Spring 5-4-2019

Human Leukocyte Antigen (HLA) Class I Molecule Components and Amyloid Precursor-Like Protein 2 (APLP2): Roles in Pancreatic Cancer Cell Migration

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HUMAN LEUKOCYTE ANTIGEN (HLA) CLASS I MOLECULE COMPONENTS AND AMYLOID PRECURSOR-LIKE PROTEIN (APLP2): ROLES IN PANCREATIC CANCER CELL MIGRATION

by

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A DISSERTATION

Presented to the Faculty of

the University of Nebraska Medical Center Graduate College

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

Cancer Research Graduate Program

Under the Supervision of Professor Joyce C. Solheim

University of Nebraska Medical Center
Omaha, Nebraska

May 2019

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ACKNOWLEDGMENTS

The journey to a Ph.D is not a solitary endeavor and I have many people to thank for my aiding in my progression to this point. Without these people, this process would not have been possible nor nearly as fun of an experience. I owe each of them many thanks for their continued and unwavering support of me throughout completion of this degree.

First, I have to thank my parents Bill and Sharon Sliker. They have shown me what it means to have excellent work ethic and I can only hope to work as hard as they do on a daily basis. They have both been there for me when things were going well and when things seemed to be too overwhelming for me to process. They have encouraged me to follow my dreams even when that meant sacrifices on their part. I wouldn't be where I am today without their continued unwavering love and support. No words can really sum up what they mean to me, but I hope I continue to make them proud.

To my mentor, Dr. Joyce Solheim, I also express my deep gratitude. I am extremely thankful that she was willing to take me on as a student in my second year of the program. Her continuous support and optimism kept me going even on days when science felt difficult and confusing. She has guided me as I continuously grow to be a better scientist and pushes me outside of my comfort zone to spur my growth. I will take many things away from this lab and I hope I can do her justice as I continue on in my scientific career.

I would also like to extend thanks to members of my Supervisory Committee, including Dr. Tony Hollingsworth, Dr. Amar Natarajan, and Dr. Jennifer Black. Their support and excellent suggestions throughout my time at UNMC have made me a more perceptive scientist. I am immensely appreciative of their willingness to always be there

to help. Additionally, I would also like to extend my thanks to Dr. Nicholas Woods who was willing to take the time to serve on my comprehensive exam committee.

To current members of the Solheim laboratory, including Dr. Ben Goetz, Brittany Poelaert, and Shelby Knoche. Without all of you, my time in the lab would not nearly have been as fun and I am lucky to consider all of you friends. Furthermore, I extend thanks to previous members of the Solheim laboratory that I had the pleasure of working with throughout my time including Nuzhat Khan, Poomy Pandey, Kaitlin Smits, Alexandra Moffitt, and Raina Barnes. Rotation and summer students that also made some of this work possible include Hannah King, Thanh Nguyen, Mohammed Awaji, and Cassie Liu. I have learned so much from all of you and I wish you all the best as you move through your scientific careers. I know you will all do extremely well.

The friends that I have made throughout my years here have made this an amazing experience both in the lab and outside of it. Brandon Griess, Lisa Richter, Nathan Booth, Caroline Robb, Stephanie (and Garrett) Barbari, Beth Neilsen, Aneesha Dasgupta, Barb Swenson, Erin Wuebben, Logan and Chelsea Bulock, Dannah Miller, among others as well as all students of the CRGP fall into this category. I am appreciative of all of the love and support each of you have provided throughout the years and I couldn't have imagined having as much fun as I have with all of you. From volleyball to fantasy football, I have made so many cherished memories with each of you that I will take with me as I move on to the next step of my scientific career. You all are amazing people and I can't wait to see what each of you accomplish.

Additional family members that offered support include Judy Kuchera, Tracy, Jeff, and Madelyn Magnuson, Anthony and Michelle Kuchera, Becky Kuchera, Ashton Ruhland, and Helen Lamberty. Bill and Lori Sliker, Jack and Jean Garcia, and Francis Lamberty as well even though some are no longer with us. To Lauren Madson, Mal Cramer, and Alex Buhler, although not blood-related, are like sisters to me. The

encouragement you guys have provided me goes beyond words. I couldn't have done it without you guys. Additional thanks to members of the Gensler and Monty families, who as my godparents, have watched me grow up and provided even more encouragement throughout all that I have done.

Final thanks is extended to Misty Pocwierz-Gaines and other administrative members of the Eppley Institute as well as to our lab funding especially the T32 Training Grant for supporting me and daily life in the lab.

EFFECTS OF HUMAN LEUKOCYTE ANTIGEN (HLA) CLASS I MOLECULE COMPONENTS ON PANCREATIC CANCER CELL MIGRATION

Bailee Helena Lamberty Sliker, Ph.D.

University of Nebraska, 2019

Supervisor: Joyce C. Solheim, Ph.D.

Human leukocyte antigen (HLA) class I molecules are composed of a light chain (beta 2-microglobulin (β_2m)) and HLA heavy chain. The heavy chains of these molecules have three different isotypes (–A, -B, and –C) and are highly polymorphic with thousands of sequence variations termed allotypes. The best-known role for these molecules is within the immune system, however, recent research implicates components of this molecule can function outside of this known immune role by contributing to cell migration. However, no studies have been published thus far investigating this non-immune function in pancreatic cancer. Therefore, I examined the role of HLA class I molecule components in pancreatic cancer cell migration.

My studies showed that when β_2m was knocked down using β_2m-specific siRNA, cell migration decreased in the S2-013 and PANC-1 cells but increased in the MIA PaCa-2 cells. Interactions with amyloid precursor-like protein 2 (APLP2) as well as total APLP2 protein expression are potential mediators of these effects. HLA-B knockdown using HLA-B-specific siRNA increased cell migration in S2-013 cells, while in both the PANC-1 and MIA PaCa-2 cells knockdown decreased migration. Alterations in integrin beta 1 (ITGB1) and focal adhesion kinase (FAK) are potential intermediates in HLA-B's effects on cell migration. Furthermore, knockdown using allotype-specific siRNA of HLA-A24 in the S2-013 cell line and HLA-A2 in PANC-1 cell line increased migration while knockdown of HLA-A2 in the S2-013 cell line and HLA-A24 in the MIA PaCa-2 cell line

reduced cell migration. Mechanistically, APLP2 C-terminal fragment (APLP2-CT) and epidermal growth factor receptor (EGFR) are likely mediators of this phenotype. Finally, the histone deacetylase (HDAC) inhibitor M344 and its effects on APLP2 processing in the S2-013 pancreatic cancer cell line was interrogated as previous studies indicated M344 treatment reduced levels of β-site APP cleaving enzyme 1 (BACE1), a proteolytic enzyme involved in APLP2 cleavage. M344 treatment of S2-013 cells led to a significant reduction in cell proliferation, migration, and APLP2-CT expression. Overall, this work sheds light on a novel role for the HLA class I molecules in pancreatic cancer outside of their known immune function and implicates M344 as a potential new therapeutic for this lethal disease.

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LIST OF ABBREVIATIONS

AICD	APP intracellular domain
APLP1	amyloid precursor-like protein 1
APLP2	amyloid precursor-like protein 2
APP	amyloid precursor protein
BACE1	beta secretase 1
CD	cluster of differentiation
CDK	cyclin-dependent kinase
CHO	Chinese hamster ovary
CIITA	class II MHC transactivator
CREB	cAMP response element binding
CT	C-terminal fragment
DC	dendritic cell
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
ERK	extracellular signal related
FAK	focal adhesion kinase
FcRN	fetal neonatal receptor
FGFR	fibroblast growth factor receptor
GAG	glycosaminoglycan
HDAC	histone deacetylase
HFE	hemostatic iron regulator

HFF	human foreskin fibroblast
HLA	human leukocyte antigen
HNC	head and neck cancer
IFN	interferon
IGF-1R	insulin-like growth factor receptor 1
IR	insulin receptor
ITGA	integrin alpha chain
ITGB	integrin beta chain
KIR	killer cell immunoglobulin-like receptor
KO	knock-out
LILR	leukocyte immunoglobulin-like receptor
MHC	major histocompatibility complex
MoDC	monocyte-derived dendritic cell
mRNA	messenger RNA
NK	natural killer
PI3K	phosphoinositide 3-kinases
PKA	protein kinase A
PLC	peptide loading complex
siRNA	short interfering siRNA
TAP	transporter associated with antigen processing
TCR	T cell receptor
TGF-β	transforming growth factor β
TH1	T helper 1
VEGF	vascular endothelial growth factor
β_2m	beta 2-microglobulin

CHAPTER 1: Introduction to the human leukocyte antigen (HLA) class I molecule

1.1 HLA class I molecule structure

The major histocompatibility complex (MHC) class I molecule in its human form is also termed the HLA class I molecule. The HLA class I molecule is present on nearly all nucleated cells and is composed of three main components (**Figure 1.1**). The first is the heavy alpha (α) chain (encoded on human chromosome 6) and the second is the small soluble beta 2-microglobulin (β_2m) that is denoted as the light chain of this structure and is encoded on chromosome 15. The final component is the peptide that is being presented, which sits in the peptide groove of the heavy chain.

The HLA class I alpha chain is composed of an α_1 and α_2 domain that form the peptide binding cleft as well as an α_3 domain, transmembrane helix, and cytoplasmic domain. β_2m, which is highly conserved in all vertebrates, is non-covalently attached to the HLA class I heavy chain. The HLA class I molecule heavy chain is known for its highly polymorphic nature and there are three main heavy chain isotypes that occur in the human population which include HLA-A, HLA-B, and HLA-C. Due to the high number of polymorphisms of these molecules, there are thousands of different possibilities for these molecules termed allotypes that include the specific sequences present in each individual (**Table 1.1**). Examples of allotypes include HLA-A2, HLA-A24, HLA-B7, and HLA-B27. Further still, sequencing has found that there are even further variations of these allotypes and these numbers can be taken out to four digits to further differentiate the variations within this molecule. Each individual inherits up to 6 different heavy chain alleles from their parents (usually 3 alleles from each parent) and this is termed a haplotype. β_2m, on the other hand, does not share this high rate of polymorphism, and there is only one human β_2m allele. These three aforementioned heavy chain isotypes and their accompanying variations are termed the classical MHC molecules while there are also non-classical forms of this molecule, which include HLA-

Figure 1.1. *Components of the HLA class I molecule.* Present on all nucleated cells, HLA class I molecules are composed of a heavy chain that has α1, α2, and α3 domains indicated in blue, transmembrane/cytoplasmic domains shown in black, a light chain (β_2m) indicated in green, and endogenous antigenic peptide indicated in orange.

Figure 1.1.

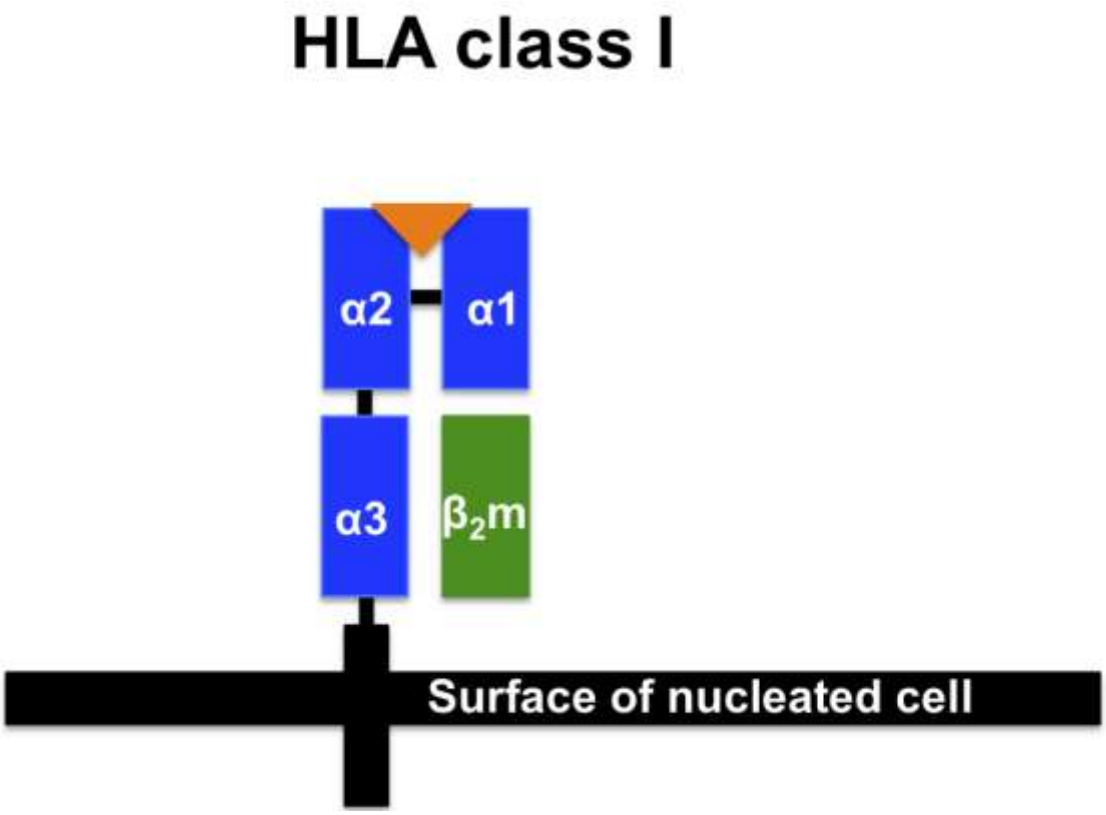


Table 1.1. *Table of the number of unique sequences for the HLA class I heavy chain isotypes present in the human population.*

Data taken from IPD-IMGT/HLA.

Table 1.1.

Genetic Locus	HLA-A	HLA-B	HLA-C
Alleles	4,846	5,881	4,654
Proteins	3,286	4,088	3,070

G and HLA-E among others. For the purposes of this dissertation, we will be focusing on the classical MHC molecules (Peaper and Cresswell 2008).

1.2 HLA class I molecule and its immune system function

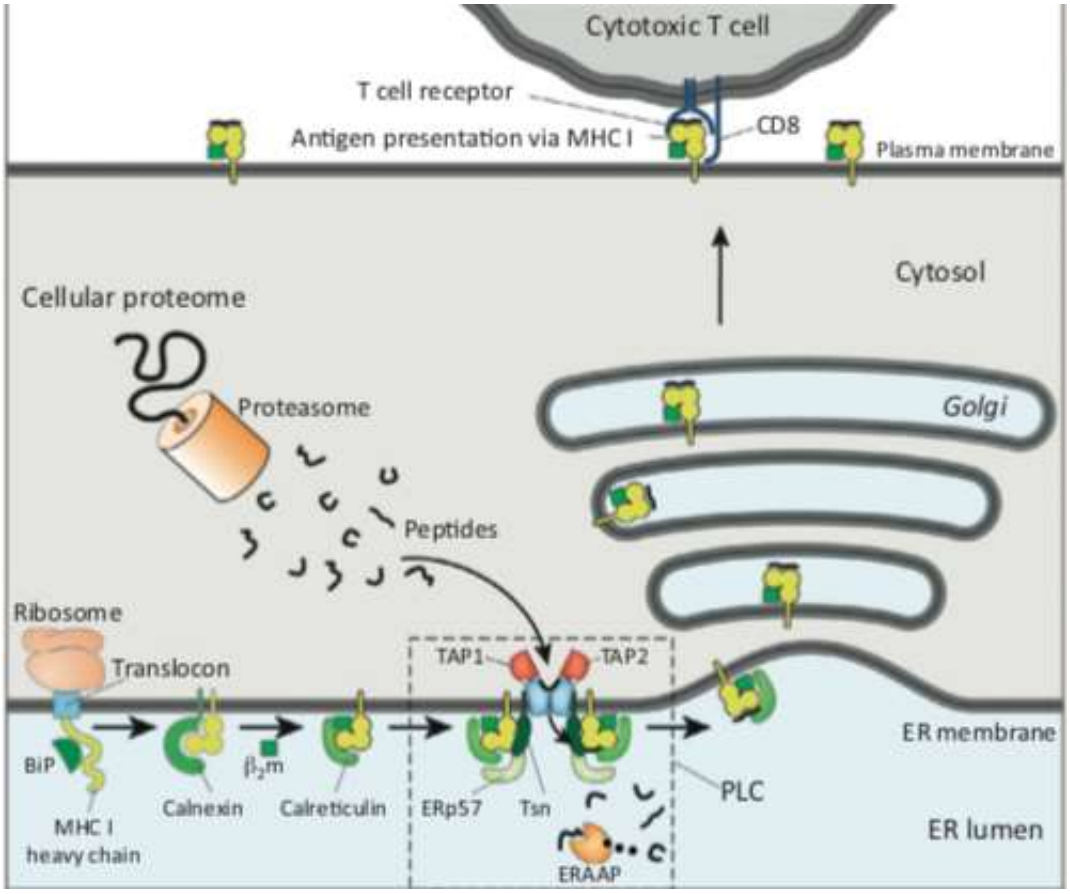
One of the main functions of the HLA class I molecule is its role in the adaptive immune system where it presents endogenous peptides to cytotoxic T cells. It does this with the help of the peptide-loading complex (PLC), which performs the general function of loading peptide onto the HLA class I molecule. The PLC also functions to perform quality control on the newly assembled HLA-peptide complexes. Peptides derived from intracellular sources are degraded by the cytosolic proteasome-ubiquitin system and are transported into the endoplasmic reticulum (ER) lumen by the transporter associated with antigen-processing (TAP) protein. Once the peptides are in the lumen of the ER, they are trimmed to a length of 8 or 9 amino acids in length by aminopeptidases. Newly formed HLA heavy chains first bind to calnexin until they begin to fold and associate with β_2m. The chaperones tapasin, calreticulin, and ERp57 work to stabilize the nascent HLA protein and this allows for proper loading of peptide into the HLA peptide binding groove as well as for association with β_2m. Once the peptide has been loaded, the complex is released and shuttled through the Golgi to the cell surface for peptide presentation. Any disruption in any of these PLC components leads to inefficient peptide presentation by HLA class I molecules. Any misfolding that occurs with the HLA class I molecule also leads to their retention in the ER until correct folding or degradation can occur (Peaper and Cresswell 2008). Of note, it has not been fully elucidated how the HLA class I molecules traffic to the cell surface but a few theories exist regarding how this happens (**Figure 1.2**) (Hulpke and Tempe 2013; Springer 2015).

Figure 1.2. *Overall schematic of peptide presentation pipeline for the HLA class I molecule.* Generally, peptides are degraded by the proteasome and loaded onto the HLA class I molecule. After peptide is loaded into the peptide-binding groove, the HLA class I molecule is shuttled to the cell surface for peptide presentation to the immune system.

Figure from:

Hulpke S, Tampe R. The MHC I loading complex: a multitasking machinery in adaptive immunity. Trends in biochemical sciences. 2013; 38(8):412-420.

Figure 1.2.


Cytotoxic T cell
T cell receptor
CD8
Antigen presentation via MHC I
Plasma membrane
Cytosol
Cellular proteome
Proteasome
Golgi
Peptides
Ribosome
Translocon
TAP1
TAP2
ER membrane
BiP
β2m
PLC
Calnexin
Calreticulin
ERp57
Tsn
MHC I
heavy chain
ERAAP
ER lumen

The best-studied function for the HLA class I molecules is their role within the immune system in which they allow cytotoxic T cells to eradicate cells that are harboring foreign, mutated, or even in some cases overexpressed, proteins. These molecules allow T cells to monitor the inside of cells and to lyse virally infected cells or cancer cells by displaying fragments of representative proteins from within the cell. When a T cell finds a peptide that it does not recognize located within the peptide groove of these molecules, it lyses this target cell (**Figure 1.3 and 1.4**). Peptides that bind the groove of the particular MHC class I heavy chain are bound both at the C-terminal and N-terminal ends with key anchor residues also present at a few locations in the sequence. The highly polymorphic nature of HLA class I molecules affects the peptide binding groove leading to enhancement of the peptide repertoire that can be presented. This supports each heavy chain allotype having the ability to bind to a different array of peptides and leads to the maximization of the number of antigenic peptides that individuals can present (Wieczorek et al., 2017).

HLA class I molecules are also ligands for natural killer (NK) cells and are recognized by their killer cell immunoglobulin-like receptors (KIR) as well as other leukocyte immunoglobulin-like receptors (LILR). These receptors are also highly polymorphic, therefore, they recognize specific HLA class I molecules. Specifically, KIRs recognize specific α_1 and α_2 domains, whereas LILRs have much broader specificity due to their recognition of β_2m and α_3 (Li and Raghaven 2010). Thus, by their interactions with NK cell receptors, as well as by their interactions with T cell receptors, HLA class I molecules have vital functions in immunity.

1.3 The HLA class I light chain (β_2m)

Figure 1.3. *Structural diagram of peptide loaded into the peptide-binding groove of the HLA class I molecule.* Diagram of the HLA-A2 molecule (α1, α2, and α3 domains of the heavy chain indicated by blue ribbons and β_2m shown in cyan ribbons) with peptide (red) in the peptide-binding groove. Green and yellow ribbons are indicative of the variable domains of the antigen-specific T cell receptor (TCR).

Figure from:

Flutter B, Gao B. MHC class I antigen presentation-recently trimmed and well presented. Cellular and Molecular Immunology. 2004; 1(1):22-30.

Figure 1.3.

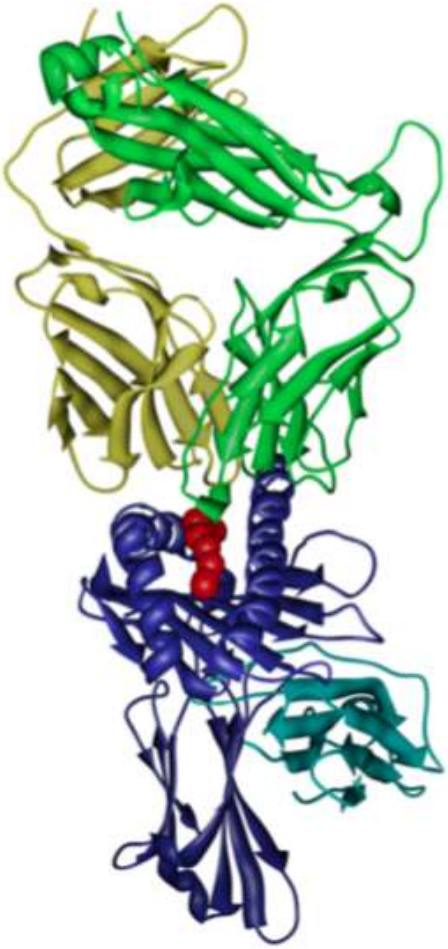
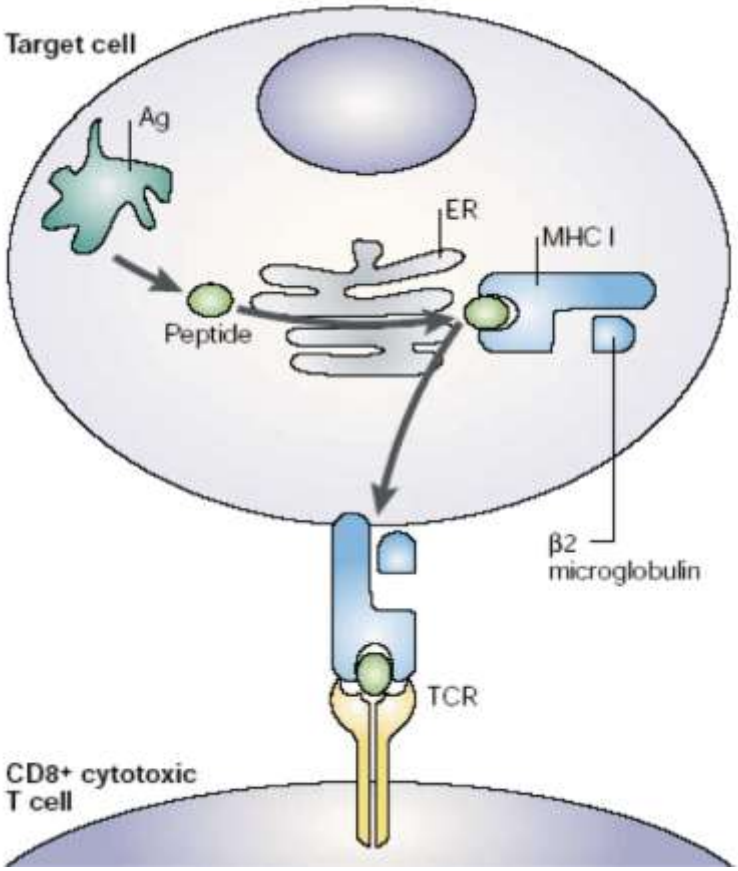


Figure 1.4. *HLA class I molecules present peptide to cytotoxic CD8+ T cells.* HLA class I molecules are best known for their role within the immune system where they present endogenous peptides to $CD8^+$ T cells. Recognition of peptide by the T cell then leads to lysis of the target cell. This function is used as a mechanism for protection against viral infection and malignant transformation.

Figure from:

Albert ML, Darnell RB. Paraneoplastic neurological degenerations: keys to tumour immunity. Nat Rev Cancer. 2004;4(1):36-44.

Figure 1.4.


Target cell
Ag
ER
MHC I
Peptide
β2
microglobulin
TCR
CD8+ cytotoxic
T cell

β_2m, the non-glycosylated invariant light chain of the HLA class I molecule, has no known variants in humans (Bjorkman and Parham 1990; Townsend et al., 1989). This protein is highly conserved among vertebrate organisms with human and mouse β_2m sharing 70% sequence similarity (Cox et al., 1982). There is no transmembrane region of this protein and it is non-covalently bound to the α chain of the HLA class I molecule, therefore, it is able to be exchanged with free β_2m in bodily fluids such as serum and blood (Bjorkman et al., 1987; Hyafil and Strominger 1979; Parker and Strominger 1985). Additionally, this protein is also present in urine and is processed through the kidneys (Karlsson et al, 1980). The β_2m protein has two tryptophans that are crucial for its function, including Trp60 which helps bind to the HLA class I molecule and Trp95 which contributes to protein stability (Esposito et al., 2008; Ricagno et al., 2008; Valleix et al., 2012).

β_2m-deficient mice are viable and are not sterile, however, they do exhibit some immune system dysfunctions. It has been found with deletion of β_2m that there is a degradation and subsequent low expression of misfolded HLA class I molecules, especially at the cell surface, due to the retention of the heavy chains in the endoplasmic reticulum. The association of β_2m with the class I heavy chain occurs very early during folding, thus the absence of β_2m precludes all of the folding and assembly steps, contributing to the lack of HLA class I expression that is observed when β_2m is deleted or mutated (Hoglund et al., 1998). Therefore, cells lacking β_2m express very low levels of HLA class I complexes on their surface, which impedes cellular immune response (Bix and Raulet 1992). Furthermore, β_2m-knockout mice have been found to have severely impaired CD8-positive T cell immunity; however, they are still able to generate CD4-positive T cell activity (Zijlstra et al., 1990). Additionally, NK cell number is normal but NK cells have impaired functions in these mice (Ardeniz et al., 2015). In a study of patients with low β_2m expression, it was found that those with heterozygous expression

do not experience as severe a phenotype as those with a homozygous mutation/deletion (Ardeniz et al., 2015). Conversely, when β_2m is overexpressed, there is an inhibition of the yield of monocyte-derived dendritic cells (MoDCs) and alterations in the cytokine secretion profiles of DCs, as well as compromised capacity of MoDCs to present antigens to activated T cells (Xie et al., 2003).

