequence of the target genes based on chin-terminating dideoxynucleotides triphosphates (ddNTPs), which lack the 3`-OF group, in addition to normal nucleotides (dNTPs) by DNA polymerase during the DNA amplification (Sanger et al 1977). It is the most efficient way to sequence these selected RTK genes via their open reading frames to confirm their disruptions. Thus, the samples will be sent to Genomic Sciences Laboratory to sequence RTK genes to validate their disruptions. Then, the growth rate will be compared using MTT cell proliferation assay (ATCC) after the CRISPR knockout of each RTK in each selected cell line compared to the cell lines with the genes intact and *KRAS* wild-type cell lines.

Expected Results

The inactivation of the selected RTK genes on the selected cell lines will affect the growth rate by reducing the rate of proliferation. The mutant *KRAS* cell lines that have the

highest expression of the selected RTK will be the most affected by the gene knockout and will have the most significant difference in the growth rate compared to wild type *KRAS* cell lines.

Studies of RTK Antagonists

This technique can also demonstrate the essential signaling through each selected RTK for mutant *KRAS*-driven pancreatic cancer by inhibiting each one separately in the selected cell lines. The antagonist molecules (inhibitors) (Table 5) that are currently available for pancreatic cancer treatment will be evaluated to determine if further future drug development is needed for the potential targets in pancreatic cancer treatment. Thus, the CRISPR will be used previously because of its high efficiency to reveal the biggest potential candidates for future drug development. With therapeutic drugs currently exist in the market, will they show the same result or is further development required? All antagonist molecules that are suspended in DMSO (Table 5) will be purchased from Selleckchem (Houston, TX).

Table 5. Antagonists of the selected RTK with their range values.

RTK	Antagonist	IC_{50} range
EGFR	Erlotinib	2.0 nM
PDGFRβ	Sunitinib	2.0 nM
HER2	Lapatinib	9.2 nM
AXL	R428 (or BGB324)	14 nM

After the molecules are obtained, the selected cell lines that have the highest expression of the selected RTK will be transferred and seeded into 96-well plates and cultured. Antagonist molecules will be then added at various concentrations according to their IC_{50}, which is a concentration of inhibitors reducing the response by half. IC_{50} is used to examine the potency of the inhibitors. The examination of each RTK with dilution series (four-fold above and below the IC_{50} of each antagonist) will be replicated three times (shown in Figure 9). Each plate will have two of six selected cell lines. After 72 h of incubation, the MTT cell proliferation assay (ATCC) will be used for the measurement of proliferation and antiproliferation activity in plates using a SpectraMax M2 microplate reader purchased from MTX Lab System (Bradenton, FL).

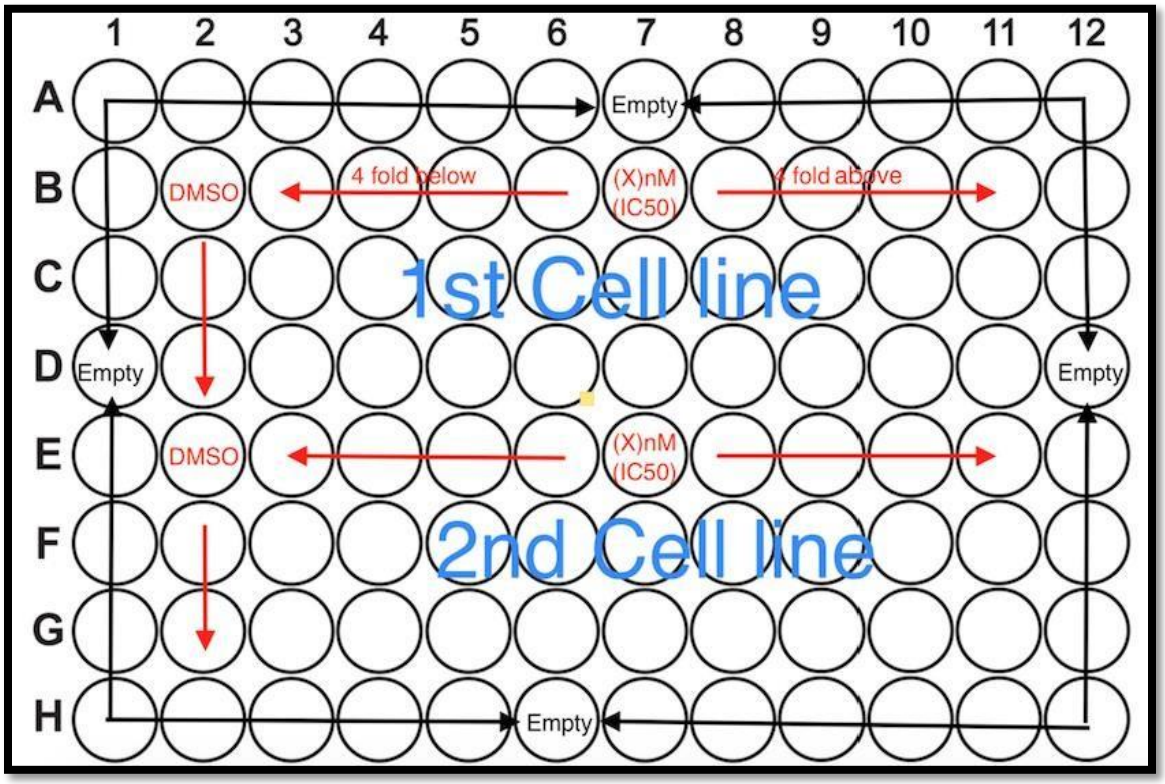

Figure 9. An example of a dilution series of the antagonist molecules for one RTK in two cell lines. Each cell line will have three times duplication.

Expected Results

The wells that have antagonists will have a lower growth rate compared to the negative wells (DMSO). The effects of antagonists may or may not reveal the need for further development of new comparison to target these proteins. Thus, the result after the knockout and inhibition may indicate that the current drugs could be improved to be more like the knockout, or the full potential of the antagonists may not get be realized with current drugs.

Tentative Timeline

Aims	Time	Activity
Aim 1	1.0 month	Obtaining the selected cell lines.
	1.0 week	Culturing and maintaining the cell lines.
	1.0 week	Obtaining the required primers and probe for qRT-PCR analysis.
	2.0 weeks	Analyzing the expression of the selected RTK.
Aim 2	1.0 week	Obtaining the specific conjugated antibodies for flow cytometry analysis.
	1.0 week	Obtaining the ELISA kits.
	2.0 weeks	Analyzing the expression of the protein cell surface using flow cytometry.

	2.0 weeks	Analyzing the expression of the total protein using ELISA
Aim 3	1.0 week	Obtaining CRISPR non-homology knockout kits.
	1.0 month	Cloning the plasmids.
	3.0 months	Selecting the positive cloning cells.
	3.0 months	Culturing single cell cloning and monitoring the proliferation rate using MTT assay
	1.0 week	Obtaining the TKIs (antagonist).
	2.0 months	Determining the IC_{50} and the cells' response

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