β_2m also non-covalently interacts with other molecules besides the HLA class I molecule and this includes many of the non-classical MHC class I molecules such as cluster of differentiation 1 (CD1), neonatal Fc receptor (FcRn), and hemostatic iron regulator (HFE), among others (Li et al., 2016). This interaction of β_2m with the non-classical MHC class I molecules leads to more problems for humans that lack this protein as compared to those that have loss of HLA class I molecules alone (Ardeniz et al., 2015). These additional issues include iron overload (due to unstable HFE protein) as well as increased catabolism of IgG and albumin due to the low expression of the FcRn (**Figure 1.5**) (Christianson et al., 1997; Ehrlich and Lemonnier 2000; Enns 2001; Schaible et al., 2002).

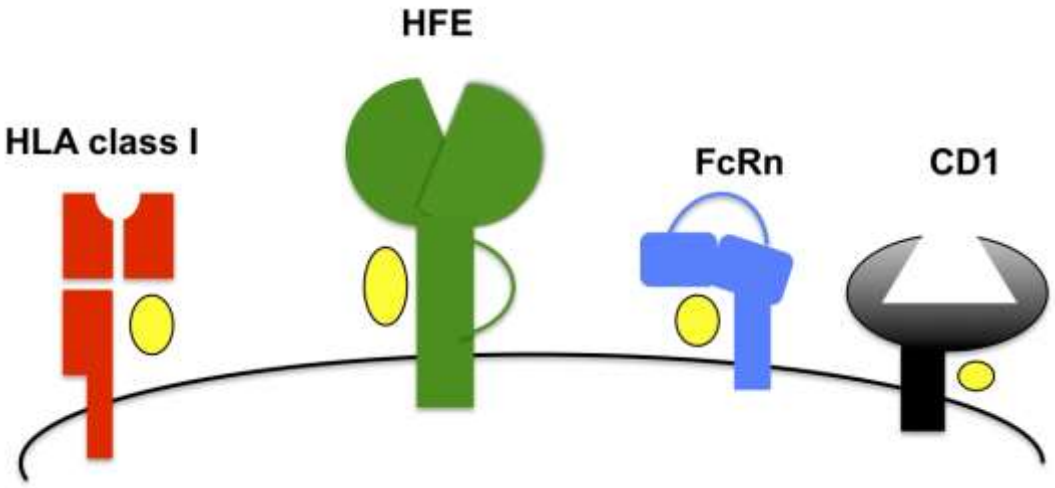
1.4 HLA class I components in cancer

1.4.1 β_2m and cancer

Studies investigating the role of β_2m in the phenotypes of cancer cells have used models of breast cancer, oral squamous cell carcinoma, renal cell carcinoma, colon cancer, prostate cancer, ovarian cancer, gastric cancers, and esophageal cancer, as well as multiple subsets of leukemia and lymphoma (Bossard et al., 2012; Chen et al., 2008; Cooper and Plesner 1980; Huang et al., 2006; Josson et al., 2011; Li et al., 2014; Molica et al., 1999; Nomura et al., 2006; Tanaka et al., 2012). Overall, in these particular types of tumors, high expression of β_2m in the cancer cells is generally

Figure 1.5. *β2m is the light chain for classical HLA class I molecules as well as other non-classical MHC molecules.* β_2m (indicated in yellow) has been found to interact not only with the classical HLA class I molecules (HLA-A, -B, and –C) but also some of the non-classical HLA molecules as well. This can cause additional defects that are observed in β_2m knockout mice.

Figure 1.5.

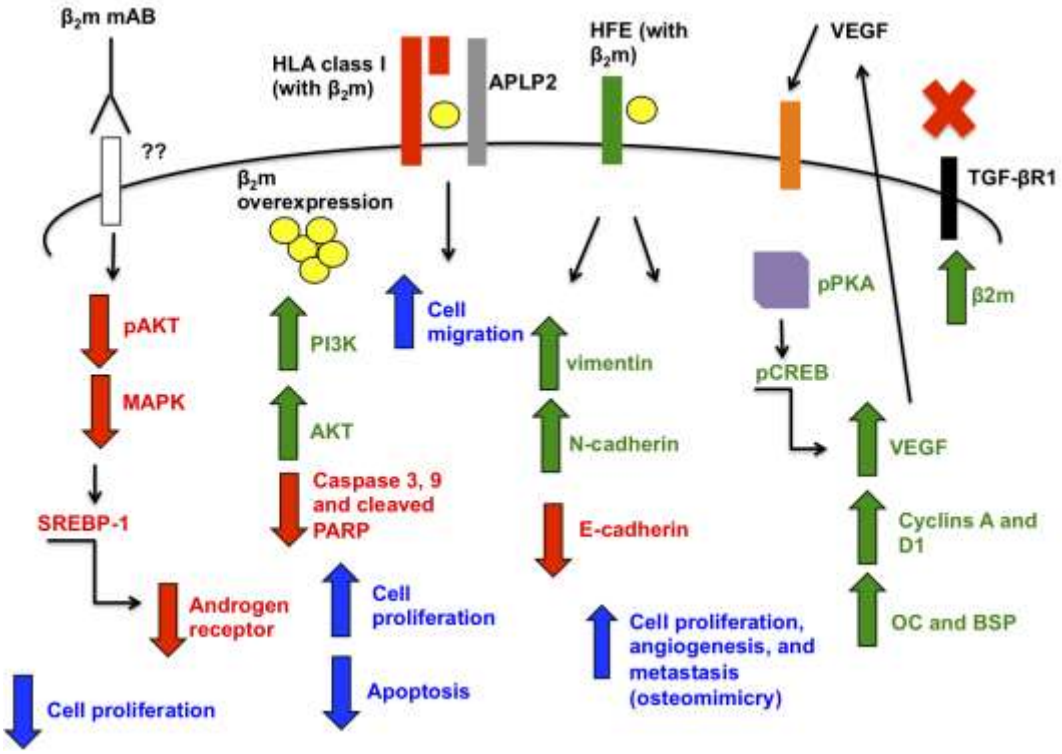


correlated with worse survival for these patients, although its effects have been found to be cell type specific. In addition to expression data, functional studies have also been performed to investigate how β_2m contributes to disease progression. It was found that β_2m plays a role in promoting cell migration and proliferation, and in preventing apoptosis, and that loss of this protein by experimental mechanisms leads to a reduction in these processes. This phenotype holds true both *in vitro* and *in vivo*. Mechanistically, these effects were caused by various pathways dependent on cancer cell type (Bossard et al., 2012; Chen et al., 2008; Cooper and Plesner 1980; Huang et al., 2006; Josson et al., 2011; Li et al., 2014; Molica et al., 1999; Nomura et al., 2006; Tanaka et al., 2012). In prostate cancer and renal cell carcinoma, it was found that β_2m causes an upregulation of p-PKA and p-CREB leading to VEGF production (Josson et al., 2011; Nomura et al., 2006). Additionally, epithelial-mesencymal transition (EMT) markers are upregulated in these models, and induction of the ERK and Akt pathways has also been noted as being upregulated in cells with overexpression of β_2m (Josson et al., 2011). Furthermore, in ovarian cancer models, the effects of β_2m were found to be correlated with the TGF-β signaling pathway (**Figure 1.6**) (Sun et al., 2016).

Similarly, the serum of cancer patients has been studied for the presence of β_2m. It has been found that patients with pancreatic cancer, breast cancer, kidney cancer, prostate cancer, and various types of leukemia and lymphoma have high expression of β_2m in their serum and β_2m has been suggested as a potential biomarker for these diseases (Gross et al., 2007; Gulec et al., 2012; Hogdall et al., 2010; Lucarelli et al., 2014; Petekkaya et al., 2014; Petekkaya et al., 2017; Pezelli et al., 1995; Prizment et al., 2016; Rashid et al., 1980). However, as the literature stands now, there are no functional or mechanistic studies that have further investigated how β_2m present in the serum of patients might have any disease significance.

Figure 1.6. *β2m has been implicated in many signaling pathways important to cancer.* β_2m has been found to play a role in many different pathways in various cancers. Many of the pathways in which it is implicated lead to increased cell proliferation and migration with a reduction in apoptosis as β_2m generally leads to cancer progression. Red arrows indicate a decrease in the expression of downstream signaling molecules by β_2m. Green arrows indicate an increase in the expression of downstream signaling molecules by β_2m. Blue arrows indicate the functional phenotypes that are altered as a results of changes due to β_2m expression or antibody binding.

Figure 1.6.


β2m mAB
??
HLA class I (with β2m)
APLP2
HFE (with β2m)
VEGF
TGF-βR1
β2m overexpression
pAKT
MAPK
SREBP-1
Androgen receptor
Cell proliferation
PI3K
AKT
Caspase 3, 9 and cleaved PARP
Cell migration
Cell proliferation
Apoptosis
vimentin
N-cadherin
E-cadherin
Cell proliferation, angiogenesis, and metastasis (osteomimicry)
pPKA
pCREB
β2m
VEGF
Cyclins A and D1
OC and BSP

1.4.2 HLA class I heavy chains and cancer

Many studies have been done looking at the role that HLA class I molecules play in cancer, specifically in regards to their function within the immune system and the immune escape of various types of cancers. In a large variety of cancers, it has been found that total HLA class I expression is reduced at the cell surface to protect cells from CD8+ T cell recognition and subsequent lysis. Many studies have indicated that high expression of HLA class I molecules in this context leads to improved survival, due to functionality of the immune system in removing cancer cells (Garrido and Ruiz Cabello 1991; Garrido et al., 1993; Ruiz Cabello et al., 1991). Further studies have found that most tumors start out as HLA class I positive, and as the disease progresses, the expression of these molecules on some tumor cells is significantly reduced (Garrido et al., 2017). This can be due to four main ways of downregulation, which include total HLA class I loss, HLA haplotype loss, HLA class I locus (A, B, or C) selective loss, or HLA class I allelic loss. These occur as a result of "hard" or "soft" genetic alterations, referring to their ability to be reversed. "Hard" alterations include point mutations or large deletions of DNA segments that can affect either β_2m or the HLA heavy chains, while "soft" alterations include transcriptional downregulation of these proteins or other proteins within the APC. These "soft" lesions can be reversed using appropriate cytokines while "hard" lesions are irreversible through these mechanisms. Haplotype loss, if it occurs, is usually a "hard" alteration, as chromosome 6 loss is the most common mechanism for this, while locus-selective loss is typically a "soft" alteration that can be restored if cells are treated with IFN-γ or other cytokines associated with T helper 1 (TH1) or an adaptive immune response. Compound phenotypes of these alterations are also frequently observed in cancer cells, as well as an overall unresponsiveness to IFN that prevents the cell's capacity to upregulate HLA class I molecules. The overall frequency of alterations to this molecule in cancer is highly variable and can range from

15-93% and can be heavily cell type dependent. Overall, studies indicate that tumors start out with positive HLA class I expression and as they progress, HLA class I expression in some cases become heterogeneous, finally ending up with negative HLA class I expression, likely as a function of immune selection. Granuloma-like formation around the tumor (immune cells surrounding the tumor but not infiltrating) occurs after HLA class I expression is lost. In addition to immune selection, it has also been shown that some cellular oncogene products can also cause a decrease in HLA class I expression as a means of escape. Furthermore, a strong, direct correlation has been found between high tumor HLA class I expression and regression of the metastasis. Those lesions that progressed were harboring defects in HLA class I that were irreversible (Aptsiauri et al., 2018; Garrido et al., 2017).

On the other hand, there have also been some studies that indicate the expression of HLA class I molecules may lead to worse cancer prognosis. In models of uveal melanoma, breast cancer, gastric cancer, esophageal cancer, medulloblastoma, and lung cancer, high expression of the HLA class I molecule led to worse prognosis in these patients while low expression was correlated with a better prognosis. Additionally, these patients experienced increased tumor stage and grade as well as more recurrence and metastasis (Madjd et al., 2005; Ramnath et al., 2006; Ueda et al., 2008). These studies hint at an additional role for the HLA class I molecule in cancers and other diseases.

1.5 HLA class I molecules and their role beyond the immune system

There has been increasing evidence that HLA class I molecules may also be playing a role beyond their immune function. This is illustrated by studies that indicate interactions with growth receptors may occur or that dimers of these molecules exist

under certain conditions. Additionally, it has been found in models of transplantation biology that HLA class I molecules can affect cell proliferation, migration, and apoptosis, likely through interaction with surface receptors as HLA class I molecules, even though they have phosphorylation sites on their cytoplasmic tails, are not able to induce intracellular signaling alone (Gur et al., 1990; Tsai and Reed 2014). The interactions that have been found to occur between the HLA class I molecule and various growth factor/surface receptors in cell types such as Chinese hamster ovary (CHO) cells, murine liver cells, and B lymphoblasts include interactions with insulin and insulin like growth factor receptor (IR and IGF-1R respectively), epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and integrins, specifically integrin beta 4 (ITGB4) (Schreiber et al., 1984; Trajkovic-Arsic et al., 2013; Tsai and Reed 2014; Zhang et al., 2010). Furthermore, cross-linking of HLA class I molecules can activate intracellular signaling cascades and certain allotypes of HLA class I molecules (e.g., HLA-B27) can form dimers with one another that contribute to disease such as ankylosing spondylitis (Campbell et al., 2012).

One of the first receptor systems that was studied in relation to interaction with the HLA class I molecule was the IR/IGF-1R system (Trajkovic-Arsic et al., 2013). Previous studies have indicated that the interactions between these receptors and HLA class I molecules have been found to occur in a variety of cell lines and that these interactions subsequently alter the downstream signaling pathways that become activated upon stimulation of these receptors (Cousin et al., 1987; Hsu and Olefsky 1993; Kittur et al., 1987; Samson et al., 1986). These downstream signaling pathways are affected through the ability of the HLA class I molecule to increase ligand sensitivity of these receptors as well as to alter receptor internalization, as found in B lymphoblasts, human and murine liver cells, and Chinese hamster ovary (CHO) cells (Cousin et al., 1987; Hsu and Olefsky 1993; Kittur et al., 1987; Samson et al., 1986). The

autophosphorylation of IGF and insulin receptors is also affected by HLA class I molecules, providing another mode of action for these molecules to affect downstream signaling pathways (Hansen et al., 1989; Ramalingam et al., 1997; Reiland and Edidin 1993; Stagsted et al., 1990; Stagsted et al., 1993). Additional research has also indicated that this interaction is likely to occur through the HLA class I α1 domain (through the use of a murine α1 peptide). Furthermore, it has been demonstrated that β_2m is able to associate with these receptors, indicating that β_2m could be necessary for these interactions and downstream signaling effects to occur (Centrella et al., 1989; Hsu and Olfesky 1993; Olson et al., 1994; Stagsted et al., 1990).

EGFR is another receptor that has been reported to interact with HLA class I molecules. This interaction was observed in A431 epidermoid carcinoma cells as well as in normal human fibroblast cells. Treatment of these cells with antibody (either monoclonal or polyclonal) against HLA class I molecules (pan-HLA class I antibody) reduced the surface display of EGFR, while binding of EGF to EGFR in turn inhibited the binding of the HLA class I antibody. Furthermore, it was noted that anti-HLA class I antibodies reduced the ability of EGF to bind to both A431 epidermoid cells and human foreskin fibroblasts (HFF) by reducing the number of receptors available for it to bind. This was shown to be an immediate effect. Interestingly, fixed cells did not display this phenomenon, and therefore this study postulated that a metabolic requirement could be at play. Further experiments indicated that divalent antibodies were needed, suggesting that cross-linking of the HLA class I molecule was necessary for this process to occur. However, use of inhibitors against patching/capping did not block these effects of HLA class I antibodies on EGF binding. Therefore, this led to the idea that HLA internalization was not leading to EGFR internalization, but likely that there is a conformational change in EGFR that affects the ability to bind to ligand (Schreiber et al., 1984).

Interestingly, it has also been found that EGFR has the ability to regulate the expression of HLA class I molecules on the cell surface, consistent with some oncogenes being able to control HLA class I expression in cancer cells. Cetuximab-mediated inhibition of EGFR in a model of head and neck cancer (HNC) led to an upregulation of HLA expression (which is more prominently seen with HLA-B/C than HLA-A), implying that the functionality of EGFR leads to down-regulation of HLA class I expression (Srivastava et al., 2015). A further study showed this same phenotype and found that these alterations in HLA class I expression may be due to effects of EGFR signaling on the expression of CIITA mRNA, which has direct effects on the promoters of both HLA class I and class II genes. It was found that the mRNA levels of CIITA were modulated by this EGFR inhibitor treatment, leading to this speculation. Further studies have confirmed this phenotype in multiple cancer types, including mesothelioma, lung cancer, gastric cancer, and esophageal cancer (Pollack et al., 2011).

An additional receptor with which HLA class I molecules have been found to interact is FGFR. This interaction was found to occur in transplantation biology models using endothelial cells and smooth muscle cells. Studies have found that when cells were treated with HLA class I antibodies, ligation of HLA class I molecules occurred. Additionally, upon ligation, there was an induction in the expression of FGFR, as well as increased binding of its ligand bFGF (Bian et al., 1998; Harris et al., 1997). This induction of FGFR expression and ligand binding in turn led to an increase in downstream signaling, ultimately leading to an increase in cell proliferation (Jin et al., 2005). It was further shown that treatment with a pan-HLA class I antibody was able to stimulate cell proliferation at a greater rate as compared to antibodies against either HLA-A or HLA-B alone (Bian et al., 1997; Bian et al., 1998; Harris et al., 1997). This indicates that the intensity of signal transduction is related to the total number of class I molecules on the cell surface and is likely not isotype specific. Further studies from this

same group investigated the downstream signaling that occurs upon treatment with HLA class I antibodies and the effect that this has on FGFR signaling. It was found that soon after treating endothelial cells with a pan-HLA class I antibody (within one minute post-treatment), there was an increase in the tyrosine phosphorylation of the proteins Src and Fyn, which also led to the phosphorylation of the protein p70 (Jin et al., 2002). This is important because it was shown previously that recruitment of these molecules usually happens upon growth factor or integrin stimulation (Jin et al., 2002). Additionally, in this same study, the molecules FAK and paxillin were found affected by Src inhibition, specifically the inhibition of Src prevented the subsequent tyrosine phosphorylation of these molecules. Downstream of FAK and paxillin, the actin cytoskeleton was indicated as being crucial in the signaling process (Jin et al., 2002). Later studies determined that the actin cytoskeletal proteins Rho and Rho-kinase were activated and increased stress fiber formation occurs upon HLA class I molecule ligation, indicating their involvement downstream of HLA class I molecule ligation by antibodies (Jin et al., 2005). Furthermore, pan-HLA class I antibody concentration and level of HLA class I surface expression also dictates the transduction of certain downstream signals. High levels of pan-HLA class I antibody trigger FGFR expression and lead to increased cell proliferation, while lower concentrations alternatively stimulate PI3K/AKt signaling and lead to the upregulation of anti-apoptotic proteins (Jindra et al., 2006). Overall, the data indicate that HLA class I clustering, mediated by the antibodies against these molecules, leads to activation of FGFR, which promotes stress fiber formation and cell proliferation through a mechanism of enhanced tyrosine phosphorylation of Src, FAK, and paxillin.

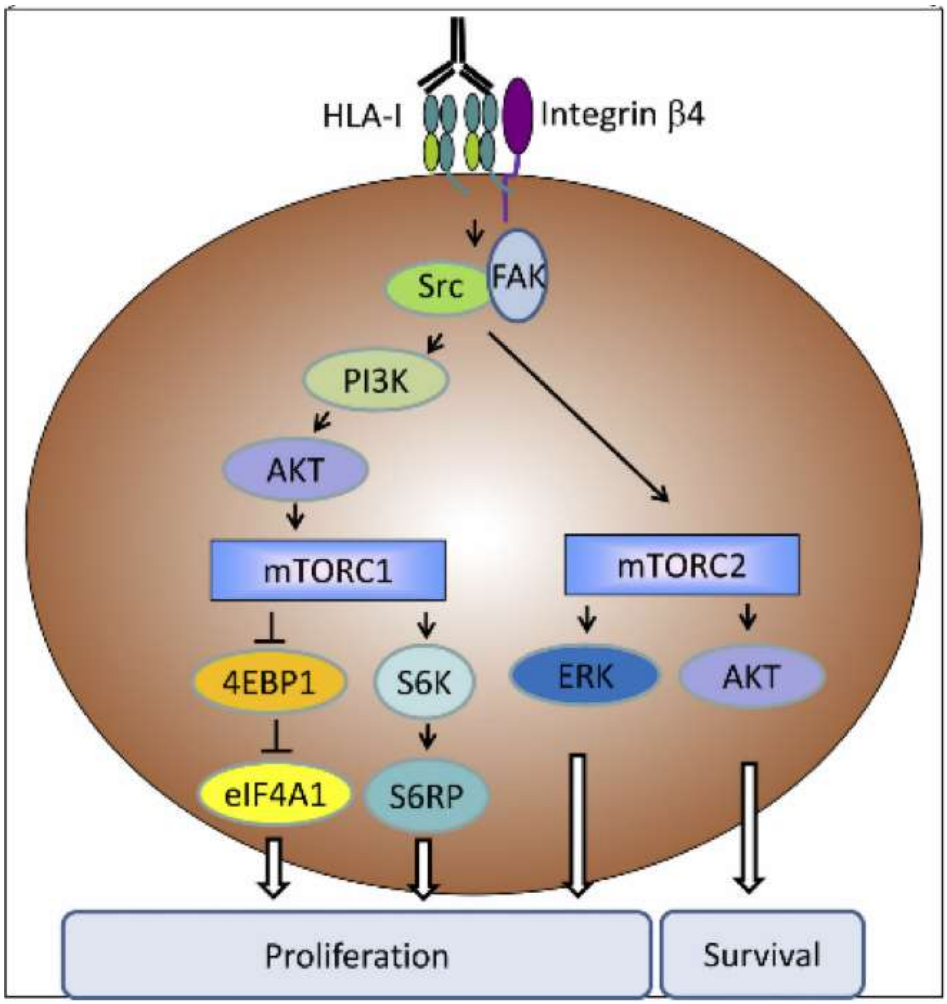
In addition to interactions with FGFR, transplantation biology models have also shown that interactions between HLA class I molecules and ITGB4 occur (**Figure 1.7**). These studies indicated that this interaction was likely occurring via the cytoplasmic tail

Figure 1.7. *Schematic of one of the signaling pathways in which HLA class I molecules are involved.* In transplantation biology models, it has been shown that HLA class I molecules interact with ITGB4 and lead to downstream signaling changes that can influence cell proliferation and survival.

Figure from:

Zhang X, Reed EF. HLA class I: an unexpected role in integrin β4 signaling in endothelial cells. Hum Immunol. 2012;73(12):1239-1244.

Figure 1.7.


HLA-I
Integrin β4
Src
FAK
PI3K
AKT
mTORC1
mTORC2
4EBP1
S6K
ERK
AKT
eIF4A1
S6RP
Proliferation
Survival

of the HLA class I molecule and that deletion of the cytoplasmic domain of this molecule impaired downstream signaling. When ITGB4 was knocked down in this endothelial cell model of transplantation, there was an abrogation of the ability of HLA class I to stimulate the phosphorylation of Akt, ERK, and Src as well as to affect cell proliferation (Zhang et al., 2010). The opposite trend was seen when the amount of HLA class I was reduced by siRNA transfection, whereby the ability of ITGB4 to stimulate the phosphorylation of ERK and the migration of cells on laminin-5 was reduced. Additionally, when using siRNA to knock down total HLA class I molecules, β_2m, or ITGB4, there was a decrease in the migration of these endothelial cells (**Figure 1.8**). These studies also indicate that these effects involved cis interactions between HLA class I and ITGB4 (Zhang et al., 2010). ITGB4-mediated migration was found to be dependent upon its interactions with HLA class I as well. It is possible then that the targeting of treatment to block the interactions between ITGB4 and HLA class I might lead to better therapeutic strategies in transplant rejection. It has been hypothesized that this may also apply to cancers, since ITGB4 has been implicated in the progression of many cancer types (Zhang and Reed 2012). The different isotypes of the HLA class I molecule are differentially expressed on various tissue types, with certain combinations of isotypes being more prevalent on some cell types compared to others. Whether or not HLA class I isotypes or various allotypes differ in their binding to growth factors or downstream signaling capabilities has not yet been fully elucidated, although some preliminary data have indicated that this may in fact be the case (Jin et al., 2011).

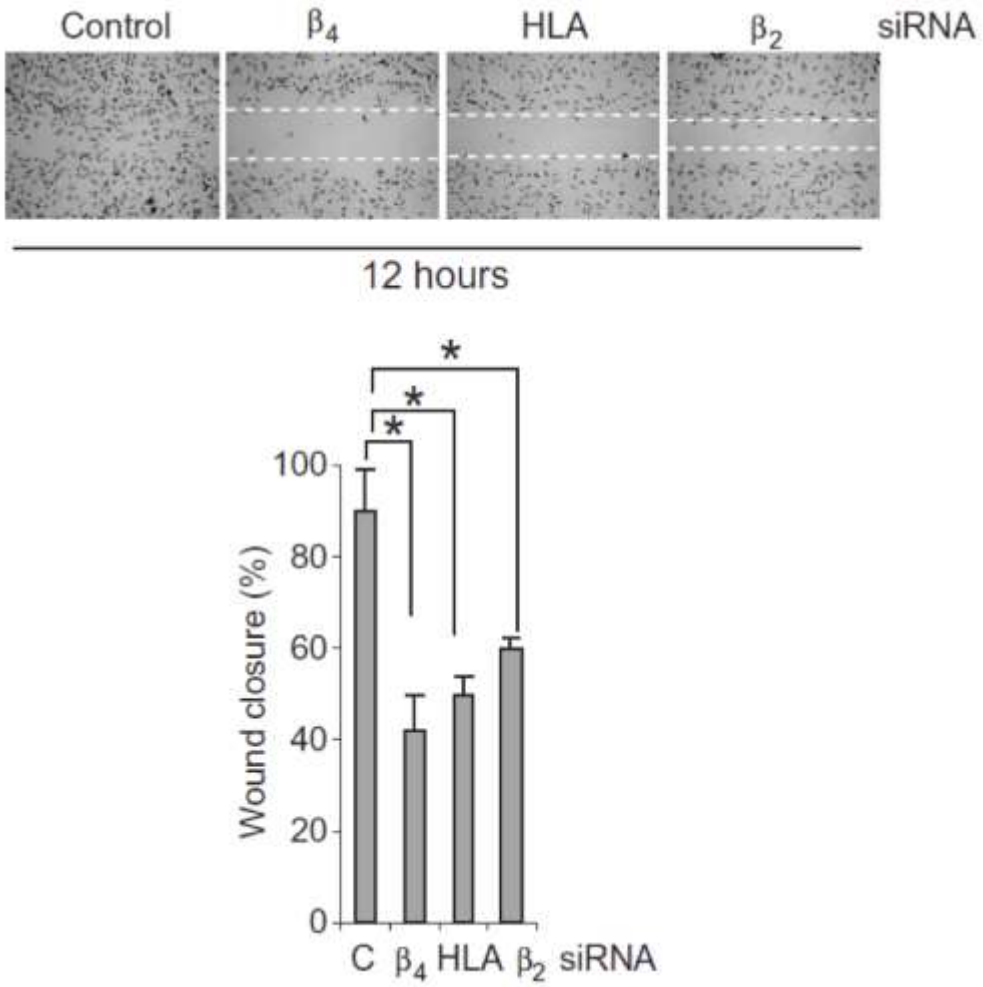
In addition to binding to other growth factor receptors, certain allotypes of the HLA class I molecules have been found to form homodimers. One disease found to be linked to certain allotypes of HLA class I molecules is ankylosing spondylitis, which is linked to HLA-B27 (Brewerton et al., 1973; Schlosstein et al., 1973). This particular allotype has been found to have the capacity to form

Figure 1.8. *HLA class I molecules and β2m affect migration of endothelial cells.* Endothelial cells were transfected with control siRNA or with siRNA against ITGB4, HLA class I heavy chain, or β_2m, and wound closure was assessed and quantified.

Figure from:

Zhang X, Rozengurt E, Reed EF. HLA class I molecules partner with integrin β4 to stimulate endothelial cell proliferation and migration. Sci Signal. 2010;3(49):ra85.

Figure 1.8.


Control
β4
HLA
β2
siRNA
12 hours
*
*
*
100
80
60
40
20
0
Wound closure (%)
C β4 HLA β2 siRNA

disulfide bonds between cysteine 67 in the peptide binding groove of two HLA-B27 heavy chains. Dimers formed between these molecules are present both on the cell surface as well as in the ER (Campbell et al., 2012). However, the role these dimers play in this particular disease has yet to be fully elucidated.

Based on the ability of HLA class I molecules to interact with cell-surface/growth factor receptors, studies in endothelial and smooth muscle cells investigating downstream signaling and functional changes related to these interactions have also been done. One study showed that exposure of endothelial cells in transplantation biology models to low concentrations of HLA class I ligating antibodies promoted cell survival via the activation of the PI3K/Akt signaling pathway, which in turn regulates the expression of anti-apoptotic proteins (Jin et al., 2004). Phosphorylation of Src and FAK is upstream of PI3K and Akt phosphorylation following ligation of HLA class molecules (Jin et al., 2007). Overall, the model that was proposed was that HLA class I molecules transduce signals that lead to the formation of a complex featuring Src, FAK, and PI3K that causes the phosphorylation of Akt and thereby increases the expression of anti-apoptotic proteins Bcl-2 and Bcl-xL and decreases the pro-death factor Bad by its sequestration into a complex with 14-3-3 protein (Jin et al., 2004). Moreover, knockdown of FAK led to an inhibition of Src, PI3K (p85 subunit), and Akt phosphorylation upon HLA class I-specific antibody treatment (Jin et al., 2007). There was also abrogation of paxillin phosphorylation seen upon HLA class I antibody treatment and a blockade of the formation of actin stress fibers. The knockdown of FAK, however, did not cause the modulation of expression of FGFR that is usually seen with HLA class I ligation, but it did block the stimulation of cell proliferation that is usually seen. Thus, FAK has been implicated as being a key regulator of HLA class I-induced cell survival and proliferation and as a key upstream tyrosine kinase controlling anti-HLA class I antibody signaling. Changes in the functions of FAK could be due to the plethora

of phosphorylation sites that it has or due to the diverse intracellular locations to which it and its binding partners localize (Jin et al., 2007). Additional studies by this group indicate that HLA class I molecules and/or integrin ligation can also lead to phosphorylation of ERK through mTORC2 (Jindra et al., 2008). Also, it was previously shown that cross-linking of the HLA class I molecule with class I specific antibodies activates Rho signaling, triggering reorganization of the cytoskeleton and formation of F-actin stress fibers. Proteomic studies using tandem mass spectrometry further revealed that CDK2 (an already known regulator of HLA class I signaling) was among the 35 kinase families that could be responsible for activation of downstream pathways. TPM4, p70S6k, and eIF4A1 were other proteins that were also found to be relevant (Zhang et al., 2012).

Overall, these studies reveal a multitude of non-immune functions for HLA class I molecules that appear to be very complex. Further investigation into the roles these molecules have outside of the immune system, especially in regards to cancer, is needed to better understand the pathobiology of this disease.

1.6 HLA class I molecules and amyloid precursor-like protein 2 (APLP2)

1.6.1 APLP2 in the nervous system

Amyloid precursor-like protein 2 (APLP2) is one of three members of the amyloid precursor protein (APP) family of proteins, along with APP and APLP1 (Jacobsen and Iverfeldt 2009; Shariati and De Strooper 2013; Walsh et al., 2007). These proteins are considered type 1 membrane glycoproteins (Slunt et al., 1994; Walsh et al., 2007). APP and APLP2 are broadly expressed while APLP1 is only present in tissues of the nervous system (Kim et al., 1995; Slunt et al., 1994). There is growing knowledge about both

APP and APLP2, with APP being the most studied of the protein family members due to its ability to yield the pathogenic amyloid beta plaque that contributes to Alzheimer's disease. This pathogenic fragment generated from APP is not formed by the other family members (Goedert 1987; Hardy and Selkoe 2002; Mawuenyeta et al., 2010; Pandey et al., 2016).

APLP2 has been most frequently studied for the various functions it plays within the nervous system and only recently has it been found to be linked with cancer. APLP2 KO mice exhibit generally subtle phenotypes but do have some retinal abnormalities likely due to abnormal morphology of the outer plexiform layer of the retina (Dinet et al., 2016; Heber et al., 2000; Muller et al., 2017; von Koch et al., 1997; Weyer et al., 2011). Additionally, APLP2 KO mice display hyperinsulinemia (Needham et al., 2008). There does seem to be some compensation between members of the family, however, since double KO of APLP2 with either APP or with APLP1, or triple KO of all three proteins, causes death within one day of birth (Klevanski et al., 2014; Klevanski et al., 2015; von Koch et al., 1997). Mice lacking both APLP1 and APP are viable and fertile (Heber et al., 2000). The APP and APLP2 double KO mice have severe neuromuscular junction deficits among various other issues, including elevated copper levels in the liver and cerebral cortex likely contributing to their early death (Caldwell et al., 2013; Klevanski et al., 2014; Klevanski et al., 2015; Wang et al., 2005; Yang et al., 2007). Interestingly, keratinocytes from these double KO mice also display decreased cell proliferation and migration (Siemes et al., 2006).

APLP2 is a multimodal protein involved in many processes. Some of the functions within the nervous system in which APLP2 participates are cell proliferation, cell adhesion, neurite outgrowth, mitosis segregation, cell signaling, metabolism and transcriptional regulation (Cappai et al., 1999; Guo et al., 1998; Li et al., 1999; Rassoulzadagan et al., 1998; Thinakaran et al., 1995). Additionally, APLP2 and APP

can function as both cell surface receptor-like proteins or even as ligands (through their secreted N-terminal forms) (Muller et al., 2017). Further studies have shown that the expression of APLP2 is regulated by subcellular localization, complex cleavage events, and post-translational modifications (Muller et al., 2017).

1.6.2 Influence of APLP2 on HLA class I molecules

Our lab has previously published many studies on the association between APLP2 and both human and mouse MHC class I molecules and how, in some cancers, this can affect MHC class I cell-surface expression. These previous studies have indicated that, in S2-013 pancreatic cancer cells, APLP2 and HLA class I molecules co-localize extensively in cytoplasmic vesicles while control receptors do not (Tuli et al., 2009b). These cytoplasmic vesicles mainly included early endosomes and, to a lesser extent, recycling endosomes. In HeLa cells, there was also association between these two proteins in early endosomes, but they were also co-localized in the Golgi complex (Tuli et al., 2008a; Tuli et al., 2009a; Tuli et al., 2009b). Additionally, APLP2 can bind to MHC class I molecules at the cell surface as well as to MHC class I molecules that have been endocytosed. Higher expression of APLP2 resulted in an increase in the endocytosis of HLA class I molecules (Tuli et al., 2009a). Previous studies indicated that APLP2 is endocytosed by a clathrin-mediated route, and that APLP2 re-channels MHC class I molecules into the clathrin-mediated endocytosis pathway, which could account for the diversity in the literature about the route of MHC class I internalization (Huet et al., 1980; Radhakrishna and Donaldson 1997). Additionally, HeLa cells that experimentally overexpress APLP2 have longer retention of MHC class I molecules within the cell, suggesting that APLP2 reduces the recycling of MHC class I molecules to the cell surface (Tuli et al., 2008a). Furthermore, inhibition of lysosome function leads to

an accumulation of MHC class I molecules in the lysosome, indicating that APLP2-associated endocytosis of the MHC class I molecules leads to their removal from the cell surface and subsequent degradation by the lysosome (Tuli et al., 2008a). The endocytic capacity of APLP2, as well as its ability to promote MHC class I endocytosis, is mediated by the NPTYKYL sequence, specifically by the middle tyrosine residue (Tuli et al., 2009b). Interestingly, mutation of this residue does not affect the ability of APLP2 to bind to MHC class I molecules. The interaction between these molecules has been mapped to the conserved α_3/transmembrane/cytoplasmic region as well as the polymorphic α_1/α_2 membrane-distal region of the MHC class I molecule (Tuli et al., 2008b) (**Figure 1.9**).

Studies from our lab also showed that APLP2 binds more strongly to certain allotypes than others in S2-013 cells. These cells express both HLA-A2 and HLA-A24; APLP2 binds both HLA-A allotypes but even more strongly to HLA-A2 than to HLA-A24 (Tuli et al., 2009b). In regards to mouse MHC heavy chain specificity, studies indicate that APLP2 binds more strongly to K^d than other murine MHC class I molecules and that β_2m is needed for this binding (Morris et al., 2003; Sester et al., 2000; Tuli et al., 2008b).

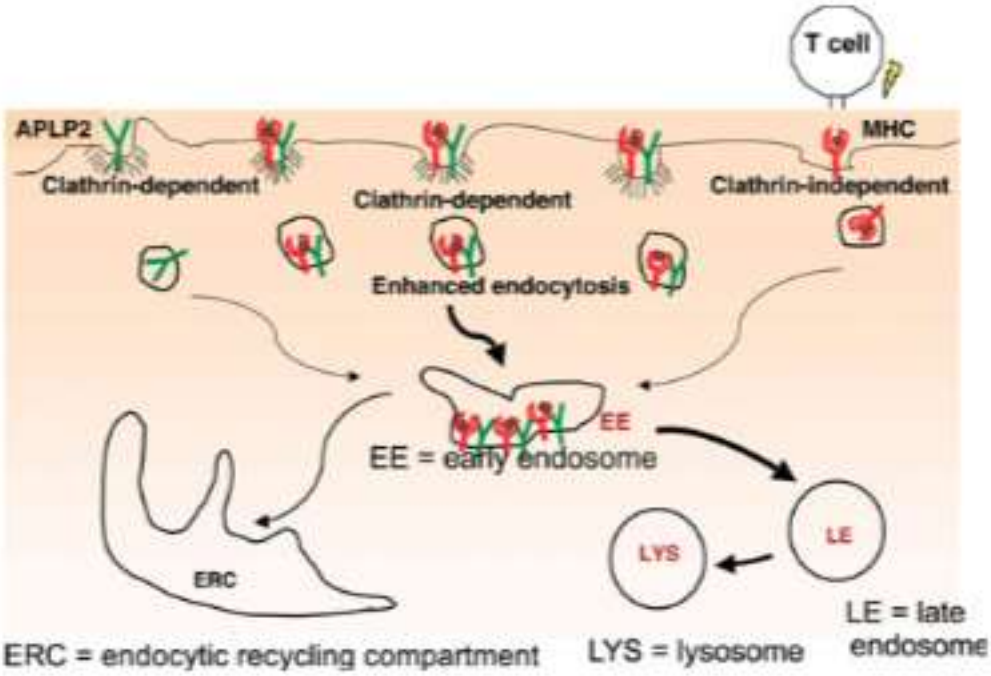
Specifically, in relation to cancer, APLP2 has been found to influence the expression of the HLA class I molecule on the surface of various cancer cell lines. When APLP2 was overexpressed in the cancer cell line MDA-MB-435S, there was a reduction in the surface expression of HLA-A24 as well as a reduction in the total cell-surface MHC class I expression. This was further confirmed in HeLa cells that were transfected with HLA-A24 and overexpressed APLP2 (Morris et al., 2003; Tuli et al., 2008a; Tuli et al., 2008b). Studies done in a pancreatic cancer cell model as well as a Ewings sarcoma model show the overexpression of APLP2 in cancer cells (Peters et al., 2012; Peters et al., 2013b). Furthermore, in Ewings sarcoma cell lines, APLP2 and HLA class I were shown to interact and, when these cells were treated with ionizing radiation,

Figure 1.9. *APLP2 affects the endocytosis of both murine and human MHC class I molecules.* APLP2 and HLA class I interact near the plasma membrane. APLP2 influences the endocytosis of the HLA class I molecule through a clathrin-dependent mechanism. Furthermore, HLA class I recycling is reduced by APLP2 by routing it to lysosomes for degradation.

Figure from:

Peters HL, Tuli A, Sharma M, Naslavsky N, Caplan S, MacDonald RG, Solheim JC. Regulation of major histocompatibility complex class I molecule expression on cancer cells by amloid precursor-like protein 2. Immunol Res. 2011;51:39-44.

Figure 1.9.


T cell
APLP2
MHC
Clathrin-dependent
Clathrin-dependent
Clathrin-independent
Enhanced endocytosis
EE
EE = early endosome
LE
LYS
ERC
LE = late endosome
ERC = endocytic recycling compartment
LYS = lysosome

it caused an increase in the amount of APLP2 at the cell surface (Peters et al., 2013a; Peters et al., 2013b). This increase in APLP2 led to a decrease in the amount of HLA class I molecules present on the cell surface. This decrease in HLA class I molecules on the cell surface was found to be abrogated with knockdown of APLP2 using siRNA (Peters et al., 2012; Peters et al., 2013b). The high expression of APLP2 typically seen in cancer cells may explain the decrease in HLA class I molecules that is characteristic of cancer cells.

1.7 Dissertation hypothesis

The immunologic functions of HLA class I molecules and their allotypes has been well studied, however, not much work has been done looking into the role that this molecule and its components play outside of the immune system, especially in cancer. Therefore, the overall aim of this dissertation was to investigate the role that the components of the HLA class I molecule (the HLA heavy chains and β_2m) play in pancreatic cancer cell migration. This was done in pancreatic cancer since pancreatic cancer cells retain relatively high expression of HLA class I molecules and the prognosis for this disease, especially for the large population with metastatic disease, is the lowest for any major solid tumor. Increased knowledge of the pathobiology of this lethal disease is, therefore, urgently needed.

Firstly, due to the interest in β_2m in cancer but the lack of studies done in pancreatic cancer specifically, the role that β_2m plays in affecting the migration of pancreatic cancer cells was investigated. Based on other studies indicating that this molecule may have a role in affecting the migration of cancer cells, it was hypothesized that β_2m promotes pancreatic cancer cell migration. Studies done to address this hypothesis were to knock down β_2m in a variety of pancreatic cancer cell lines and a

non-transformed pancreas cell line and evaluate changes in migration. Previous studies from our lab have indicated that APLP2 is a protein that binds to HLA class I molecules in pancreatic cancer, that β_2m is necessary for this interaction, and that decreases in expression of this protein contribute to pancreatic cancer metastasis. Therefore, interactions with APLP2 as well as changes in APLP2 expression were also investigated as a potential mechanism for any migratory changes that were observed upon knockdown of β_2m.

Additionally, as has been shown in transplantation biology models, HLA class I heavy chains possess the ability to promote migration of smooth muscle cells and endothelial cells. Studies have further indicated that this function may also extend to a medulloblastoma model. Therefore, it was further hypothesized that HLA class I molecules will not only be amply expressed in pancreatic cancer cells but that their presence will lead to alterations in pancreatic cancer cell migration. Due to sequence differences between isotypes as well as allotypes and the fact that expression of certain allotypes has been linked to worse disease in cancer patients, it was further hypothesized that the effects of the HLA class I molecule on migration will not only be heavy chain isotype dependent, but will also be dependent on specific heavy chain allotypes. To test this hypothesis, transient transfections using isotype-specific siRNA to knock down HLA class I isotypes as well as transfections of designed siRNA to knock down specific allotypes were performed, and migration was assessed in multiple pancreatic cancer cell lines. Furthermore, as it has been previously shown, the HLA class I molecule itself cannot cause downstream signaling, and it is necessary for it to interact with other receptors on the cell surface to promote downstream processes, such as cellular migration. The receptors that were investigated were integrins which have been previously studied in relation to cancer cell migration, and EGFR, another protein known to affect pancreatic cancer cell migration. Both of these proteins have been

previously mentioned to either interact with or be affected by HLA class I molecules. Thus, integrins and EGFR were hypothesized to interact with HLA class I molecules and dictate any changes observed in cell migration upon knockdown of the different forms of the HLA class I molecule.

Finally, the effects of the HDAC inhibitor M344 on APLP2 processing as well as the expression of HLA class I molecules in pancreatic cancer cells was determined. M344 has previously been shown to affect the expression of the BACE1 proteolytic enzyme, which cleaves APLP2 and leads to formation of C-terminal fragments. As these C-terminal fragments have been shown to affect pancreatic cancer cell proliferation and migration as well as to interact with HLA class I molecules in this model, it was hypothesized that treatment of the S2-013 pancreatic cancer cell line with M344 will lead to a reduction in APLP2 C-terminal fragments which will, in turn, cause a decrease in proliferation and migration in these cells. Additionally, as previously shown by our laboratory, reduction in APLP2 as well as its C-terminal fragment leads to an increase in the expression of HLA class I molecules, therefore, it is expected that HLA class I molecules will increase in S2-013 cells upon M344 treatment. This could potentially have important implications for immunotherapy treatment in this disease.

CHAPTER 2: Materials and Methods

2.1 Cell Lines and Culture Conditions

2.1.1 Human Pancreatic Cancer Cell Lines

S2-013 and BxPC-3 pancreatic cancer cells (Iwamura and Hollingsworth 1999) were grown in RPMI base medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 x MEM NEAA, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 µg/mL streptomycin, and 10% heat inactivated FBS. S2-013 cells are a well-characterized subclone of the SUIT2 pancreatic cancer cell line (Taniguchi et al., 1992). Both cell lines were generously donated by Dr. Tony Hollingsworth (UNMC). PANC-1 and MIA PaCa-2 cell lines (Iwamura and Hollingsworth 1999) were grown in DMEM base medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 1 x MEM NEAA, 1 mM sodium pyruvate, 100 units/mL penicillin and 100 µg/mL streptomycin, and 10% heat inactivated FBS. PANC-1 cells were generously donated by Dr. Michel Ouellette (UNMC) and MIA PaCa-2 cells were purchased directly from ATCC. All cell lines were tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit. Additionally, all cells were authenticated by short tandem repeat deoxyribonucleic acid profiling analysis (UNMC Molecular Diagnostics Facility) or purchased directly from ATCC.

2.1.2 Human Pancreatic Ductal Cell Lines

hTERT-HPNE cells were cultured as previously described (Lee et al., 2003). Briefly, this includes 75% glucose-free DMEM, 25% Medium M3, 5% heat inactivated fetal bovine serum, 5.5 mM glucose, 10 ng/ml epidermal growth factor, and 50 µg/mL Geneticin. These cells were generously donated by Dr. Michel Ouellette who also

generated this cell line at UNMC. Cells were tested for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit.

2.1.3 Treatment of Pancreatic Cancer Cells with Growth Factors

Pancreatic cancer cell lines were treated with either epidermal growth factor (EGF) or transforming growth factor β (TGF- β). The day after transfection, medium on transfected cells was replaced with either fresh maintenance medium alone or fresh maintenance medium with either 20 ng/ml EGF or 10 ng/ml TGF-β. 48 hours post-treatment, cells were either collected for signaling experiments or were replated per the migration assay protocol listed below to assess changes in migration.

2.1.4 Treatment of Pancreatic Cells with the HDAC Inhibitor M344

Pancreatic cancer cell lines were treated with the HDAC inhibitor M344 at the indicated concentrations and timepoints. Briefly, 24 hours after cell plating, cells were treated with inhibitor for the indicated timepoints and assessed for cell proliferation, migration and viability.

2.2 Genetic manipulation

2.2.1 Short-interfering (si)RNA constructs

The β_2m-specific siRNA used for β_2m downregulation was the ON-TARGETplus SMARTpool siRNA which contains four separate β_2m-specific siRNA purchased from Thermo Scientific Dharmacon (L-004366-00-0020). Pan siRNA specific to either HLA-A and HLA-B heavy chain isotypes were also ON-TARGETplus SMARTpool siRNA purchased from Themo Scientific Dharmacon (L-012850-01-0020 and L-013203-00-

0020 respectively). siRNA targeting specific allotypes of the HLA class I molecule were designed with the help of Nuzhat Khan and were ordered customly. The following sequences were used for each: siRNA sequence against HLA-A2: GCAUAUGACUCACCACGCUUU, siRNA sequence against HLA-A24: CCAAGACACAUAUGACCCAUU, siRNA sequence against HLA-B7: GGGCAUGACCAGUACGCCUUU, siRNA sequence against HLA-B59: AGACCAACACACAGACUUAUU. ON-TARGETplus non-targeting siRNA pool (Thermo Scientific Dharmacon D-001810-10-50) was used as a negative control.

2.2.2 Transient Transfection of siRNA

Transfections were performed following the siRNA manufacturer's instructions for cells in base maintenance medium. Briefly, cells were plated 24 hours prior to transfection at a concentration of $1x10^5$ cells/well (or $1.5x10^5$ cells/well for hTERT-HPNE cells due to slower growth) in a 6 well plate. On the day of transfections, cells were starved in base maintenance medium for approximately three hours. DharmaFECT Transfection Reagent. No. 1 (Thermo Scientific Dharmacon T-2001-03) was incubated with 0.4 pmol of siRNA for β_2m or 0.2 pmol of siRNA for all forms of the HLA heavy chain siRNA for 30 minutes at room temperature and the mixture was added drop-wise to the well. Four hours post-transfection, serum-containing medium was added to each well. Confirmation of knockdown of respective proteins was evaluated at indicated times post-transfection by immunoblot analysis of cell lysates or by flow cytometry for HLA class I allotypes.

2.3 Antibodies

2.3.1 Anti-HLA class I component antibodies

Total HLA class I molecules for flow cytometry were detected by the W6/32 antibody which recognizes all isotypes of HLA class I molecules when bound to β_2m. This antibody was provided courtesy of Dr. Ted Hansen (Shields and Ribaudo 1998). To recognize the HLA-A isotype for western blot, the HLA-A specific rabbit monoclonal antibody was used (Abcam). For flow cytometry, HLA-A2 was recognized by BB7.2 was produced from a hybridoma obtained from ATCC (Parham and Brodsky 1981). HLA-A24 was detected by flow cytometry using an HLA-A24 (and HLA-A23) specific antibody purchased from OneLambda. To recognize HLA-B heavy chain isotypes for western blot, an HLA-B specific rabbit monoclonal antibody (Novus) or the HC-10 antibody that recognized HLA-B and HLA-C isotypes and some HLA-A allotypes were used. The HC-10 antibody was also kindly provided by Dr. Ted Hansen (Carreno and Hansen 1994; Stam et al., 1990). The BB7.1 antibody (Thermo Scientific) was used to detect HLA-B7 by flow cytometry. The antibody 30-5-7 (which recognized the mouse H-2L^d molecule and not HLA class I molecules) was also kindly provided by Dr. Ted Hansen (Washington University School of Medicine, St. Louis, MO, USA) (Ozato et al., 1980; Sernee et al., 1998; Solheim et al., 1993; Solheim et al., 1995). The 64-3-7 monoclonal antibody (recognizes L^d that has an open, peptide-free groove and not HLA class I molecules) was also kindly provided by Dr. Ted Hansen (Washington University School of Medicine, St. Louis, MO, USA) (Cox et al., 1991; Smith et al., 1993; Smith et al., 1995) To detect β_2m, a β_2m-specific antibody from Cell Signaling was used.

2.3.2 Additional antibodies

APLP2 full length protein antibodies that detect both the full length protein as well as GAG-APLP2 was purchased from R&D Systems. An antibody specific to the C-terminal cleaved fragment of APLP2 was made for the Solheim lab by Thermo Scientific.

EGFR and ITGB1 antibodies were purchased from Bethyl Laboratories and Santa Cruz Biotechnology. Antibodies against pFAK Y397, FAK, and BACE1 were purchased from Cell Signaling Technologies. Loading control antibodies against Hsc70 was purchased from Enzo LifeSciences as well as pan-actin purchased from Cell Signaling Technologies.

2.4 Cell Lysis

2.4.1 Cell lysate collection for Western blotting

Cells were harvested and washed once with cold phosphate-buffered saline (PBS) and then resuspended in cell lysis buffer. Cell lysis buffer is composed of 1 mM EGTA, 1 mM EDTA, 50 mM Tris-HCL pH 7.5, 1% Triton X-100, 1 mM Na_3VO_4, 2 mM DTT, 0.1 mM PMSF, and 1 µg/ml Halt Cocktail. Cell lysates were stored at -80°C overnight, then thawed on ice and centrifuged at 13,000 rpm for 30 min at 4°C. Supernatants were transferred to new tubes and stored at -80°C.

2.4.2 Cell pellet collection for immunoprecipitation

Cells at approximately $1x10^7$ cells were trypsinized and collected in 15 ml conical tubes. Cell pellets were then washed with a solution of 20 mM IAA in PBS three times spinning at 1500 rpm at 4°C for 5 mins. After the final wash, supernatant was completely removed and cell pellets were stored at -80°C until use.

2.5 Immunoprecipitation

Cell pellets were lysed in 1% CHAPS lysis buffer (1% CHAPS in PBS (pH 7.4), 200 mM IAA, and 0.2 mM PMSF) and incubated along with primary antibody for 3 hours while rotating at 4°C. Following this incubation, cells were spun at 4°C for 30 minutes at 13,000 rpm. Cells were then incubated with either Protein A Sepharose beads alone (mouse primary antibody) or a mixture of 75% Protein A and 25% Protein G beads (rabbit primary antibody) for 1 hour gently mixing every 10 minutes. Cells were then washed 4 times with ice-cold wash buffer (0.1% CHAPS, 200 mM iodoacetamide in Tris-buffered saline pH 7.4). The first two washes and centrifugations (at 1,100 rpm for 5 minutes at 4°C) were done immediately after cell resuspension, and the last 2 washes included a 10 minute incubation on ice followed by centrifugation at 1,100 rpm for 5 minutes at 4°C. Elution buffer (125 mM Tris pH 6.4, 2% w/v SDS, 12% v/v glycerol, and 0.02% bromophenol blue) was then added to the Protein A-Sepharose bead mixture and boiled for 5 minutes followed by a final spin at 1100 rpm for 5 minutes at 4°C. The supernatant was then collected followed by a quick spin to pellet any leftover beads. Samples were then stored at -80 C until electrophoresis.

2.6 Electrophoresis and Western Blotting

Aliquots of lysate supernatants were mixed with 5 x sodium dodecyl sulfate loading dye (250 mM Tris-HCL pH 6.8, 10% w/v sodium dodecyl sulfate, 30% v/v glycerol, 5% v/v β-mercaptoethanol, 0.02% bromophenol blue) and boiled for 5 minutes prior to loading. Samples were then brought up to a volume of 15 ul with 1x SDS loading buffer. Samples were then loaded on Invitrogen Novex Tris-glycine polyacrylamide pre-cast 4-20% or 10-20% gels (Thermo Fisher Scientific). Electrophoresis was performed at 90 V at room temperature and proteins were then transferred to PVDF membranes at room temperature at 40 volts for either 1.5-2.5 hours

depending on weight of the proteins of interest. The Kaleidescope protein marker was used to determine the running length of the proteins of interest. Following the transfer, the membranes were blocked in 5% w/v solution of nonfat dry milk in PBST for one hour at room temperature while gently rocking. Membranes were then incubated with indicated primary antibodies overnight while rocking at 4°C. After primary antibody incubation, membranes were washed 3 times for 5 minutes at room temperature with 1% Tween-20 in PBS solution. Subsequently, membranes were incubated with the appropriate HRP-conjugated secondary antibodies for one hour at room temperature with gentle rocking and were again washed with 1% Tween-20 in PBS. For protein visualization, membranes were incubated in Pierce ECL Western Blotting Substrate and exposed to Carestream BioMax MR film. The intensity of the protein bands were quantified by densitometry using the LiCOR Image Studio Lite software.

2.7 Simple Western

Cell lysates were prepared as seen above and β_2m protein levels were evaluated by a Protein Simple Peggy Sue instrument per the manufacturer's instructions. Briefly, lysates were mixed with Simple Western sample dilution buffer (Protein Simple) that contains a reducing agent and fluorescent standards, heated at 95°C for 5 minutes and were loaded at a final protein concentration of 0.04 mg/ml. Lysates, primary antibody against β_2m and actin (Cell Signaling Technologies 12851 and 4968 respectively, both used at a concentration of 1:50), horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Protein Simple, used at the manufacturer's dilution), separation and stacking matrix along with the chemiluminescent substrate were distributed into the appropriate wells in a 384 well plate which was then placed into the Peggy Sue instrument allowing for automatic running of 8 cycles. Run settings were adjusted to a

35 minute run time from the default of 40 minutes with the 12-230 size separation module used. Compass software (Protein Simple) was used for analysis of the generated data.

2.8 Flow Cytometry

Cells were collected at $1x10^6$ cells/ml and 100 ul were added to each well at a concentration of $1x10^5$. Cells were then spun down at 1500 rpm for 5 minutes at 4°C and were resuspended in FACS buffer (0.2% BSA and 0.1% sodium azide). Cells were incubated with primary antibody for 30 minutes at 4°C. Following primary antibody incubation, cells were washed three times with FACS buffer and spun at 1500 rpm for 5 minutes at 4°C between each wash. Cells were incubated with a PE-conjugated secondary antibody or with a PE-conjugated isotype control for 30 minutes at 4°C. Cells were again washed three times followed by centrifugations at 1500 rpm for 5 minutes at 4°C. Samples were then transferred to tubes and analyzed on a BD FACSCalibur or LSRII instrument with the assistance of the UNMC Cell Analysis Facility.

2.9 Analysis of Cell Migration

2.9.1 Transwell assay

Post-transfection, cells were analyzed for changes in migration by using a transwell assay. Briefly, 72 hours post-transfection, cells were plated at $1x10^5$ cells in base maintenance media into a 8-μm insert and were incubated for 24 hours at 37°C. After 24 hours, inserts were stained using the Hema 3 Stat Pack (Thermo Fisher) and mounted onto slides. Three random fields of each insert were taken and all cells in each

field were counted. For analysis of cells treated with M344, cells were treated with M344 for 24 h and then replated in inserts in serum free base maintanence media without drug. The same protocol was then followed for M344 drug treated migration assays.

2.9.2 Scratch assay

Cells were seeded at $1.5x10^5$ cells in a 6 well plate and were treated with 10 μM of M344 for 24 hours. Plates were then scratched with a 200 μl pipette time and media was replaced with drug-free maintenance media contained 10% serum. Pictures of approximately the same location were taken at time of scratch as well as 8, 12, and 26 hours post-scratch.

2.10 Analysis of Cell Viability

Cell viability was assessed at the various indicated timepoints post-transfection by use of trypan blue staining. Briefly, cells were collected and resuspended with trypan blue stain and counted using a hemacytometer. Both live and dead cells were counted and analysis was performed with percentage being total number of live cells by the total number of cells (both live and dead).

2.11 Analysis of Cell Growth and Proliferation

2.11.1 MTT assay

Cell growth and proliferation were assessed by MTT assay. Briefly, 48 hours post-transfection cells were replated at 2500 cells/100 μl in a 96 well plate. Lysates were collected at this timepoint to confirm knockdown of the protein of interest and a 0

hour timepoint MTT was performed at this time to confirm accurate cell plating between conditions. At the indicated timepoints post-replating MTT assays were performed to assess cell proliferation. For the actual assay, 50 ul of MTT reagent was added to 100 ul of media and incubated at 37 C for two hours. Post-incubation, all media was removed and 100 ul of DMSO was added and plates were read at 570 nm.

For antibody-treatment MTTs and M344-treatment MTTs, cells were plated at 2500 cells/100 µl and incubated for 24 h. Cells were then treated with antibody or drug for the indicated timepoints and the same protocol was then followed for plate reading.

2.11.2 Trypan Blue exclusion assay

To measure cell proliferation by trypan blue staining, the same protocol was followed as for measuring cell viability except only live cells were counted at the indicated timpoints.

2.12 Statistical Analysis

All statistical analysis was performed using the two-tailed Students t-test with the criterion set for significance at $p \leq 0.05$. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Error bars are representative of standard error of the mean (SEM).

CHAPTER 3: Function of β_2m in pancreatic cancer cell migration

Data in this chapter have been published in the following manuscript:

Sliker BH, Goetz BT, Peters HL, Poelaert BJ, Borgstahl GEO, Solheim JC. Beta 2-microglobulin regulates amyloid precursor-like protein 2 expression and the migration of pancreatic cancer cells. Cancer Biol Ther. 2019;epub ahead of print.

3.1 Introduction to β_2m and APLP2

β_2m is a 12-kD light chain that non-covalently binds and stabilizes the MHC class I heavy chain, thereby enabling its presentation of an antigenic tumor-derived or pathogen-derived peptide to a cytotoxic T cell. The presentation of the antigenic peptide leads to lysis of the targeted malignant or infected cell bearing the MHC molecule (Collins et al., 1995; Krangel et al., 1979; Townsend A et al., 1989). Surprisingly, β_2m's expression has been noted to be maintained (and in some cases even elevated), instead of being down-regulated, within a variety of different solid tumors, including renal cell carcinoma, oral squamous cell carcinoma, breast cancer, prostate cancer, colorectal cancer, and esophageal cancer (Bossard et al., 2012; Chen et al., 2008; Huang et al., 2006; Li et al., 2014; Nomura et al., 2006; Tanaka et al., 2012). β_2m is also elevated in the serum of patients with lymphoid malignancies, including lymphomas, leukemias, and multiple myeloma (Cooper and Plesner 1980; Josson et al., 2011; Molica et al., 1999). For esophageal, colorectal, and oral cancers, as well as hematological malignancies, several publications have shown that higher β_2m expression within the tumor is correlated with worse disease and poorer prognosis (Bataille et al., 1983; Bossard et al., 2012; Chen et al., 2008; Cooper and Plesner 1980; Molica et al., 1999; Tanaka et al., 2012). For renal cell carcinoma and oral squamous cell carcinoma, β_2m increases tumor cell migration and possibly epithelial-to-mesenchymal transition (Bataille et al., 1983; Chen et al., 2008; Nomura et al., 2006). Thus, for these abovementioned cancers, the data suggest that besides its immunological, anti-cancer function in antigen presentation, β_2m could also potentially have a pro-tumor function. However, despite the reputation of pancreatic cancer as a highly metastatic disease, whether or not the migration of pancreatic cancer cells is increased by β_2m has not thus far been reported.

APLP2, a member of the amyloid precursor protein family of proteins, is expressed in a transmembrane form and as cleaved forms, with secretion of the large outer domain and retention within the cell of the small C-terminal portions (Walsh et al., 2007). Some splice forms of APLP2 are post-translationally modified on the extracellular domain by a chondroitin sulfate glycosaminoglycan (GAG) (Walsh et al., 2007). APLP2 expression has been noted to be high in pancreatic cancer, as well as in some other cancers (Abba et al., 2004; Moss et al., 2007; Peters et al., 2012; Peters et al., 2013). Previous studies from our laboratory have indicated that APLP2 associates with mouse and human MHC class I molecules and reduces MHC class I cell-surface expression (Peters et al., 2011; Sester et al., 2000; Tuli et al., 2009a; Tuli et al., 2009b). Furthermore, we demonstrated that APLP2 increases pancreatic cancer cell migration *in vitro* and causes more metastasis to distant organ sites in a mouse orthotopic pancreatic cancer xenograft model (Pandey et al., 2015). Whether APLP2's pro-migratory effect on pancreatic cancer cells is linked to the interactions of APLP2 with any component of MHC class I molecules, including β_2m, is a question that has not previously been addressed.

Thus, the focus of this study was to investigate whether β_2m influences the migration of pancreatic cancer cells, and, if so, to assess potential involvement of APLP2 in the mechanism. The human pancreatic cancer cell lines that we analyzed were found to express substantial levels of β_2m. When pancreatic cancer cell expression of β_2m was experimentally down regulated by siRNA transfection, the migration of S2-013 and PANC-1 pancreatic cancer cells was significantly decreased, but in contrast the migration of MIA PaCa-2 was significantly increased. The β_2m/HLA class I/peptide complexes in the S2-013 and PANC-1 pancreatic cancer cell lines, but not the MIA PaCa-2 cell line, associate with APLP2. Reduction in β_2m, by siRNA transfection, in turn down regulated the expression of APLP2 in S2-013 and PANC-1. However, knockdown

of β_2m by siRNA transfection in MIA PaCa-2 cells up regulated the expression of APLP2 in that cell line, in accordance with the effect of β_2m knockdown on migration capability. Thus, our data indicate that β_2m is amply expressed in pancreatic cancer cells, and that β_2m regulates APLP2 expression, and, correspondingly, affects the migration of pancreatic cancer cells. Therefore, our findings suggest that β_2m could be a potential factor influencing pancreatic cancer metastasis, acting via APLP2.

3.2 β_2m in pancreatic cancer cell migration

3.2.1 β_2m is amply expressed in pancreatic cancer cells

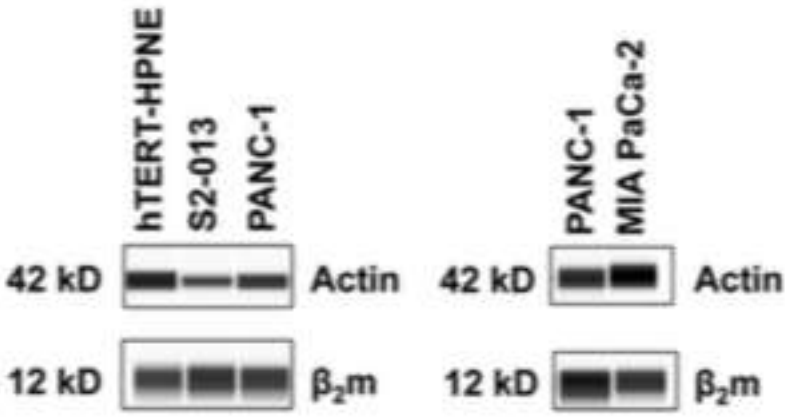
To assess the level of β_2m expressed in pancreatic cancer cell lines, we analyzed lysates of several pancreatic cancer cell lines (S2-013, PANC-1, and MIA PaCa-2) along with an immortalized, non-transformed pancreas cell line (hTERT-HPNE) for comparison. As shown in **Figure 3.1**, all of the cell lines express β_2m at a level comparable to the non-transformed hTERT-HPNE cell line. These data suggest that, despite its immunological function as a component of the HLA class I molecule, β_2m expression is not necessarily lost or down regulated in human pancreatic cancer cell lines.

3.2.2 β_2m's influence on pancreatic cancer cell migration

To investigate whether β_2m increases pancreatic cancer cell migration, we transfected S2-013, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines with siRNA specific for β_2m (or with control scrambled siRNA). As a control, the non-transformed pancreatic cell line hTERT-HPNE was also transfected with β_2m-specific or scrambled

Figure 3.1. *β2m is amply expressed in human pancreatic cancer cell lines.* Simple Westerns to detect actin (as a control), as well as for β_2m, were performed on cell lysates from hTERT-HPNE (an immortalized but not transformed pancreatic cell line) (*left panel*) and from pancreatic cancer cell lines (*left panel*: S2-013, PANC-1; *right panel*: PANC-1, MIA PaCa-2). The results shown are representative of experiments performed on 3 separate lysates of each cell type to assess the levels of β_2m in these cell lines.

Figure 3.1.


hTERT-HPNE
S2-013
PANC-1
PANC-1
MIA PaCa-2
42 kD
Actin
42 kD
Actin
12 kD
β2m
12 kD
β2m

siRNA. After siRNA transfection, the down regulation of β_2m protein in the cell lines was verified (**Figure 3.2**). By transwell assay, we determined that the migration rates of S2-013 and PANC-1 were significantly impaired when β_2m expression was down regulated (**Figure 3.2**). In contrast, when we down regulated β_2m expression in MIA PaCa-2 cells with siRNA, the migration rate increased significantly (**Figure 3.2**). The hTERT-HPNE pancreatic cell line is less migratory than the pancreatic cancer cell lines, and there was not a significant diminishment nor enhancement of migration when β_2m was knocked down in these cells (**Figure 3.2**). These findings indicate that β_2m influences the migration rate of pancreatic cancer cells, but not of normal pancreatic cells.

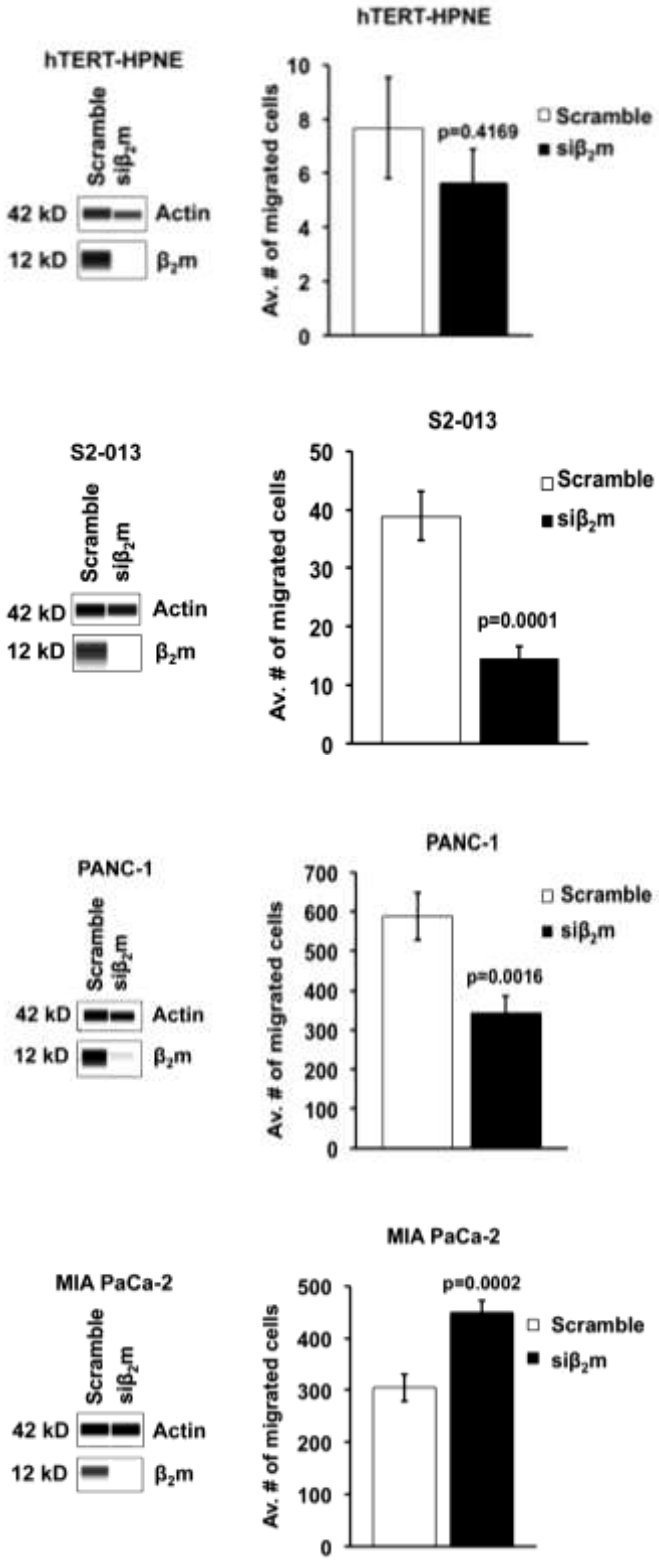
3.3 β_2m and APLP2 in pancreatic cancer

3.3.1 HLA class I molecules (in complex with β_2m) interact with APLP2

Earlier studies in our laboratory showed that APLP2 is highly expressed in pancreatic cancer cells, and that APLP2 increases pancreatic cancer cell migration *in vitro* and facilitates metastasis in an orthotopic mouse xenograft model (Pandey et al., 2015; Peters et al., 2012). In addition, we had previously demonstrated that APLP2 associates with β_2m/HLA class I/peptide complexes in S2-013 cells (Tuli et al., 2009a) To investigate if a relationship exists between APLP2 and β_2m in the promotion of pancreatic cancer cell migration, we first performed co-immunoprecipitation experiments on lysates of pancreatic cancer cell lines (S2-013, PANC-1, and MIA PaCa-2). The antibody W6/32 is very well established as having specificity for β_2m/HLA class I/peptide complexes (Carreno and Hansen 1994; Ladasky et al., 1999; Parham et al., 1979; Shields et al., 1998). Immunoprecipitations were done with the W6/32 antibody (and control immunoprecipitations were also performed). The immunoprecipitates were

Figure 3.2. *β2m knockdown by siRNA decreases the migration rate of S2-013 and PANC-1 pancreatic cancer cells, increases the migration rate of MIA PaCa-2 pancreatic cancer cells, and does not significantly alter the migration of non-transformed hTERT-HPNE pancreatic cells.* Simple Westerns were performed on lysates of the indicated cells to confirm knockdown of β_2m at 72 h post-transfection. Actin was used as the control. To assess migration, transwell assays were performed with each of the cell lines. Cells were replated 72 h post-transfection into 8-μm inserts and incubated for 24 h. Three random fields were photographed and counted and graphed based on the average numbers of migrated cells. For the S2-013 cell line, the results from 9 separate migration experiments were compiled, for PANC-1, data were acquired in 8 separate experiments, and for MIA PaCa-2 there were 5 repetitions of the migration experiment performed. The protein expression data shown correspond to one of the migration experiments with each cell line, and are representative of the whole set of data obtained to confirm knockdown for each of the experiments. The statistical significance of the results was analyzed with Student's two-tailed t test and the P values are shown on the graphs. Each error bar represents the standard error of the mean.

Figure 3.2.


hTERT-HPNE
Scramble
siβ2m
42 kD
Actin
12 kD
β2m
Av. # of migrated cells
p=0.4169
Scramble
siβ2m
S2-013
p=0.0001
PANC-1
p=0.0016
MIA PaCa-2
p=0.0002

immunoblotted with an antibody for APLP2, along with an antibody for the HLA class I heavy chain, as a control to verify the success of the immunoprecipitation. As shown in **Figure 3.3**, the immunoblots demonstrate the association of APLP2 with the HLA class I heavy chain/β_2m/peptide complex in S2-013 (as previously reported) (Tuli et al., 2009a) and in PANC-1 cells, but not in MIA PaCa-2 cells. Association of higher molecular weight GAG-APLP2 with the HLA class I molecules was not observed in any of these cell lines (data not shown). HLA class I heavy chains are highly polymorphic, (Robinson et al., 2015; Robinson et al., 2016) and their extensive polymorphism may account for the association of APLP2 with HLA class I molecules in the S2-013 and PANC-1 pancreatic cancer cell lines but not the MIA PaCa-2 cell line.

3.3.2 Effects of β_2m knockdown on APLP2 protein levels

To further investigate if a linkage might exist between APLP2 and β_2m in facilitating pancreatic cancer cell migration, we next evaluated the effect of β_2m on the level of APLP2 in the pancreatic cancer cell lines. We observed a diminution of both GAG-APLP2 and APLP2 expression when β_2m siRNA was transfected into the S2-013 pancreatic cancer cell line (**Figure 3.4**). In addition, it was also evident that β_2m knockdown reduced the level of APLP2 in the PANC-1 cell line (**Figure 3.4**). The PANC-1 cell line expresses very little GAG-APLP2 (**Figure 3.4**). Knockdown of β_2m in MIA PaCa-2 had a distinctly different effect, causing a substantial increase, rather than a decline in both APLP2 and GAG-APLP2 levels. When the hTERT-HPNE cells were transfected with β_2m siRNA, there was neither substantial upregulation nor downregulation of APLP2 or GAG-APLP2 expression (**Figure 3.4**).

3.3.3 Effects of β_2m knockdown on HLA class I heavy chains

Figure 3.3. *APLP2 associates with β2m/HLA class I heavy chain/peptide complexes in S2-013 and PANC-1 but not MIA PaCa-2.* The W6/32 antibody which only recognizes HLA class I heavy chains when they are bound to β_2m and peptide, was used for immunoprecipitations of HLA class I molecules from lysates of S2-013, PANC-1, or MIA PaCa-2 cells. The lanes marked (+) indicate immunoprecipitations in which cell lysates were included, and lanes marked (-) indicate control mock immunoprecipitations in which cell lysates were omitted. As a negative control, an isotype-matched mouse IgG antibody (the 30-5-7 antibody) was used, which has specificity for the mouse MHC class I molecule L^d and not for HLA class I molecules. After electrophoresis, the proteins on the gel were transferred to a membrane and probed with anti-APLP2 antiserum to reveal APLP2 (or the higher molecular weight GAG-APLP2) or with the HC10 antibody to verify HLA class I immunoprecipitation.

Figure 3.3.

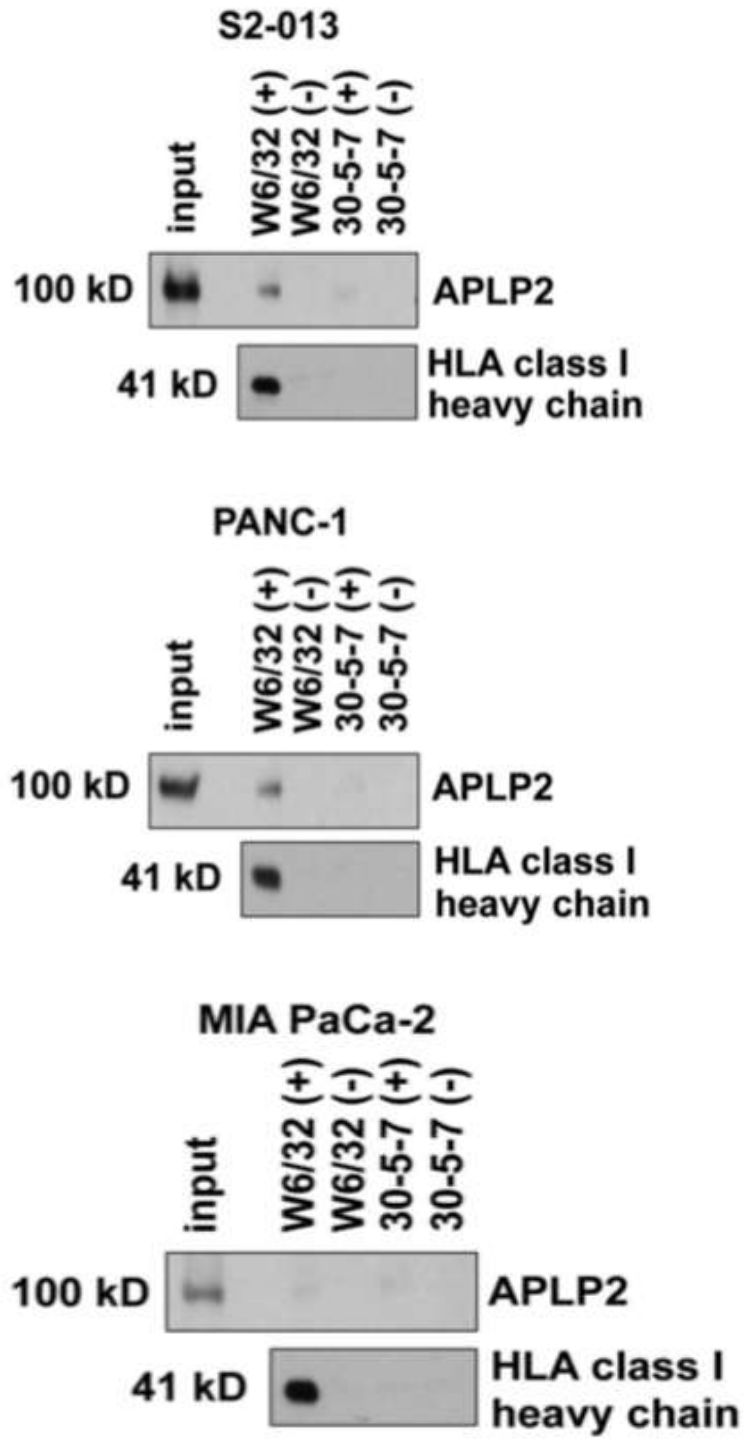
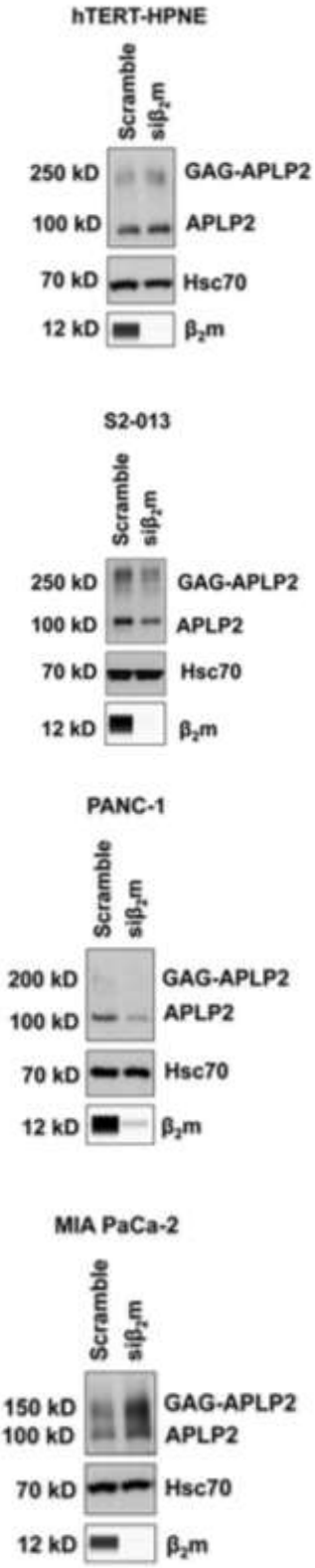

S2-013
input
W6/32 (+)
W6/32 (-)
30-5-7 (+)
30-5-7 (-)
100 kD
APLP2
41 kD
HLA class I heavy chain
PANC-1
input
W6/32 (+)
W6/32 (-)
30-5-7 (+)
30-5-7 (-)
100 kD
APLP2
41 kD
HLA class I heavy chain
MIA PaCa-2
input
W6/32 (+)
W6/32 (-)
30-5-7 (+)
30-5-7 (-)
100 kD
APLP2
41 kD
HLA class I heavy chain

Figure 3.4. *β2m knockdown by siRNA reduces the APLP2 expression level in S2-013 and PANC-1 pancreatic cancer cells, elevates APLP2 expression in MIA PaCa-2 pancreatic cancer cells, and does not significantly change the level of APLP2 in non-transformed hTERT-HPNE pancreatic cells.* The indicated cell lines were cultured with β_2m siRNA (or scrambled sequence control siRNA) for 72 h and cell lysates were collected and tested by Simple Western for β_2m (to verify β_2m knockdown by siRNA) and immunoblotted with an antibody for APLP2 or for Hsc70 (as the control). The APLP2-specific antibody recognizes both APLP2 and the high molecular weight GAG-modified form of APLP2. The results shown are representative of 5 independent experiments for S2-013, PANC-1, and MIA PaCa-2, and of 2 independent experiments for hTERT-HPNE.

Figure 3.4.


hTERT-HPNE
Scramble
siβ2m
250 kD
GAG-APLP2
100 kD
APLP2
70 kD
Hsc70
12 kD
β2m
S2-013
Scramble
siβ2m
250 kD
GAG-APLP2
100 kD
APLP2
70 kD
Hsc70
12 kD
β2m
PANC-1
Scramble
siβ2m
200 kD
GAG-APLP2
100 kD
APLP2
70 kD
Hsc70
12 kD
β2m
MIA PaCa-2
Scramble
siβ2m
150 kD
GAG-APLP2
100 kD
APLP2
70 kD
Hsc70
12 kD
β2m

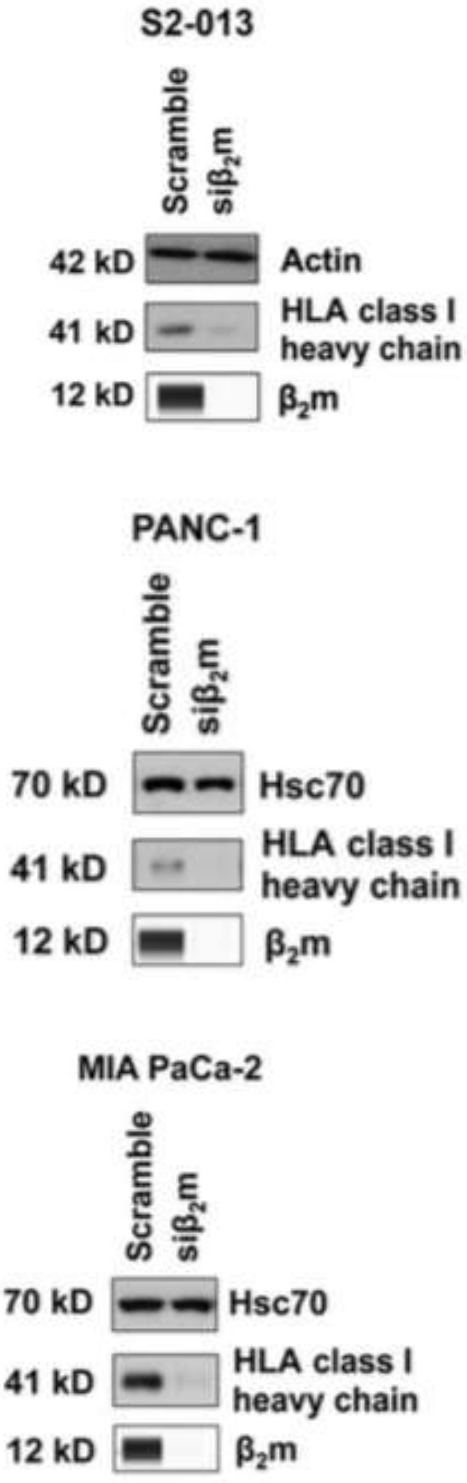
For comparison, we also examined the impact on the HLA class I heavy chain level when β_2m expression was knocked down in pancreatic cancer cell lines. When β_2m expression was down regulated by siRNA, the HLA class I heavy chain expression was lower for all three of the pancreatic cancer cell lines tested (PANC-1, MIA PaCa-2, and S2-013) (**Figure 3.5**). Thus, HLA class I heavy chain expression is linked to β_2m expression and consistently decreases in coordination with β_2m, likely due to rapid turnover of the HLA class I heavy chains when β_2m is not available to stabilize it. These findings indicate that the increase in APLP2 (and GAG-APLP2) in MIA PaCa-2 is not due to a global rise in protein expression when the cells are deficient in β_2m, since the expression of the control Hsc70 protein in unchanged (**Figure 3.4**) and the expression of the HLA class I heavy chain is decreased (**Figure 3.5**).

3.4 Discussion

Even though β_2m is a subunit of the HLA class I molecule that presents tumor antigens marking pancreatic cancer cells as cytotoxic T cell targets, our findings with pancreatic cancer cell lines (**Figure 3.1**) suggest that β_2m is not typically down regulated in pancreatic cancer. A study utilizing immunohistochemical staining demonstrated that β_2m is amply expressed in human pancreatic cancer tissue (Liu et al., 2015). In addition, two data sets on β_2m protein expression in pancreatic tumor cells are included in the Human Protein Atlas (https://www.proteinatlas.org/ENSG00000166710-B2M/pathology) (Uhlen et al., 2010; Uhlen et al., 2015). Of these sets, the first reports that 10 patients had tumors expressing β_2m (with β_2m not detected in 2 other patients' tumors), and the second set reports that 11 of 11 patients' tumors expressed β_2m (6 with high, 4 with medium, and 1 with low expression). These results on human pancreatic

Figure 3.5. *β2m knockdown by siRNA causes a deficiency in HLA class I heavy chain expression in all three pancreatic cancer cell lines tested.* S2-013, PANC-1, and MIA PaCa-2 cells were cultured with β_2m siRNA (or scrambled sequence control siRNA) for 72 h and cell lysates were collected. The lysates were tested for β_2m by Simple Western (to verify β_2m knockdown by siRNA) and immunoblotted for the HLA class I heavy chain (with antibody HC10) or for a control protein (actin for S2-013 and Hsc70 for PANC-1 and MIA PaCa-2). The results shown are representative of 8 independent experiments for S2-013 and of 3 separate experiments for PANC-1 and MIA PaCa-2.

Figure 3.5.


S2-013
Scramble
siβ2m
42 kD
Actin
41 kD
HLA class I heavy chain
12 kD
β2m
PANC-1
Scramble
siβ2m
70 kD
Hsc70
41 kD
HLA class I heavy chain
12 kD
β2m
MIA PaCa-2
Scramble
siβ2m
70 kD
Hsc70
41 kD
HLA class I heavy chain
12 kD
β2m

cancer tissue are commensurate with our findings of β_2m at substantial levels in pancreatic cancer cell lines.

We found that β_2m increases the migration of two pancreatic cancer cell lines (PANC-1 and S2-013), and decreases the migration of a third (MIA PaCa-2) (**Figure 3.2**). In our analysis of the mechanism by which β_2m exerts these effects on pancreatic cancer cell migration, we assessed the possible role of APLP2 in its effects, since we had previously shown APLP2/HLA class I molecule association in S2-013 cells and we had demonstrated by transwell and wound healing assays that APLP2 is a pro-migratory and pro-metastasis protein in pancreatic cancer cells (Pandey et al., 2015; Tuli et al., 2009a; Wen et al., 2003). Consistent with our theory that APLP2 might be linked to β_2m's effect on pancreatic cancer cell migration, we observed that APLP2 co-immunoprecipitates with the β_2m/HLA class I heavy chain/peptide complex specifically in the same two pancreatic cancer cell lines for which β_2m increases migration (PANC-1 and S2-013) (**Figure 3.3**). Upon knockdown of β_2m, the expression of APLP2 was also decreased selectively in S2-013 and PANC-1, but increased in MIA PaCa-2 (**Figure 3.4**). In contrast, knockdown of β_2m was not selective in regard to its effect on HLA class I expression, as it caused a decline in the level of HLA class I heavy chain in all three cell lines (**Figure 3.5**).

Although APLP2 is known to regulate the migration of pancreatic cancer cells (Pandey et al., 2015), our understanding of how it does so is incomplete. We previously showed that APLP2 knockdown in a pancreatic cancer cell line has a dramatic impact on the actin cytoskeleton (Pandey et al., 2015). Therefore, APLP2 regulation of actin polymerization may be responsible for its ability to increase the rate of migration (Pandey et al., 2015). In *Drosophila*, APLP2 was also recently reported to facilitate cell migration via c-Jun N-terminal kinase phosphorylation (Wang et al., 2018). APLP2 (called YWK-II in this case), when bound by Müllerian-inhibiting substance in YWK-II-

transfected CHO cells, triggers signaling that results in ERK1/2 activation (Huang et al., 2000; Yin et al., 2007). In a separate study that used APLP2-transfected CHO cells, APLP2 was found to act as an adhesion protein in response to fibronectin and type IV collagen, which are both present in the extracellular matrix. Furthermore, in some publications, the GAG group on APLP2 has been suggested to contribute to APLP2's ability to assist in migration (Guo et al., 1998; Li et al., 1999), and so the GAG moiety may contribute, potentially by altering extracellular matrix interactions *in vivo*.

APLP2 and MHC class I molecules associate in a variety of cell types (Feuerbach and Burgert 1993; Morris et al., 2003; Peters et al., 2013; Tuli et al., 2008a; Tuli et al., 2008b; Tuli et al., 2009a; Tuli et al., 2009b). The subcellular itinerary of associated APLP2 and MHC class I molecules has been analyzed in earlier studies. More APLP2 is associated with MHC class I molecules when the adenovirus E3/19K protein (which has a natural endoplasmic reticulum retention sequence) is present in cells (Feuerbach and Burgert 1993; Morris et al., 2003; Sester et al., 2000), which indicates that APLP2/MHC association starts in the endoplasmic reticulum. APLP2 interaction with MHC class I molecules is dependent on the presence of β_2m (Sester et al., 2000). We have also demonstrated that APLP2 is co-localized with MHC class I molecules in the Golgi and in early endosomes and recycling endosomes (Tuli et al., 2008a; Tuli et al., 2009a; Tuli et al., 2009b). In addition, in our previous work, we found that APLP2 and MHC class I molecules are associated at the cell surface and internalize together into early endosomes by a process that requires Tyrosine 755 in the APLP2 C-terminal region (Tuli et al., 2009a)

Together, these findings suggest that β_2m (as part of the complete HLA class I molecule) potentially exerts a stabilizing effect on HLA-associated APLP2 proteins in S2-013 and PANC-1 at the cell surface, where APLP2 could contribute to migration via interactions with the extracellular milieu. Alternatively, since MHC class I molecules co-

localize with APLP2 in the recycling endosome and in the lysosome, (Tuli et al., 2009a; Tuli et al., 2009b) β_2m (as an MHC class I molecule component) that is associated with APLP2 may delay the release of secreted APLP2 at the surface from recycled endosomes and/or retard the degradation of APLP2 in the lysosome.

Notably, in MIA PaCa-2 cells, in which APLP2 and β_2m/HLA class I/peptide complexes do not detectably associate, the level of APLP2 is still influenced by β_2m; however, both APLP2 expression and cell migration are actually increased when β_2m expression is knocked down in these cells (**Figure 3.2 and Figure 3.4**). In MIA PaCa-2, APLP2 and β_2m/HLA class I/peptide complexes may be interacting but in a more unstable or transient manner, preventing detection. The knockdown of β_2m by siRNA might cause a distinctive change in the localization of APLP2, in comparison to its localization in S2-013 and PANC-1, with subsequent effects on the APLP2 level and cell migration. With or without inducing an alteration in APLP2 subcellular localization, the absence of β_2m might alter APLP2 interaction with other intracellular proteins, perhaps due to the resultant increased level of APLP2. Besides the MHC class I molecule, APLP2 has been shown to associate with several other proteins. For example, in brain tissue APLP2 interacts with RAC1, PP2ac, and RHOA, which are also expressed in the pancreas and have known functions in pancreatic cancer cell migration. (Bai et al., 2008; Ungefroren et al., 2018; Melzer et al., 2017; Mu et al., 2018; Witte et al., 2017; Wu et al., 2014). Furthermore, a small (6-kilodalton) C-terminal fragment of APLP2 associates with the CP2 transcription factor and stimulates the expression of glycogen synthase kinase (GSK)-3β (Xu et al., 2007). Although this APLP2/GSK-3β study was not done with pancreatic cancer cells, its findings could potentially have a relationship to pancreatic cancer too, as GSK-3β is a protein that has been linked to pancreatic cancer invasiveness (Kitano et al., 2013).

It may be relevant to our findings that MIA PaCa-2 differs from PANC-1 and S2-013 in the type of K-ras mutation and TP53 mutation. MIA PaCa-2 has Kras Gly12Cys and mutant TP53 Arg248Trp mutations, whereas S2-013 and PANC-1 have alternative mutations in Kras and TP53 that are more commonly found in pancreatic cancer cells (ExPASy Bioinformatics Resource Portal https://web.expasy.org/cellosaurus). The presence of the Gly12Cys mutation in Kras has been previously noted in other cancer cell types to alter signaling pathways. In non-small cell lung cancer cells, Kras Gly12Cys leads to activated Ral signaling and a decline in growth factor-dependent Akt activation, whereas Kras Gly12Asp cells have activated PI3K and MEK signaling (Ihle et al., 2012). Additionally, MIA PaCa-2 but not PANC-1 is sensitive to a MEK inhibitor used without concurrent Akt inhibitor, due to the MIA PaCa-2 Kras Gly12Cys mutation (Brauswetter et al., 2017). Thus, MIA PaCa-2 utilizes signaling pathways that are separate from those of S2-013 and PANC-1, and that may intersect with APLP2 in different manners and with distinct outcomes.

HLA class I molecules are encoded by three genetic loci (HLA-A, B, and C); thus, there are three HLA class I isotypes. Furthermore, as mentioned above, HLA class I heavy chains are extremely polymorphic, with an enormous number of allele products (protein allotypes), (Robinson et al., 2015; Robinson et al., 2016) and such polymorphism may influence the presence or absence of association of APLP2 with specific β_2m/peptide/HLA class I allotype complexes that are disparate among pancreatic cancer cell lines. To determine whether MHC class I polymorphism dictates whether or not APLP2 can associate with specific β_2m/HLA class I/peptide complexes in pancreatic cancer cells, we have begun an examination of the impact of individual HLA class I heavy chains allotypes on APLP2 expression in pancreatic cancer cells. Preliminary results from our experiments that are underway show very interesting patterns of HLA class I heavy chain isotype-specific and polymorphic allotype-specific

effects on APLP2 expression, but these are sufficiently complex as to be beyond this scope of this report.

In total, our discoveries indicate that β_2m significantly affects the migration of pancreatic cancer cells, and so may play a role in regulating pancreatic cancer metastasis. The Human Protein Atlas indicates a significantly lower survival probability for pancreatic cancer patients who have higher levels of tumor β_2m expression (https://www.proteinatlas.org/ENSG00000166710B2M/pathology/tissue/pancreatic+cancer#ihc), which is consistent with our findings of increased migration for the S2-013 and PANC-1 cells when β_2m is not knocked down. Pancreatic cancer is extremely metastatic, and it is a high and rising cause of cancer-related mortality (Rahib et al., 2014; Siegel et al., 2018). The five-year survival rate is already among the worst for any major cancer, with only ~7% of patients in 2018 expected to survive past this mark (Siegel et al., 2018). Acquisition of information about the factors likely to add to the metastatic pathobiology of this disease, from studies such as this one, will improve our comprehension of pancreatic cancer and potentially contribute to the development of better therapeutic approaches to target it.

CHAPTER 4: HLA-B and its effects on pancreatic cancer cell migration

4.1 Introduction to HLA-B and integrins

HLA class I molecules are composed of a heavy chain, the light chain β_2m, and endogenous peptide (Krangel et al., 1979). These heavy chains are encoded by three separate genetic loci that encode for three similar yet not completely identical sequences, which are termed isotypes. These isotypes include an –A, -B, and –C isotype (Townsend et al., 1989). These molecules are best known for being part of the adaptive immune system and presenting antigens to cytotoxic T cells. Peptide presentation to T cells leads to lysis of the target cell and this allows for protection against viral infection or malignant transformation (Benacerraf 1981). Recently, in the field of transplantation biology, HLA class I molecules have been implicated in affecting processes such as cell migration (Zhang et al., 2010). In terms of cancer, although these molecules can be down-regulated as a form of immune escape, there have been studies in various cancer types, including medulloblastoma and breast, gastric, and lung cancer have shown that high expression of these molecules can be associated with poor prognosis (Madjd et al., 2005; Ramnath et al., 2006; Smith et al., 2011; Ueda et al., 2008). Furthermore, HLA class I molecules were found to promote increased medulloblastoma cell migration upon addition of exogenous β_2m, which stabilizes cell-surface HLA class I heavy chains that have released their β_2m light chains after arrival at the plasma membrane, through an ERK1/2-mediated mechanism (Smith et al., 2009; Smith et al., 2011). However, even though pancreatic cancer is known for its high rate of metastasis, an investigation of the role HLA class I molecules in cell migration in this disease has not been reported. Additionally, the function that specific isotypes have in this process has also never been fully addressed.

A few previous studies in transplantation biology models have indicated that integrins interact with HLA class I molecules and that this interaction can contribute to

cell migration in these models (Zhang et al., 2010; Zhang and Reed 2012). Integrins are cell surface molecules that function as heterodimers and that are present on nearly all nucleated cells. There are 8 α subunits and 18 β subunits that can pair together to form 24 distinct functional heterodimers. A specific range of heterodimers can exist on particular cell types and they function mainly to bind to certain components of the extracellular matrix (ECM) (Bianconi et al., 2016; Ginsberg 2014; Hynes 2002). The α2β1 heterodimer binds mainly to collagen but also has the ability to bind to laminin, fibronectin, and E-cadherin (Giancotti and Ruoslahti 1999; Gout et al., 2001; Plow et al., 2000; Stipp 2010; Tuckwell et al., 1995; Whittard et al., 2002). This heterodimer is overexpressed in many cancer types including pancreatic cancer and is associated with an aggressive disease phenotype (Danen 2005; Grzesiak et al., 2006; Grzesiak et al., 2007; Guo and Giancotti 2004; Madamanchi et al., 2010; McCall-Culbreath and Zutter 2008; Mizejewshi 1999). The α2β1 heterodimer also was shown to promote the migration of pancreatic cancer cells as well as various other cancer cell types such as gastric cancer and prostate cancer (Bassaganas et al., 2014; Chuang et al., 2018; Grzesiak et al., 2006; Grzesiak et al., 2011; Hall et al., 2008; Yang et al., 2003). Whether an interaction exists between integrins and HLA class I molecules in pancreatic cancer and if this interaction contributes to pancreatic cancer cell migration has yet to be addressed.

Thus, the focus of this study was to investigate whether the specific HLA-B isotype influences the migration of pancreatic cancer cells, and if so, to assess the potential involvement of integrin β1 (ITGB1) and integrin α2 (ITGA2) in this mechanism. The human pancreatic cancer cell lines that we analyzed were found to express substantial levels of HLA-B. When expression of HLA-B was experimentally down-regulated by siRNA transfection using a pan-HLA-B siRNA in pancreatic cancer cell lines, the migration of S2-013 cells was significantly increased while in contrast the

migration of PANC-1 and MIA PaCa-2 cells was significantly reduced. From examination of overall protein expression of ITGB1, it was found that in S2-013 cells, there was an increase in ITGB1 expression when HLA-B expression was knocked down. In the PANC-1 and MIA PaCa-2 cells, there was conversely a decrease in ITGB1 expression. Similar results were observed for the S2-013 cell line when surface expression of ITGB1 was investigated. Since ITGB1 is able to form a heterodimer with ITGA2, ITGA2 was also investigated. Interestingly, total protein levels of ITGA2 did not change in the S2-013 or PANC-1 cell line but surface expression followed the same expression pattern as ITGB1, increasing in S2-013 cells upon HLA-B knockdown. MIA PaCa-2 cells, however, do not express ITGA2. A downstream effector of integrin signaling, focal adhesion kinase (FAK), show similar changes in total level of expression and in its phosphorylation status, indicating alterations to this pathway that have the ability to affect cell migration.

These data thus indicate that HLA-B is amply expressed in pancreatic cancer cells and influences their migration, and that HLA-B regulates the expression of ITGB1 and correspondingly ITGA2, which together have been previously established to affect the migration of pancreatic cancer cells. Therefore, our findings suggest that HLA-B could be a factor that alters the migration of pancreatic cancer cells through its effects on the α2β1 heterodimer.

4.2 HLA-B in pancreatic cancer cell migration

4.2.1 HLA-B is amply expressed in pancreatic cancer cell lines

To assess levels of HLA-B in a panel of pancreatic cancer cell lines by immunoblotting, we analyzed lysates from S2-013, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines and compared them to hTERT-HPNE cells, an immortalized but not

transformed pancreas ductal cell line. As shown in **Figure 4.1**, all of the cell lines tested have expression of HLA-B and the expression is comparable to that in hTERT-HPNE cells. These data suggest that, despite the well-known anti-cancer immunological function of this molecule, the HLA-B isotype is not necessarily lost or down-regulated in human pancreatic cancer cell lines.

4.2.2 HLA-B's influence on pancreatic cancer cell migration

To investigate whether the HLA-B isotype affects pancreatic cancer cell migration, we transfected S2-013, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines with siRNA specific for pan-HLA-B (or with control scrambled siRNA). After siRNA transfection, the reduction of HLA-B protein levels was confirmed in the pancreatic cancer cell lines tested (**Figure 4.2**). By transwell assay, the migration rate of the S2-013 cell line was determined and was found to be significantly increased when HLA-B expression was knocked down. In contrast, when we down-regulated the expression of HLA-B in PANC-1 and MIA PaCa-2 cells, the migration rate was significantly impaired (**Figure 4.2**). These findings indicate that HLA-B affects the migration rate of pancreatic cancer cells.

4.3 Integrins and HLA-B in pancreatic cancer cells

4.3.1 Effects of HLA-B knockdown on integrin total protein expression

To examine what could potentially be mediating the differential effects that HLA-B exerts on pancreatic cancer cell migration, integrin expression was interrogated as integrins have a major role in cancer cell migration, including pancreatic cancer cell

Figure 4.1. *The HLA heavy chain isotype HLA-B remains abundantly expressed in human pancreatic cancer cell lines.* Immunoblots to detect HLA-B and Hsc70 (as an internal control) were performed on an untransformed pancreas duct cell line (hTERT-HPNE) and S2-013, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines. The results shown are representative of three experimental replicates to assess HLA-B in lysates from these cell lines.

Figure 4.1.

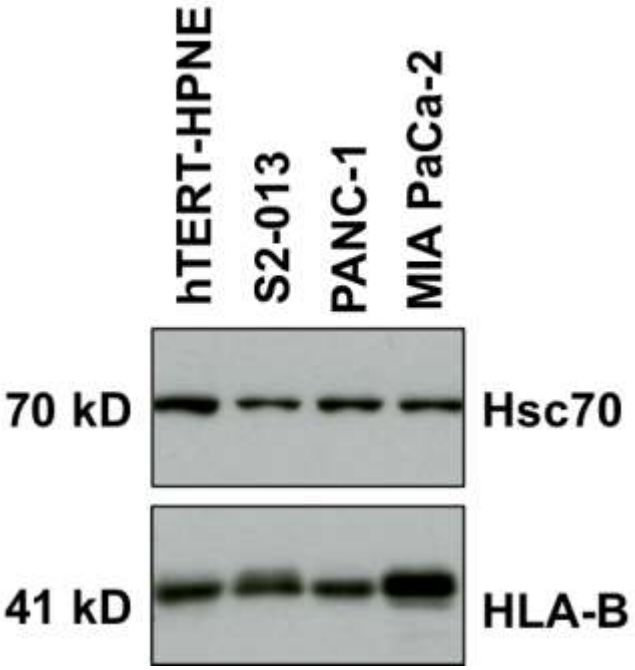
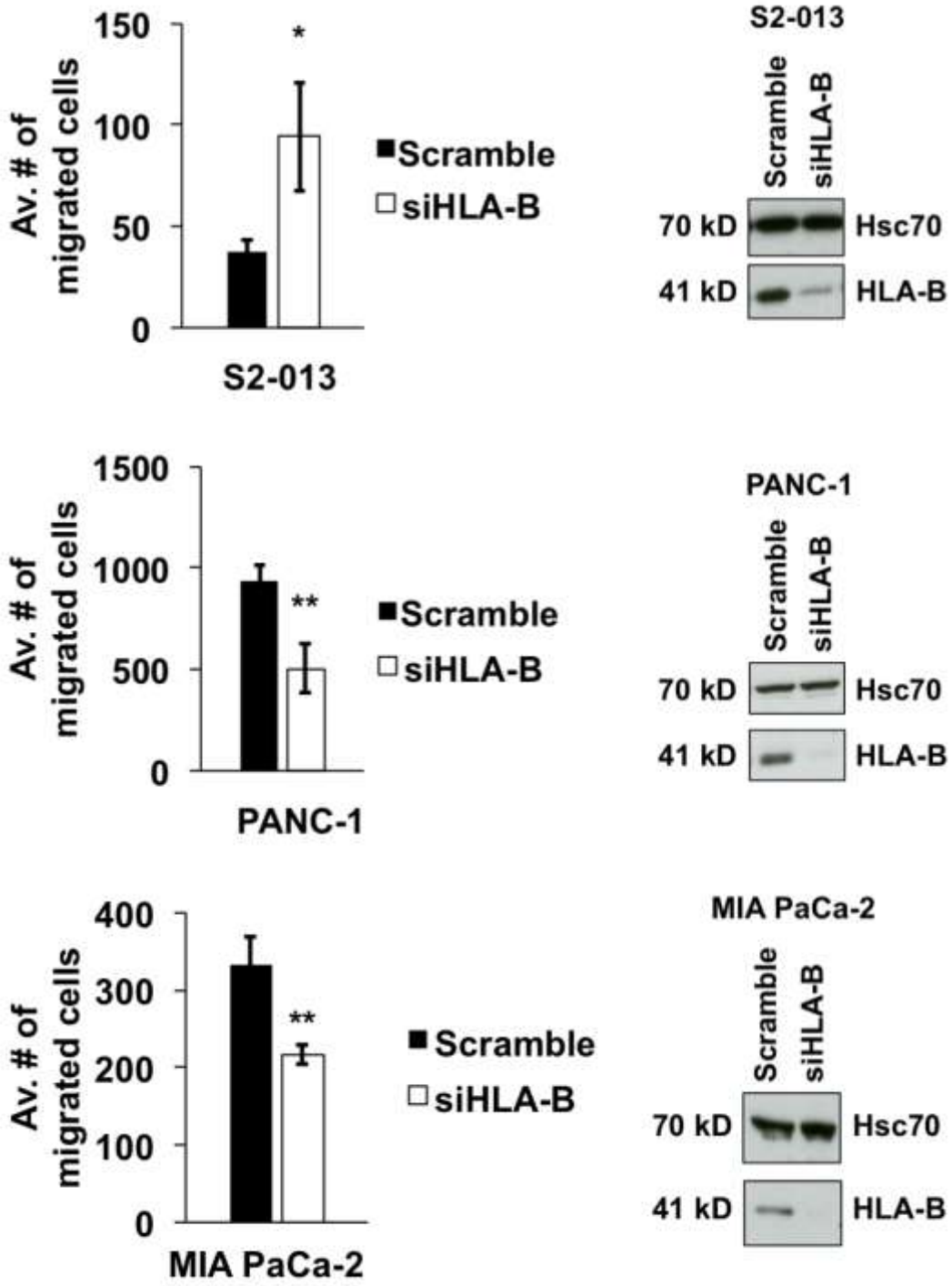


Figure 4.2. *The siRNA knockdown of HLA-B increases migration of S2-013 pancreatic cancer cells but diminishes the migration of PANC-1 and MIA PaCa-2 pancreatic cancer cells.* Immunoblots were performed on lysates from the same cell lines used for migration assays to verify knockdown of total HLA-B 72 h post-transfection. Hsc70 was used as a loading control. The knockdown immunoblotting results shown are representative of all experiments performed for each cell line. The migration of each pancreatic cancer cell line was assessed by transwell assay. At 72 h post-transfection, the cells were replated into 8-μm inserts and incubated for 24 h. Photographs were taken of three random fields, the cells in each of the fields were counted, and the results were graphed as average number of cells migrated. For each cell line, 5 repetitions were performed. Error bars represent standard error of the mean. Student's two-tailed t-test was used for assessment of statistical significance, and *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 4.2.


Av. # of migrated cells
150
100
50
0
*
Scramble
siHLA-B
S2-013
S2-013
Scramble
siHLA-B
70 kD
Hsc70
41 kD
HLA-B
1500
1000
500
0
**
PANC-1
PANC-1
MIA PaCa-2
400
300
200
100
0
**
MIA PaCa-2

migration (Bassaganas et al., 2014; Chuang et al., 2018; Grzesiak et al., 2006; Grzesiaket al., 2011; Hall et al., 2008; Yang et al., 2003). Due to their high prevalence in heterodimeric form in pancreatic cancer, ITGA2 and ITGB1 were chosen to be investigated (Danen 2005; Grzesiak et al., 2006; Grzesiak et al., 2007; Guo and Giancotti 2004; Madamanchi et al., 2010; McCall-Culbreath and Zutter 2008; Mizejewshi 1999). Firstly, the expression levels of ITGB1 and ITGA2 were analyzed in pancreatic cancer cell lines, tested in comparison to the hTERT-HPNE cells. Expression of both molecules was confirmed in all pancreatic cancer cell lines tested with the exception of MIA PaCa-2 cells, which lack expression of ITGA2 (**Figure 4.3**). Expression of these integrins were then assessed upon HLA-B siRNA transfection. In the S2-013 cells, when HLA-B was knocked down, an increase in the protein expression of ITGB1 was observed (**Figure 4.4A**). Interestingly, however, no significant change in ITGA2 protein expression was observed in this cell line when HLA-B was knocked down (**Figure 4.4A**). Conversely, in PANC-1 and MIA PaCa-2 cells, there was a decrease in ITGB1 expression when HLA-B was knocked down in both of these cell lines (**Figure 4.4B-C**). ITGA2 also remained unchanged in the PANC-1 cells when HLA-B was knocked down (**Figure 4.4C**). MIA PaCa-2 cells do not express ITGA2 and no changes were observed in this cell line (**Figure 4.3**).

4.3.2 Effects of HLA-B knockdown on integrin surface expression

Most of the effects of integrin molecules take place at the cell surface; therefore, we interrogated if the surface expression of these molecules also follows the same trend as total protein level. By flow cytometry, surface expression of both ITGB1 and ITGA2 was investigated on the S2-013 pancreatic cancer cell line. In these cells, when HLA-B was knocked down, an increase in ITGB1 at the cell surface was also seen (**Figure 4.5A**). Interestingly, there was also an increase in ITGA2 at the cell surface,

Figure 4.3. *Expression of ITGA2 in pancreatic cancer cell lines.* Lysates from hTERT-HPNE non-transformed cells as well as from a panel of pancreatic cancer cell lines were collected and immunoblotted for ITGA2 and Hsc70 (loading control). The results are representative of experiments performed on 2 separate lysates of each cell type.

Figure 4.3.

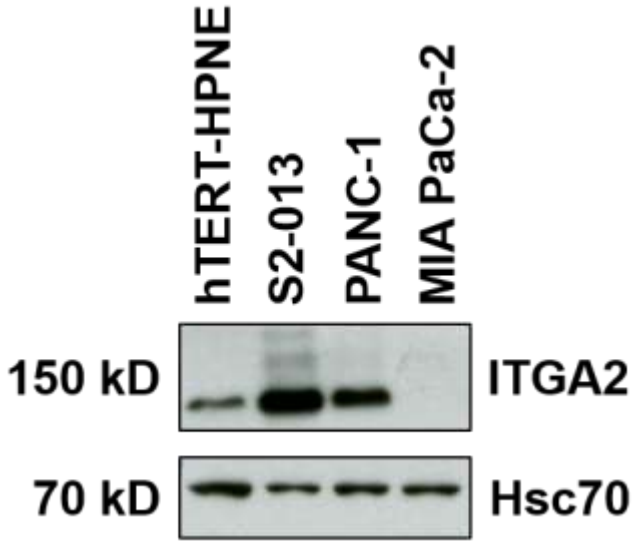
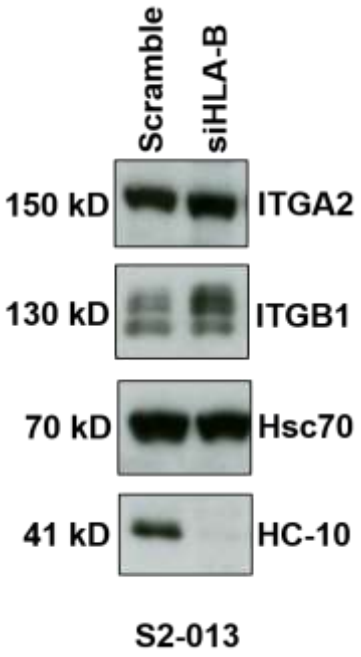


Figure 4.4. *Total expression of ITGB1 is increased in HLA-B knockdown in S2-013 cells but decreased in expression in PANC-1 and MIA PaCa-2 cells. ITGA2 total expression is not affected by HLA-B knockdown in either S2-013 or PANC-1 cells.* Cell lysates were collected from the abovementioned pancreatic cancer cell lines 72 h post-transfection. Lysates were immunoblotted for ITGB1, Hsc70 (loading control), and HLA-B (by HC-10 antibody) to confirm knockdown of this protein. The results shown are representative of (**A)** 2 separate experiments for S2-013. (**B**) For MIA PaCa-2 cells, results shown are from one experiment. (**C**) For PANC-1 cells, results are shown from 2 separate experiments.

Figure 4.4. (A)


Scramble
siHLA-B
150 kD
ITGA2
130 kD
ITGB1
70 kD
Hsc70
41 kD
HC-10
S2-013

(B)

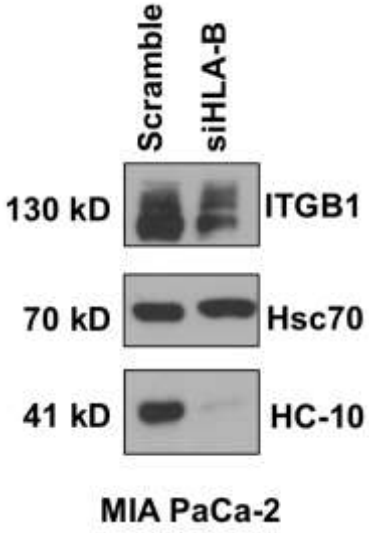
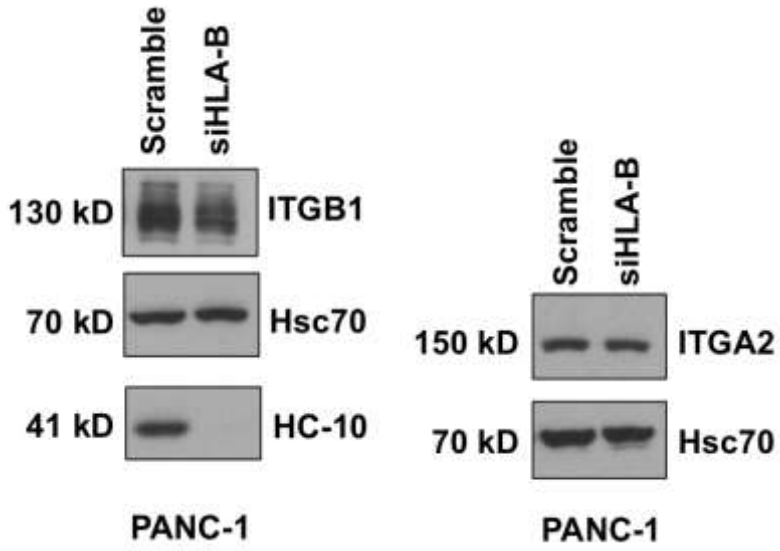

Scramble
siHLA-B
130 kD
ITGB1
70 kD
Hsc70
41 kD
HC-10
MIA PaCa-2

Figure 4.4. (C)


Scramble
siHLA-B
130 kD
ITGB1
70 kD
Hsc70
41 kD
HC-10
PANC-1
Scramble
siHLA-B
150 kD
ITGA2
70 kD
Hsc70
PANC-1

which is in contrast to total expression levels where no change was observed (**Figure 4.5B**).

4.4 Downstream signaling of HLA class I molecules and Integrins

One of the major and most well studied signaling molecules downstream of integrins is FAK (Bianconi et al., 2016). FAK is a cytoplasmic non-receptor tyrosine kinase (Parsons et al., 2000). Ligand binding leads to clustering of integrins at which point activation of FAK by autophosphorylation at Tyr397 can occur. This position on FAK is a binding site for other kinases including Src and PI3K (Chen et al., 1996; Cobb et al., 1994; Schaller et al., 1994). The integrin-FAK pathway has been associated with many aspects of pancreatic cancer metastasis such as adhesion, migration, and invasion (Furuyama et al., 2006; Itoh et al., 2004; Kanteti et al., 2016; Miyazaki et al., 2003). Therefore, to investigate further downstream signaling controlled by the HLA class I molecules and the α2β1 heterodimer, pFAK Tyr397 and total FAK expression was investigated in pancreatic cancer cell lines upon knockdown of HLA-B. HLA-B knockdown in S2-013 pancreatic cancer cells led to an increase in pFAK Y397 similarly to the increase in ITGB1 that was also observed as a result of HLA-B knockdown (**Figure 4.6**). PANC-1 cells, which had a decrease in ITGB1, also had a decrease in pFAK Y397 (**Figure 4.6**). Interestingly, in all cell types, total FAK expression followed the same pattern as pFAK Y397 for all cell lines tested (**Figure 4.6**). Total FAK protein expression has been found to be upregulated in many different cancer types including pancreatic cancer and diminished expression of this protein can contribute to decreased cell migration through reduction in the amount of FAK available for activation (Furuyama et al., 2006; Itoh et al., 2004; Jiang et al., 2016; Kanteti et al., 2016; Miyazaki et al., 2003).

Figure 4.5. *ITGB1 is increased in expression upon HLA-B knockdown in S2-013 cells. The surface expression of ITGA2 is also higher on S2-013 cells in which HLA-B expression has been knocked down.* Cells were collected from the mentioned pancreatic cancer cell lines 72 h post-transfection and were stained for ITGB1. Flow cytometry was performed on cells and results were analyzed by DIVA software. The results are from one experimental replicate.

Figure 4.5.

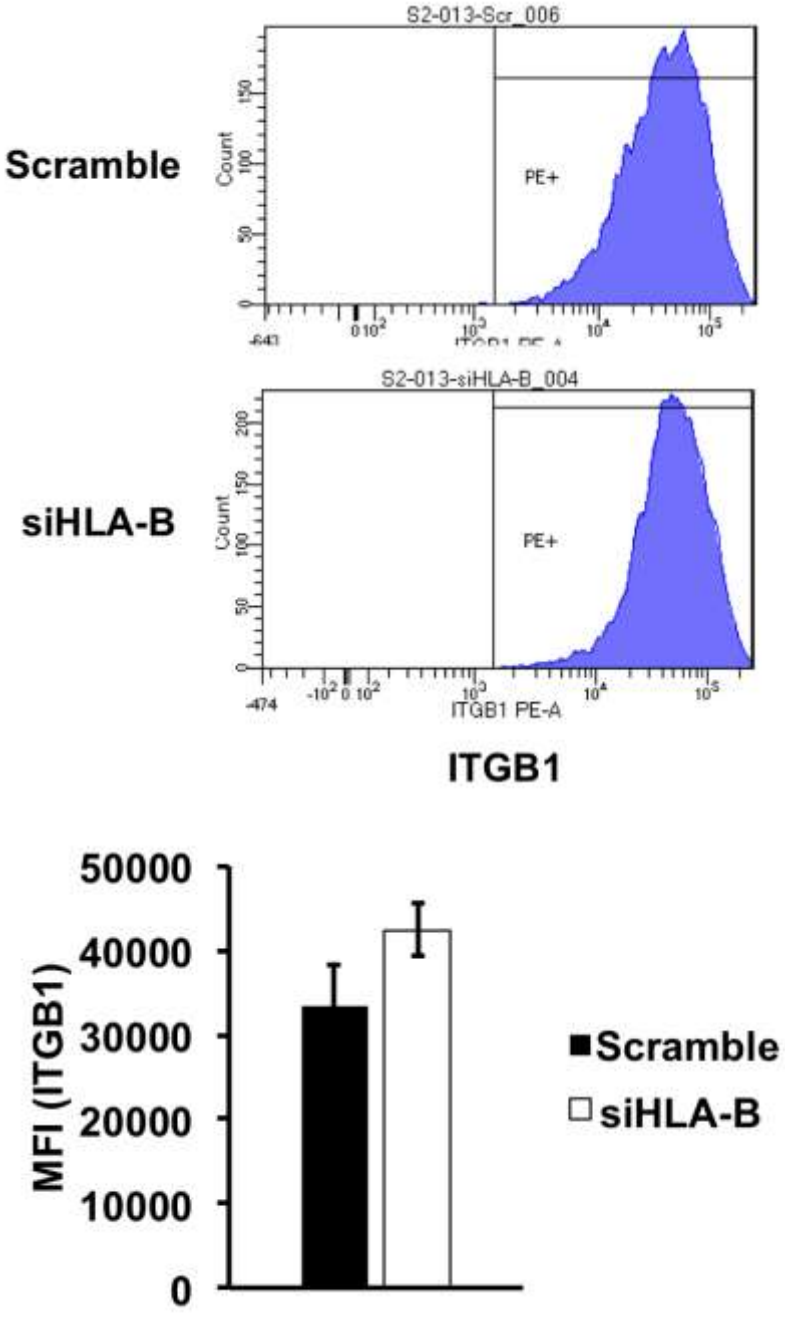

Scramble
S2-013-Scr_006
Count
PE+
siHLA-B
S2-013-siHLA-B_004
Count
PE+
ITGB1 PE-A
ITGB1
MFI (ITGB1)
50000
40000
30000
20000
10000
0
Scramble
siHLA-B

Figure 4.5.

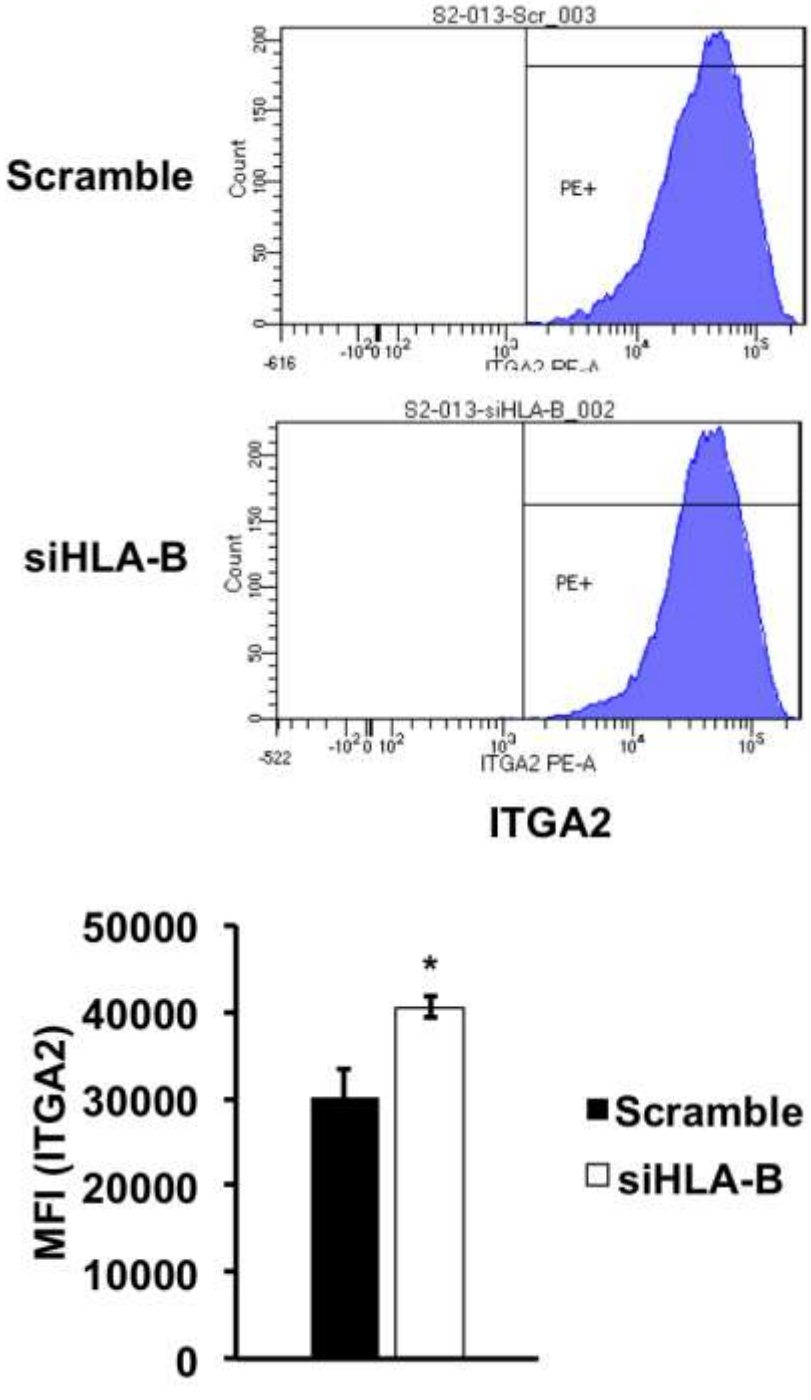
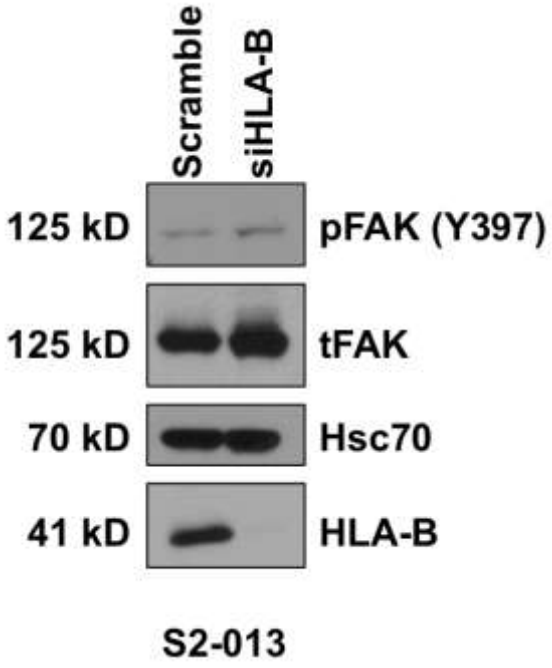
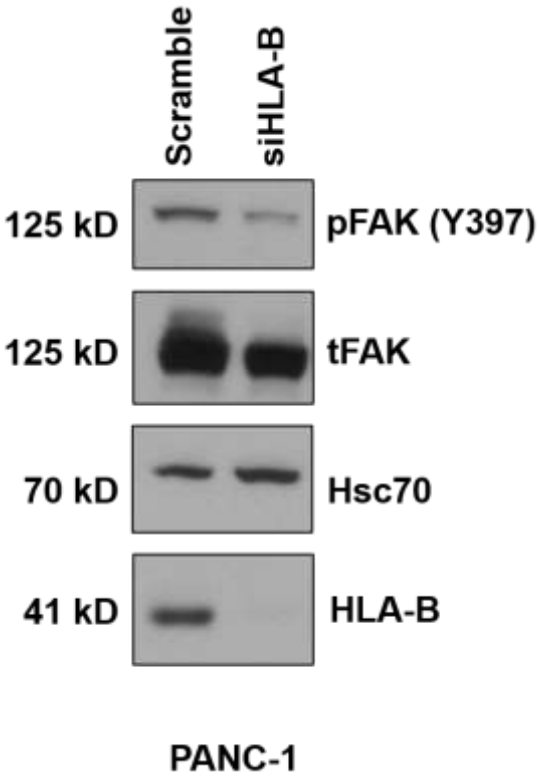

Scramble
S2-013-Scr_003
Count
PE+
siHLA-B
S2-013-siHLA-B_002
Count
PE+
ITGA2 PE-A
ITGA2
MFI (ITGA2)
50000
40000
30000
20000
10000
0
*
Scramble
siHLA-B

Figure 4.6. *Expression of FAK is increased in expression upon knockdown of HLA-B in S2-013 cells but FAK is decreased in expression in PANC-1 that have undergone knockdown of HLA-B.* Cell lysates were collected from the aforementioned pancreatic cancer cell lines 72 h post-transfection. Lysates were immunoblotted for pFAK (Y397), total FAK, Hsc70 (loading control), and HLA-B to confirm knockdown of this protein. The results are representative of experiments performed on 7 separate lysates for the S2-013 cell line and 2 separate lysates for the PANC-1 cell line.


Scramble
siHLA-B
125 kD
pFAK (Y397)
125 kD
tFAK
70 kD
Hsc70
41 kD
HLA-B
S2-013


Scramble
siHLA-B
125 kD
pFAK (Y397)
125 kD
tFAK
70 kD
Hsc70
41 kD
HLA-B
PANC-1

4.5 Discussion

Our findings suggest that HLA-B, even though having the capability of presenting tumor antigens to cytotoxic T cells, remains highly expressed in pancreatic cancer cell lines. A majority of studies investigating HLA class I molecule expression in pancreatic cancer interrogated total HLA class I expression but did not assess differences in expression among HLA class I isotypes in normal tissue as compared to malignant tissue (Pandha et al., 2006; Ryschich et al., 2004; Ryschich et al., 2005; Scupoli et al., 1996). Our findings for the first time indicate that the HLA-B isotype is not robustly down-regulated in pancreatic cancer cell lines (**Figure 4.1**).

Additionally, we found that HLA-B slows the migration of a pancreatic cancer cell line (S2-013) while it increases the migration of two other pancreatic cancer cell lines (PANC-1 and MIA PaCa-2) (**Figure 4.2**). In our analysis of the mechanism by which HLA-B exerts these effects on pancreatic cancer cell migration, we assessed the possible role of integrins, since some integrin molecules have been demonstrated to interact with HLA class I molecules and contribute to cell migration in an endothelial cell model of transplantation biology (Zhang et al., 2010). ITGB1 and ITGA2 were chosen as these proteins form a heterodimer commonly seen in pancreatic cancer (Grzesiak et al., 2006; Grzesiak et al., 2007). Consistent with our hypothesis that the α2β1 heterodimer is linked to HLA-B's effect on pancreatic cancer cell migration, we observed that ITGB1 total protein expression is reduced upon knockdown of HLA-B in PANC-1 and MIA PaCa-2 cells. Additionally, ITGB1 total protein expression was induced by HLA-B knockdown in S2-013 cells. Furthermore, following HLA-B knockdown, α2β1 surface expression is induced in the S2-013 cells, supporting the observed changes in migration upon HLA-B knockdown. Downstream effector molecule pFAK Y397 and total FAK expression followed the same trends as ITGB1in each cell line as those observed with

indicating the induction or reduction of this downstream pathway consistent with the observed changes in cell migration.

Although ITGA2/ITGB1 heterodimers are known to regulate pancreatic cancer cell migration, understanding of the role that these molecules together play with HLA class I molecules, specifically HLA-B, is incomplete. Previous studies have shown that HLA class I molecules on the cell surface can interact with ITGB4 after stimulation of cells with HLA class I antibody (Zhang et al., 2010). Loss of either HLA class I molecules or ITGB4 altered signaling pathways and downstream effectors such as pFAK, pSrc, pERK, and pAKT (Zhang et al., 2010). Pancreatic cancer cells express other integrin heterodimers in addition to ITGA2/ITGB1; therefore, certain integrin heterodimers not explored in this study could also be contributing to the differential effects of HLA-B on pancreatic cancer cell migration. The potential influence of other integrins is particularly relevant in the case of the MIA PaCa-2 cell line, which does not express ITGA2. ITGA3, another integrin alpha chain found to heterodimerize in pancreatic cancer, might also be mediating effects on migration under the influence of HLA-B in MIA PaCa-2 cells. Furthermore, ITGA6, which binds to both ITGB1 and ITGB4, also could be playing a role in conjunction with HLA-B class I molecules in pancreatic cancer cells.

Notably, in the S2-013 cell line, HLA-B expression represses cell migration as compared to the PANC-1 and MIA PaCa-2 cell lines, in which HLA-B was found to induce cell migration. Interestingly, S2-013 cells express two distinct allotypes of HLA-B while PANC-1 and MIA PaCa-2 cells express only one allotype likely having lost the other allotype during oncogenic transformation (**Table 4.1**). The presence of multiple allotypes as well as the certain sequences of the particular allotypes expressed could lead to these differential effects on integrin molecules. Previous studies indicate that the interaction between integrins and HLA class I molecules is mediated by the cytoplasmic

Table 4.1. HLA-B allotypes present in the S2-013, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines.

Data generated in collaboration with the Hildebrand lab at the University of Oklahoma Health Science Center for the S2-013 cell line. The HLA-B allotype information for the PANC-1 and MIA PaCa-2 cells was obtained from the website: http://celllines.tron-mainz.de/

Table 4.1.

	HLA-B allele 1	HLA-B allele 2
S2-013	HLA-B7	HLA-B59
PANC-1	HLA-B38	
MIA PaCa-2	HLA-B14	

tail of the HLA class I molecule (Zhang et al., 2010). A few allotypes of HLA-B, such as HLA-B27, have also previously been found to be able to form interchain disulfide bonds that cause homodimerization via cysteine residues in their extracellular sequences and formation of “redox-induced dimers” of certain HLA class I molecules can occur that involve cysteine residues in the cytoplasmic domain (Campbell et al., 2011; Campbell et al., 2012; Lenart et al., 2012). The particular allotypes expressed by these pancreatic cancer cell lines have not been fully investigated for the ability to form these bonds, but HLA-B7 shares the cysteine residue present in HLA-B27 that forms these bonds. Thus, particular sequences expressed by the specific allotypes of HLA-B present in pancreatic cancer cells could be potential mediators of the differences observed in the effects of HLA-B on pancreatic cancer cell migration.

Moreover, crosstalk has been found to exist between growth factor receptors and integrins in some types of cancer such as breast and pancreatic cancer. Growth factor receptors that intersect with integrins include EGFR, FGFR, and IGF-1R. These intersections have also been implicated in the effects that integrins have on cancer cell migration (Bianconi et al., 2016; Carpenter et al., 2015; Freeman et al., 2003; Fujita et al., 2013; Morello et al., 2011). These growth factor receptors, as indicated in Chapter 1, have also been implicated as interacting with HLA class I molecules adding another layer of complexity to what could be occurring when HLA class I molecules are knocked down. This intersection may be another potential mechanism that could explain the differential effects of HLA-B on the migration of various pancreatic cancer cell lines.

Together, these findings suggest that the specific HLA class I heavy chain isotype HLA-B influences pancreatic cancer cell migration through effects on the α2β1 integrin heterodimer and downstream signaling molecule pFAK Y397 and total FAK protein. These discoveries indicate that HLA-B may be playing a role in pancreatic cancer metastasis as well. Due to the high metastatic rate of pancreatic cancer and its

continually dismal 5-year survival rate, garnering information about factors that contribute to the migration of pancreatic cancer cells from studies such as this one will aid in the comprehension of this dreadful disease, and may potentially contribute to the generation of better treatments for pancreatic cancer.

CHAPTER 5: HLA-A allotypes and their influence on pancreatic cancer cell migration

5.1 Introduction to HLA-A allotypes and EGFR

HLA class I molecules are best known for their function within the human immune system where they present antigenic peptides to cytotoxic T cells (Benacerraf 1981). These molecules are composed of three components: the light chain β_2m, heavy chain, and endogenous antigenic peptide (Townsend et al., 1989). One unique aspect of these molecules is the high rate of polymorphisms that exist for the heavy chains. There are thousands of different sequence variations for each heavy chain, which are termed allotypes (Robinson et al., 2015; Robinson et al., 2016). Recent studies in transplantation biology models have indicated that HLA class I molecules are able to alter cell migration (Zhang et al., 2010). Studies have also shown that the HLA class I molecules can interact with various growth factor receptors on the surface of cells, including EGFR on the surface of fibroblasts, and that the interaction between EGFR and HLA class I molecules affects ligand binding to EGFR (Schreiber et al., 1984). No publications to date, however, have reported interaction between EGFR and HLA class I molecules in pancreatic cancer cells, nor have investigations into how certain HLA class I allotypes affect such interactions been conducted.

Thus, the focus of this current study was to investigate the intersection of HLA-A allotypes and EGFR in pancreatic cancer cells and to examine how their associations contribute to cell migration. Human pancreatic cancer cell lines (S2-013, PANC-1, and MIA PaCa-2) were analyzed for total HLA-A expression and were found to express substantial levels of HLA-A as compared to hTERT-HPNE (an immortalized but not transformed pancreas ductal cell line). S2-013 cells have the HLA-A allotypes HLA-A2 and HLA-A24. The PANC-1 and MIA PaCa-2 cell lines each share one HLA-A allotype with S2-013 expressing HLA-A2 and HLA-A24 respectively. Specifically designed siRNAs targeting each HLA-A allotype individually were transfected into S2-013, PANC-

1, and MIA PaCa-2 cells, and knockdown of each HLA-A allotype in the respective cell lines was confirmed by flow cytometry. In the S2-013 cells, when HLA-A2 was knocked down, a decrease in migration was observed, while when HLA-A24 was knocked down, there was a highly significant induction of cell migration. Conversely, the other two cell lines had the opposite trend, i.e., knockdown of HLA-A2 in the PANC-1 cells induced migration while HLA-A24 knockdown in the MIA PaCa-2 cells significantly reduced cell migration. EGFR expression was slightly induced upon loss of HLA-A2 in PANC-1 cells, while the opposite occurred in S2-013 cells. Thus, our data indicate that HLA-A and its allotypes are amply expressed on pancreatic cancer cells and that HLA-A2 potentially regulates EGFR protein expression and correspondingly affects the migration of pancreatic cancer cells. Therefore, our findings suggest that allotypes of HLA-A could be potential factors that influence pancreatic cancer migration and metastasis, acting partially through EGFR.

5.2 HLA-A and its allotypes in pancreatic cancer cell migration

5.2.1 The HLA-A isotype is robustly expressed in pancreatic cancer cells

The levels of the HLA-A isotype were analyzed in several pancreatic cancer cell lines as compared to hTERT-HPNE cells, an immortalized but not transformed pancreas ductal cell line. As shown in **Figure 5.1**, all pancreatic cancer cell lines tested had levels of HLA-A comparable to the level in the non-transformed hTERT-HPNE cells. These data suggest that, despite the immunological function that this molecule is best known for, HLA-A expression is not necessarily lost or robustly down regulated in human pancreatic cancer cell lines.

5.2.2 Effects of the HLA-allotypes on pancreatic cancer cell migration

To investigate the effects that the allotypes HLA-A2 and HLA-A24 have on pancreatic cancer cell migration, the S2-013, PANC-1, and MIA PaCa-2 pancreatic cancer cell lines were transfected with siRNA against their respective allotypes or with control siRNA and knockdown was confirmed by flow cytometry (**Figue 5.2A**). Cell migration upon knockdown of HLA-A allotypes was tested by transwell assay. It was observed that in the S2-013 cells, when HLA-A2 was knocked down, there was a significant reduction in cell migration (**Figure 5.2B**), while HLA-A24 knockdown in this same cell line led to a highly significant induction in cell migration (**Figure 5.2C**). Conversely, the other two cell lines showed the exact opposite trends in cell migration compared to the S2-013 cells. In the PANC-1 cells when HLA-A2 was knocked down, there was a significant increase in cell migration (**Figure 5.2B**) whereas the HLA-A24 knockdown in the MIA PaCa-2 cell line correlated with a significant decrease in migratory capability of these cells (**Figure 5.2C**). These findings indicate that pancreatic cancer cell migration is affected by HLA-A in an allotype-specific manner, but that there are also additional factors that influence the impact of HLA-A allotypes in the various pancreatic cancer cell lines.

5.3 HLA-A allotypes and EGFR in pancreatic cancer

5.3.1 EGFR interacts with HLA-A2 in PANC-1 cells but not with HLA-A2 in S2-013 cells

Earlier studies published by others have shown that HLA class I molecules and EGFR associate and that this association can affect ligand binding to EGFR (Schreiber et al., 1984). In our study, firstly, the levels of EGFR were assessed in a panel of pancreatic cancer cell lines compared to the non-transformed hTERT-HPNE cells, and

Figure 5.1. *HLA-A is robustly expressed in pancreatic cancer cell lines.* Cell lysates from hTERT-HPNE cells as well as the S2-013, PANC-1, and MIA PaCa-2 cell lines were collected and immunoblotted for HLA-A using a pan-HLA-A antibody. Hsc70 was used as a loading control. The results shown are representative of experiments performed on 3 separate lysates of each cell type.

Figure 5.1.

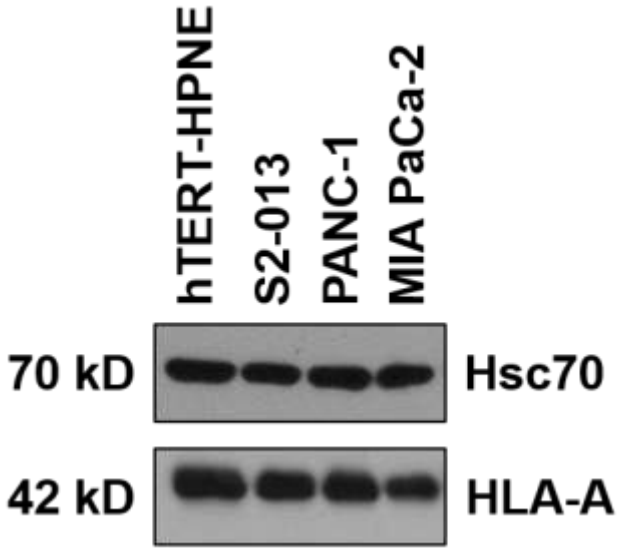


Figure 5.2. *Knockdown of HLA-A allotypes affects pancreatic cancer cell migration.* **(A)** Pancreatic cancer cell lines were transfected with HLA-A2 or HLA-A24 siRNA, and at 72 h post-transfection the cells were probed with HLA-A2 or HLA-A24 specific antibodies and flow cytometry was performed to confirm knockdown of allotypes in each cell lines. The solid black peak represents isotype control, the black line represents cells transfected with scramble siRNA and the dotted line represents cells transfected with HLA-A allotype-specific siRNA. Data are representative of 2 separate experiments. **(B)** S2-013 and PANC-1 cells were transfected with HLA-A2 siRNA and 72 h post-transfection, cells were plated in 8-μm inserts and incubated for 24 h. Inserts were then fixed, stained, and photographed. Pictures of 3 random fields were taken and used to determine the average number of cells that had migrated. Results for the S2-013 cell line are a compilation of 7 separate migration experiments and data for the PANC-1 cells are representative of 4 separate experiments. The error bars indicate the standard error of the mean, and the standard for statistical significance was set at $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(C)** S2-013 and MIA PaCa-2 cells were transfected with HLA-A24 siRNA, and at 72 h post-transfection the cells were plated in 8-μm inserts and incubated for 24 h. Inserts were then fixed, stained, and photographed. Pictures of 3 random fields were taken and used to determine the average number of cells that migrated. Results for the S2-013 cell line are a compilation of 5 separate migration experiments and data for the MIA PaCa-2 cells are representative of 3 separate experiments. The error bars indicate the standard error of the mean, and the standard for statistical significance was set at $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 5.2. (A)

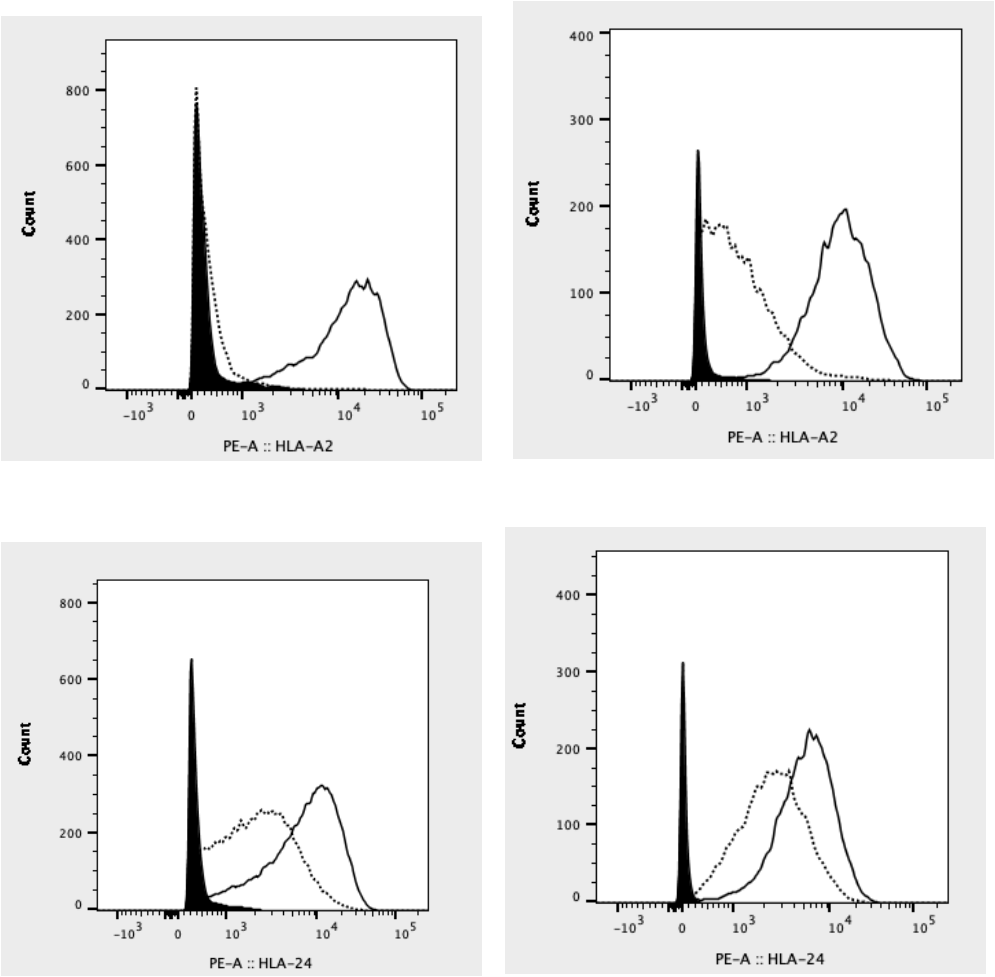

Count
PE-A :: HLA-A2
Count
PE-A :: HLA-A2
Count
PE-A :: HLA-24
Count
PE-A :: HLA-24

Figure 5.2. (B)

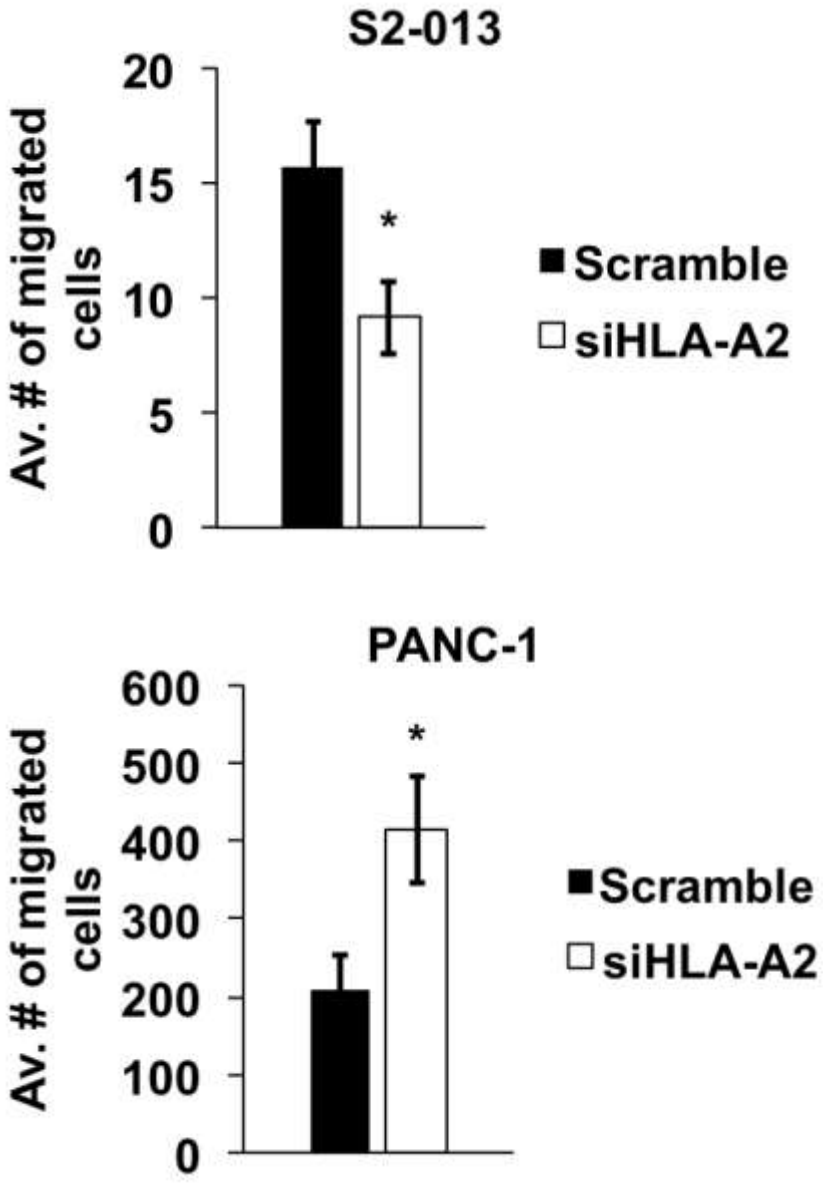
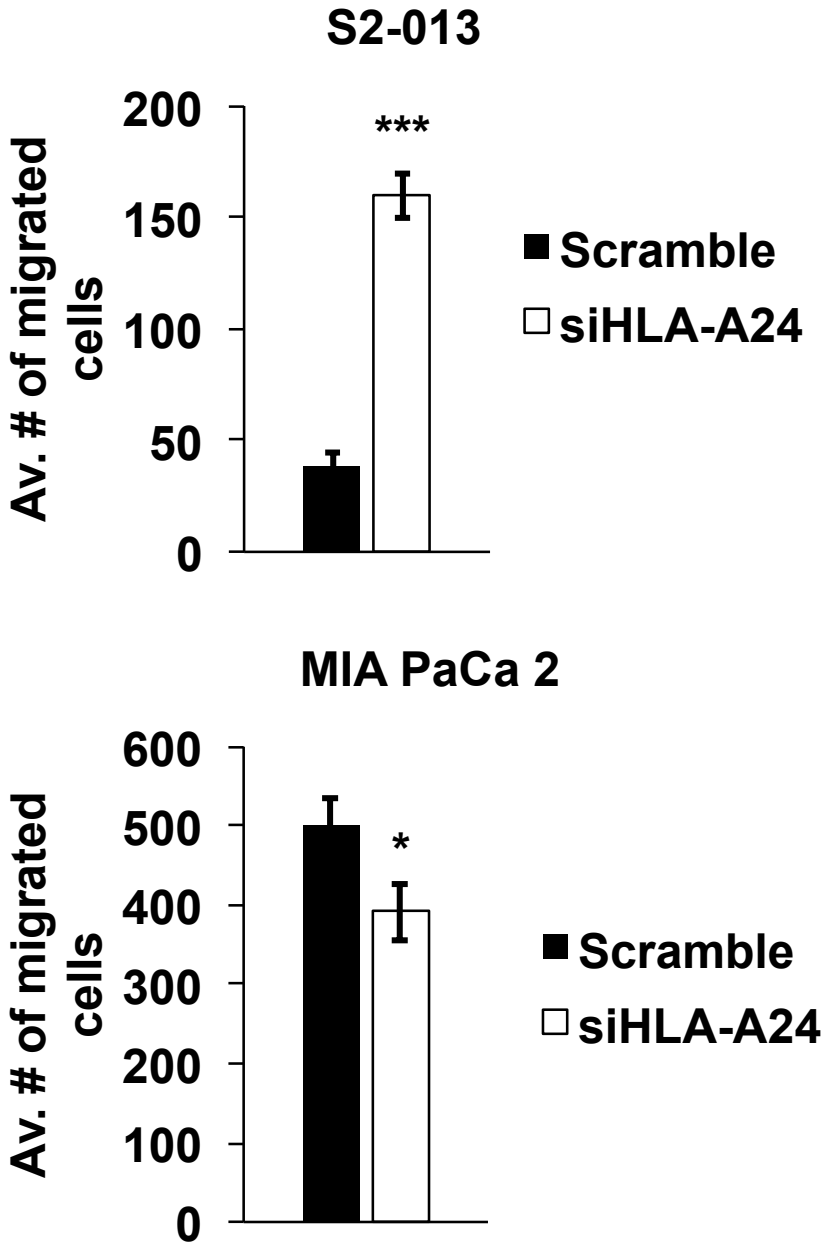

S2-013
Av. # of migrated cells
20
15
10
5
0
*
Scramble
siHLA-A2
PANC-1
Av. # of migrated cells
600
500
400
300
200
100
0
*
Scramble
siHLA-A2

Figure 5.2. (C)


S2-013
Av. # of migrated cells
200
150
100
50
0
***
Scramble
siHLA-A24
MIA PaCa 2
Av. # of migrated cells
600
500
400
300
200
100
0
*
Scramble
siHLA-A24

high levels of EGFR were observed in all cell lines tested (**Figure 5.3**). MIA PaCa-2 cells had slightly less expression of EGFR as compared to the other cell lines tested (**Figure 5.3**). To investigate if a relationship exists between HLA-A allotypes and EGFR in pancreatic cancer cell lines, co-immunoprecipitation experiments were first performed on lysates of the S2-013 and PANC-1 pancreatic cancer cell lines. The BB7.2 antibody is a well established antibody specific for the HLA-A2 heavy chain. Immunoprecipitations of HLA-A2 with BB7.2 were performed on lysates of S2-013 and PANC-1 cells (as both cell lines express this specific HLA-A allotype), and the immunoprecipitates were immunoblotted with an antibody specific for EGFR. An antibody specific to the pan-HLA-A isotype was used as a control to verify the success of the immunoprecipitation. An association between EGFR and HLA-A2 in PANC-1 cells was observed but this same interaction was not present in the S2-013 cells (**Figure 5.4**).

5.3.2 Effects on EGFR protein levels when HLA-A2 is knocked down

In addition, the impact of HLA-A2 knockdown on EGFR expression levels in pancreatic cancer cell lines was examined. When HLA-A2 was knocked down in the S2-013 cells, there was an observable decrease in EGFR total protein expression (**Figure 5.5**). Densitometry indicates that there is up to a 40% reduction in EGFR expression in the S2-013 cells in which HLA-A2 was knocked down as compared to the S2-013 cells transfected with scramble control siRNA (**Figure 5.5**). Conversely, when HLA-A2 was knocked down in the PANC-1 cell line there was a slight increase in total EGFR expression (**Figure 5.5**). When taking the loading control into account, densitometry indicated that there was about a 15% induction of EGFR in PANC-1 cells (**Figure 5.5**).

Figure 5.3. *EGFR is highly expressed in most pancreatic cancer cell lines.* Cell lysates from hTERT-HPNE cells as well as the S2-013, PANC-1, and MIA PaCa-2 cell lines were collected and immunoblotted for EGFR using an EGFR-specific antibody. Hsc70 was used as a loading control. The results shown are representative of experiments performed on 2 separate lysates of each cell type.

Figure 5.3.

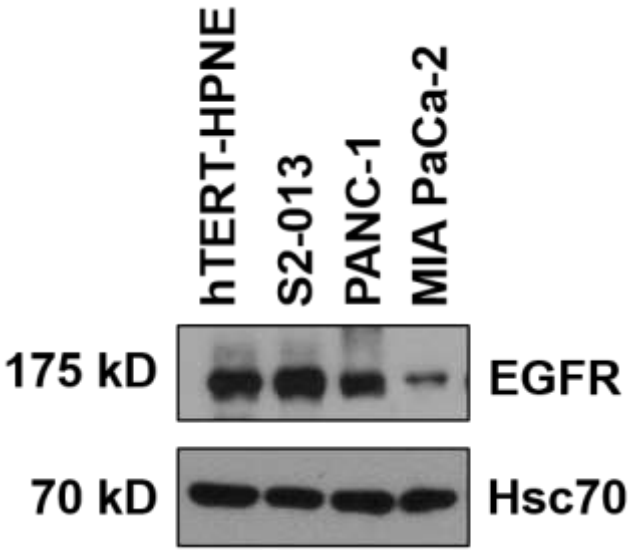


Figure 5.4. *EGFR associates with HLA-A2 in PANC-1 cells but not in S2-013 cells.* BB7.2 antibody, which is specific to HLA-A2, was used for immunoprecipitations. 64-3-7 (which recognizes the mouse MHC class I molecule H2-L^d but not HLA class I molecules) was used as a non-specific isotype control antibody. (+) indicates that cell lysates were present while (-) indicates no cell lysates were present and antibody alone was used as a control. Immunoblots were then performed for EGFR after the immunoprecipitations. A pan-HLA-A antibody was used to confirm success of the immunoprecipitation. The results shown from one experiment.

Figure 5.4.

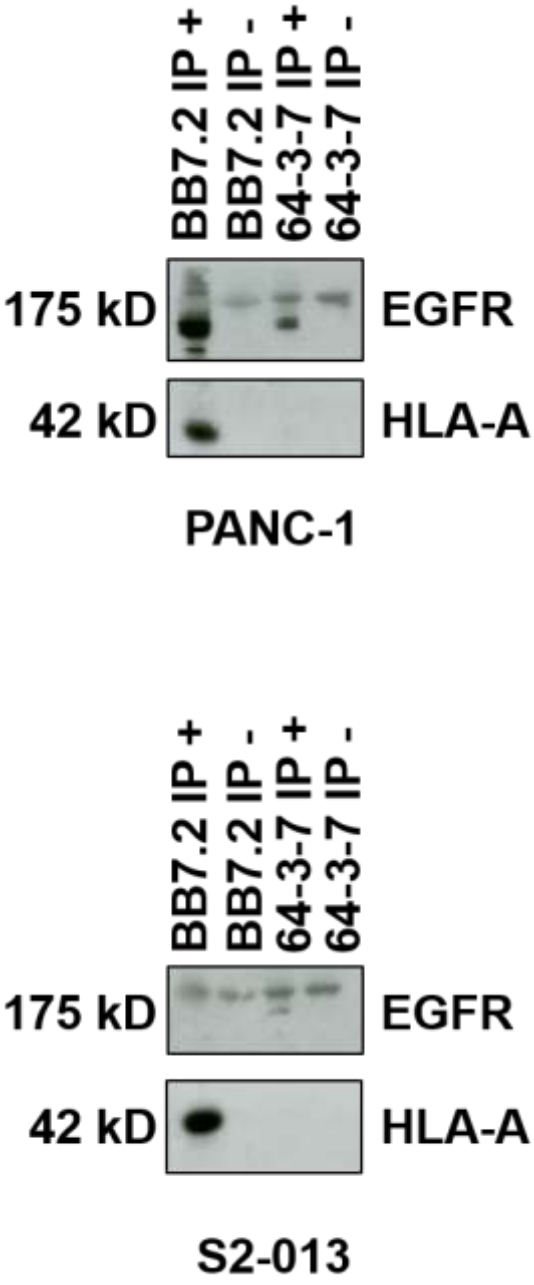
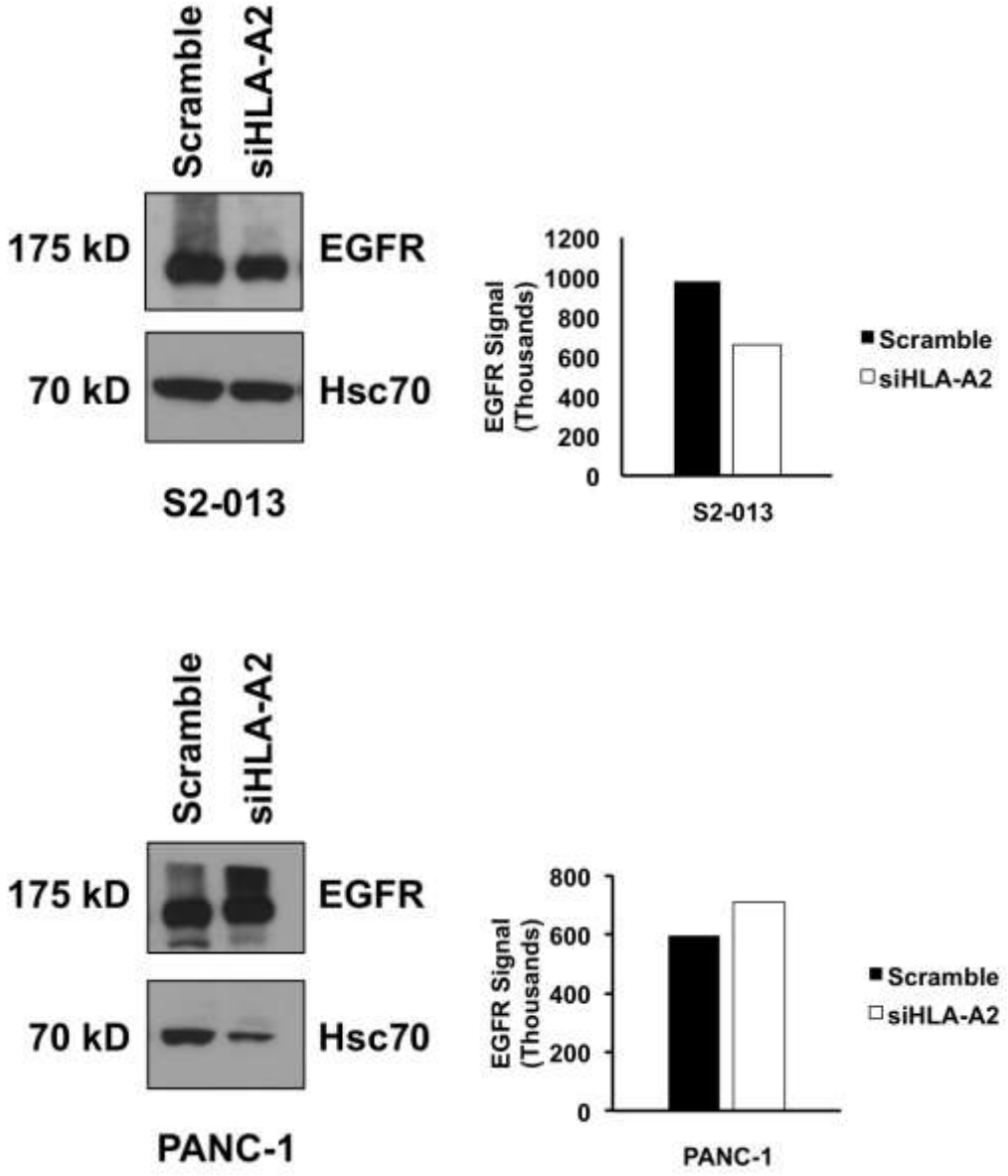

BB7.2 IP +
BB7.2 IP -
64-3-7 IP +
64-3-7 IP -
175 kD
EGFR
42 kD
HLA-A
PANC-1
BB7.2 IP +
BB7.2 IP -
64-3-7 IP +
64-3-7 IP -
175 kD
EGFR
42 kD
HLA-A
S2-013

Figure 5.5. *EGFR expression is decreased in S2-013 HLA-A2 knockdown while it is slightly increased in PANC-1 cells that have HLA-A2 knocked down.* The indicated cell lines were cultured with HLA-A2 specific siRNA (or scramble control siRNA) for 72 h and cell lysates were collected and immunoblotted for EGFR or Hsc70 (loading control). Densitometry was performed using Image Studio Lite software by Li-COR Biosciences. The results shown are representative of experiments performed on 2 separate lysates for S2-013 and are shown from one set of experiments for PANC-1 cells.

Figure 5.5.


Scramble
siHLA-A2
175 kD
EGFR
70 kD
Hsc70
S2-013
EGFR Signal (Thousands)
1200
1000
800
600
400
200
0
Scramble
siHLA-A2
S2-013
Scramble
siHLA-A2
175 kD
EGFR
70 kD
Hsc70
PANC-1
EGFR Signal (Thousands)
800
600
400
200
0
Scramble
siHLA-A2
PANC-1

5.4 Discussion

Even though HLA-A and its allotypes are best known for presenting endogenous antigenic peptides to cytotoxic T cells, our findings in pancreatic cancer cell lines indicate that the HLA-A allotypes HLA-A2 and HLA-A24 are still amply expressed by pancreatic cancer cell lines. The results from this study build upon the supposition that particular HLA-A allotypes might be important in disease prognosis (Nagata et al., 2009; Orgad et al., 1998), and suggest the possibility that HLA-A allotypes may affect pancreatic cancer prognosis through regulation of the ability of cells to migrate and metastasize.

We found that HLA-A allotypes differentially affect the migration of pancreatic cancer cell lines in a cell type-dependent manner. HLA-A2 in S2-013 cells induces cell migration while HLA-A2 in PANC-1 cells reduces cell migration. For HLA-A24, this particular allotype slows the migration of S2-013 cell line while it increases the migration of the MIA PaCa-2 cell line. Since there are many molecular subytpes of HLA-A2 and of A24, performing detailed molecular typing of the HLA-A2 and A24 heavy chains expressed by each of these cell lines would reveal whether they are identical at the molecular level or only closely related, and would perhaps provide new clues as to the mechanism whereby these HLA-A allotypes cause divergent effects on the migration of different cell lines.

In our analysis of the mechanism by which these HLA-A allotypes exert these effects on pancreatic cancer cell migration, we assessed the possible role of EGFR in these differential effects. EGFR was hypothesized to exert the effects of the HLA-A allotypes on pancreatic cancer cell migration since other studies have previously shown that EGFR and HLA class I molecules interact with one another in epidermoid carcinoma cells (Schreiber et al., 1984). Additionally, EGFR is highly expressed in our panel of

pancreatic cancer cells (**Figure 5.3**), and it has been shown to be involved in pancreatic cancer cell migration (Stock et al., 2014). Consistent with our hypothesis that EGFR might be linked to the differential effects of the HLA-A allotypes on pancreatic cancer cell migration, HLA-A2 and EGFR are associated in PANC-1 cells, while in the S2-013 cells no association was observed between these two molecules. Additionally, EGFR protein expression was found to be reduced in S2-013 with HLA-A2 knockdown and increased slightly by HLA-A2 knockdown in PANC-1 cells. Studies investigating whether a similar mechanism mediates the changes observed when the expression of the allotype HLA-A24 is knocked down in pancreatic cancer cells is currently underway.

Furthermore, previous studies have shown that there is a relationship between EGFR and APLP2. Two studies have been published thus far that demonstrated an inverse correlation between the expression levels of these two proteins, indicating that loss of APLP2 is associated with an increase in EGFR (Aydin et al., 2011; Zhang et al., 2007). Specifically, in a model of Alzheimer's disease, the mRNA of EGFR was found to be increased in APLP2$^{-/-}$ mice (Aydin et al., 2011). Furthermore, in fibroblasts that had double knockout of both APP and APLP2, there was an increase in EGFR expression (Zhang et al., 2007). The APP intracellular domain (AICD) was found to regulate EGFR expression, as indicated by overexpression of the AICD leading to a decrease in EGFR (Zhang et al. 2007). AICD has been shown to regulate the transcription of multiple genes, and the AICD and the intracellular domain of APLP2 share considerable sequence homology indicating that there could be overlap of the functions of these fragments (Baek et al., 2002; Kim et al., 2003; Pardossi-Piquard et al., 2005; von Rotz et al., 2004). The AICD, in the fibroblast cell model, is thought to bind to promoter regions of EGFR to promote its expression. This is hypothesized to occur via Fe65, which binds and stabilizes AICD (Cao and Sudhof 2001; Kimberly et al., 2001) and that is thought to

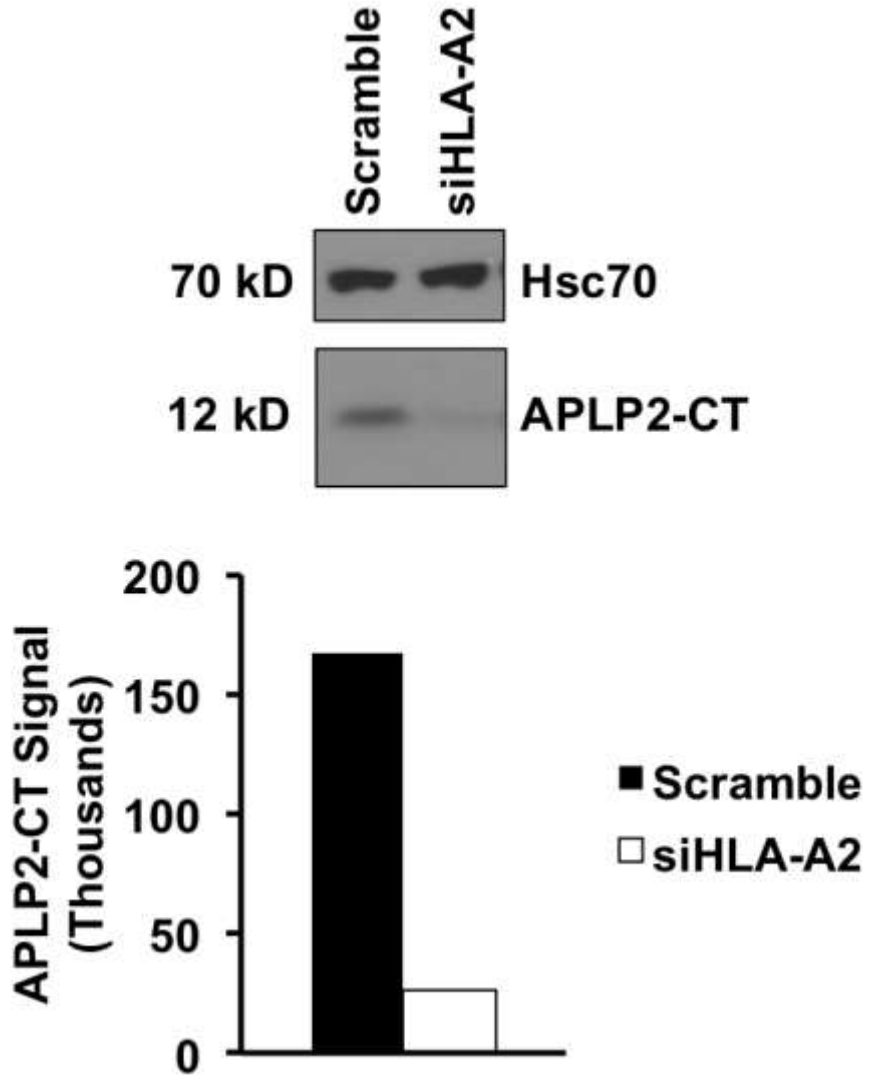
potentially allow translocation of AICD to the nucleus where AICD can bind to the promoter of EGFR.

As we have previously shown, APLP2 and its C-terminal fragment are highly expressed in pancreatic cancer cells and are increased in expression upon oncogenic transformation (Peters et al. 2012). In S2-013 pancreatic cancer cells, interaction between both the full-length form of APLP2 and the APLP2-CTF with HLA class I molecules has been detected (Sliker et al., 2019; Tuli et al., 2009b). Earlier studies from our laboratory found that these interactions could be allotype-specific, as HLA-A2 was found to more strongly interact with APLP2 than HLA-A24 (Tuli et al., 2009b). Additionally, previous studies by our laboratory have found that APLP2 promotes pancreatic cancer cell migration, and that β_2m, the light chain of the HLA class I molecule, may mediate its effect on pancreatic cancer cell migration through this molecule (Pandey et al., 2015; Sliker et al., 2019). EGFR has further been established as a regulator of pancreatic cancer cell migration (Stock et al., 2014). No studies have been published, however, that indicate how APLP2-CT, EGFR, and HLA-A allotypes may interact with one another. Preliminary studies into a potential relationship between HLA-A allotypes, APLP2-CT, and EGFR have indicated that knockdown of HLA-A2 can induce changes in APLP2-CT, with HLA-A2 knockdown in PANC-1 pancreatic cancer cells leading to a substantial reduction in the level of APLP2-CT (**Figure 5.6**).

Further reasons for the differences in the effects of disparate HLA-A allotypes on pancreatic cancer cell migration could be minor sequence variations between these allotypes. At this point, the necessary sequences required for the association of HLA-A with EGFR remain unknown. Additionally, as previously mentioned, the APP intracellular domain also plays a similar role in affecting EGFR expression (Zhang et al., 2007). APP is highly expressed in addition to APLP2 in all of the pancreatic cancer cell lines tested (Peters et al., 2012); therefore, it is possible that part of the differential role

Figure 5.6. *APLP2-CT expression is decreased in HLA-A2 knockdown in PANC-1 cells.* PANC-1 pancreatic cancer cells were cultured with HLA-A2 specific siRNA (or scramble control siRNA) for 72 h and cell lysates were collected and immunoblotted for APLP2-CT or Hsc70 (loading control). Densitometry was performed using Image Studio Lite software by Li-COR Biosciences. The results shown are from 1 experimental replicate for PANC-1 cells.

Figure 5.6.


Scramble
siHLA-A2
70 kD
Hsc70
12 kD
APLP2-CT
APLP2-CT Signal (Thousands)
200
150
100
50
0
Scramble
siHLA-A2

that is seen in these cell lines could be due to an unknown input by APP. Furthermore, HLA class I molecules can interact with other growth factor receptors such as IGF-1R (Hsu and Olefsky 1993). APLP2 has also been found to be regulated by IGF-1 (Jacobsen et al., 2010), and therefore preferential interactions with other growth factors receptors such as IGF-1R could also contribute to the differences among allotypes in the same cell line as well as between cell lines.

Together, the findings from this study suggest that HLA-A allotypes, especially HLA-A2, potentially affect pancreatic cancer cell migration through EGFR expression regulation. These discoveries, in total, indicate that HLA-A allotypes significantly affect the migration of pancreatic cancer cells and so may play a role in regulating pancreatic cancer metastasis. Pancreatic cancer has the lowest 5-year overall survival rate for any major solid tumor and is currently the 3rd leading causing of cancer related deaths in the United States (Rahib et al., 2014; Siegel et al., 2018). Acquisition of information from studies such as this one about the factors that could potentially be affecting the metastatic pathobiology of this disease will improve the overall understanding of pancreatic cancer and could potentially contribute to the development of better treatments for this disease.

CHAPTER 6: The HDAC inhibitor M344 and its effect on APLP2 processing

6.1 Introduction to APLP2 processing

APLP2, along with the other members of this protein family (APP and APLP1), undergoes cleavage by proteolytic enzymes called secretases (Walsh et al., 2007). Sites of cleavage by the alpha-secretase ADAM10, beta-secretase BACE1, and gamma-secretase have been identified within APLP2 (Hogl et al., 2011; Jacobsen and Iverfeldt 2009; Walsh et al., 2007; Zheng and Koo 2006). This cleavage results in the release of a large extracellular domain as well as smaller C-terminal fragments (Eggert et al., 2004; Vassar et al., 2009). Specifically, cleavage of APLP2 with either alpha or beta-secretases allows the generation of an approximately 70 kD soluble ectodomain. This cleavage is usually N-terminal to the transmembrane domain. Post-cleavage with either alpha or beta-secretase, this leaves a transmembrane fragment approximately 10-15 kD in size (Walsh et al., 2007). Additional cleavage of this C-terminal fragments can occur by a gamma-secretase that creates an intracellular domain (ICD) fragment that has the capacity to translocate to the nucleus and regulate gene transcription of actin cytoskeletal elements and proteins such as GSK-3β (Kitano et al., 2013). Previous studies conducted by our laboratory have shown that the 10-15 kD APLP2 C-terminal fragment (APLP2-CT) is highly expressed in pancreatic cancer cells and that the expression of this cleavage fragment is increased upon oncogenic transformation (Peters et al., 2012). These studies also indicated that blockade of beta-secretase activity using chemical inhibitors caused a reduction in the APLP2-CT level as well as a loss of viability of pancreatic cancer cells. Conversely, the same beta-secretase inhibitor treatment of a non-transformed pancreas cell line did not result in any observable changes in growth or survival (Peters et al., 2012).

Of further relevance is the ability of several isoforms of APLP2 to undergo glycosylation. Only those isoforms that exclude exon 14 are modified at Ser614 by a

unique O-linked glycan that can be extended by chondroitin sulfate glycosaminoglycans (Thinkaran and Sisodia 1994; Thinkaran et al., 1995). Our studies have indicated that this glycosaminoglycan (GAG)-modified form is highly expressed in pancreatic cancer cells (Peters et al., 2012). Additionally, earlier studies by other groups using corneal epithelial cells and Chinese hamster ovary (CHO) cells indicated that expression of GAG-modified APLP2 was correlated with an increase in cell migration (Li et al., 1999). Another study implicated this modified form of APLP2 in epithelial wound healing (Guo et al., 1998).

6.2 Introduction to the histone deacetylase (HDAC) inhibitor M344

HDACs function to repress transcription by removing acetyl moieties from lysine residues located on histones. HDAC inhibitors work to suppress this function and are being used as new anti-cancer treatments (Donadelli et a., 2003; Sambucetti et al., 1999; Xu et al, 2007). Studies using these inhibitors in cancer have indicated that they lead to inhibition of cancer cell growth through either induction of apoptosis or reduction in progression through the cell cycle (Huang et al., 2000; Konstantinopoulos et al., 2007; Sowa et al., 1997). One new HDAC inhibitor is M344 (4-dimethylamino-N-(6-hydroxycarbamoylhexyl)-benzamide. This drug has been found to be an anti-cancer agent in a variety of different cancers including endometrial and ovarian carcinomas as well as medulloblastoma and neuroblastoma. Furthermore, M344 has the ability to increase the therapeutic response to radiation therapy in a squamous carcinoma cell model (Furchert et al., 2007; Jung et al., 1999; Takai et al., 2006; Zhang et al., 2004). Further studies have also indicated that M344 has an anti-proliferative impact on breast cancer cells through the induction of apoptosis (Yeung et al., 2012). Of particular

interest to this study is the ability of M344 to decrease the gene expression of the beta-secretase BACE1 in a model of Alzheimer's disease (Volmar et al., 2017).

Thus, the aim of this study was to investigate the effects of M344 treatment on pancreatic cancer cell proliferation and migration through its effects on APLP2 processing. We found that M344 treatment of the S2-013 pancreatic cancer cell line led to a significant decrease in proliferation that corresponded with increasing concentrations of M344. This decrease in proliferation occurred at 24, 48, and 72 hours post-treatment. Interestingly, however, there were no overall changes in cell viability with treatment. Additionally, M344 treatment led to a highly significant decrease in the ability of S2-103 cells to migrate. When the protein expression levels of APLP2, GAG-APLP2, and APLP2-CT were investigated post-M344 treatment, it was found that APLP2 expression did not change but there was a significant reduction in APLP2-CT upon increasing concentrations of M344. Unexpectedly, GAG-APLP2 was found to undergo a reduction in molecular weight from a size of 250 kD to a size of about 150 kD, suggesting that there was a modification in the structure of the GAG moiety. Therefore, our findings suggest that M344 affects pancreatic cancer cell proliferation and migration, and that these effects may be mediated through the reduction of APLP2-CT expression and changes in the GAG modification of APLP2.

6.3 M344 treatment affects processing of the APLP2 protein

6.3.1 Treatment of pancreatic cancer cells with M344 decreases the production of the C-terminal fragment of APLP2

Previous studies using M344 in an *in vitro* model of Alzheimer's disease indicated that treatment of cells with this drug led to a reduction in the protein expression

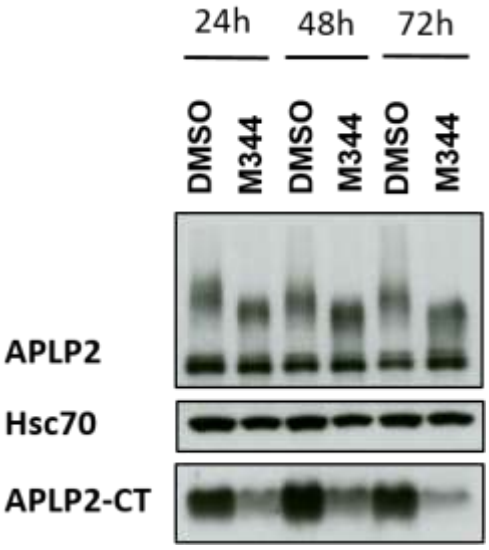
of BACE1 (Volmar et al., 2017). BACE1 has been found to be amply present in pancreas tissue and has been found to cleave APLP2 (Figueroa et al., 2001; Hogl et al., 2011; Stutzer et al., 2013). Past studies in our lab have indicated that treatment of pancreatic cancer cells with a BACE1 inhibitor led to inhibition of cell proliferation and viability, concurrent with blockade of APLP2 cleavage (Peters et al., 2012). Therefore, we first investigated if M344 was also affecting APLP2-CT expression in pancreatic cancer cells. When S2-013 cells were treated with 10 μM of the M344 drug for 24, 48, and 72 hours, there was a significant reduction in APLP2-CT at all time points examined (**Figure 6.1A**). When this reduction was quantified, there was up to a 90% reduction in APLP2-CT at 72 h post-treatment (**Figure 6.1B**). No observable change occurs in the full length form of APLP2, however, indicating that the decrease observed in APLP2-CT does not simply reflect a reduction in expression of total APLP2 but is more likely related to alterations in its cleavage (**Figure 6.1A**).

6.3.2 M344 treatment reduces the molecular weight of the GAG-modified form of APLP2

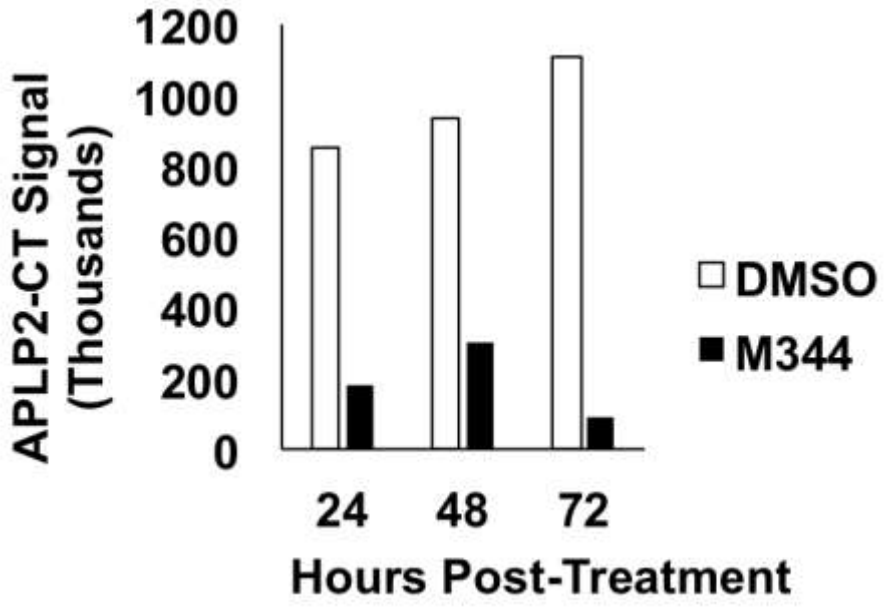
APLP2 and GAG-APLP2 can be simultaneously detected by immunoblotting with the same antibody, and by immunoblotting the GAG-APLP2 present in S2-013 pancreatic cancer cells is found to be approximately 250 kD in molecular weight. Interestingly, when S2-013 cells were treated with 10 μM of M344, there was a substantial reduction in the molecular weight of GAG-APLP2 (**Figure 6.1A**). In S2-013 cells, upon M344 treatment the molecular weight of the GAG-APLP2 band shifts down to approximately 150 kD. GAG-APLP2 has been implicated in cell migration (Guo et al., 1998; Li et al., 1999), and therefore this shift in GAG-APLP2 size could be a potential mediator of any observed changes in S2-013 pancreatic cancer cell migration. However, by what mechanism this downward shift occurs has yet to be elucidated.

Figure 6.1. *APLP2 processing is affected by M344 treatment.* **(A)** S2-013 cells were treated with 10 μM of M344 or equal volume of DMSO for the indicated times. Post-treatment, cell lysates were collected and immunoblotted for APLP2 (with an antibody that also recognizes GAG-APLP2), APLP2-CT, and Hsc70 (loading control). The results shown are representative of experiments performed on 3 separate cell lysates treated with M344. **(B)** Densitometric analysis of APLP2-CT from the bands in A.

A.



B.



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Further investigations into the question of whether BACE1 mediates this phenotype, or if this is occurring by an alternate mechanism mediated by M344, are currently underway.

6.4 M344 and its effect on pancreatic cancer cell proliferation and migration

6.4.1 M344 treatment decreases pancreatic cancer cell proliferation but has no effect on viability

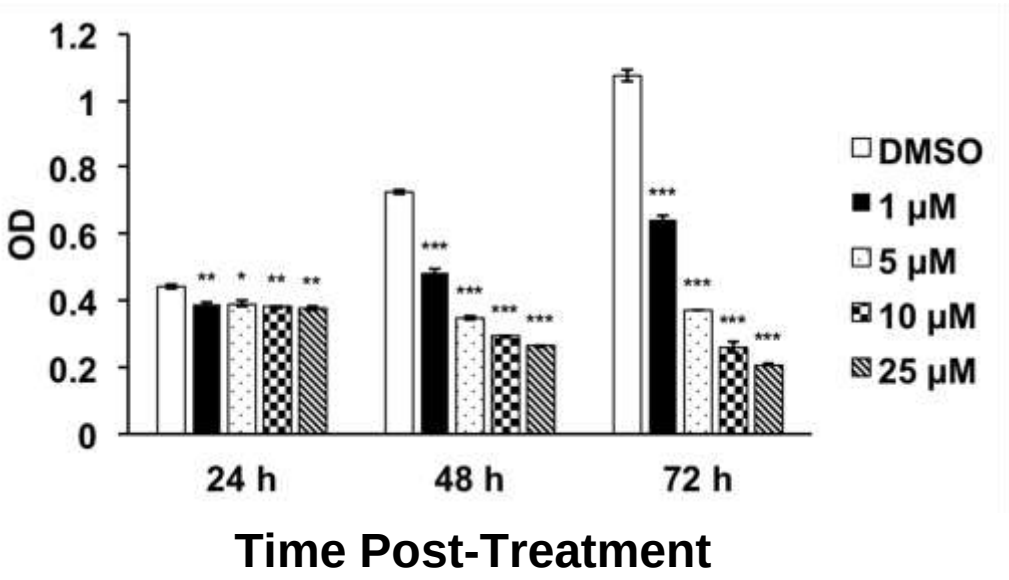
The M344 drug has been shown to affect proliferation of the MCF-7 breast cancer cell line by promoting apoptosis in these cells (Yeung et al., 2012). Therefore, to assess the effect of M344 treatment on pancreatic cancer cells, both proliferation and viability of the S2-103 cells were investigated. Using an MTT assay, it was found that at all time points and all drug concentrations tested, there was a significant reduction in the proliferation of S2-013 cells as compared to DMSO control (**Figure 6.2A**). In contrast, studies of the effect of these same drug concentrations and treatment durations on S2-013 cell viability showed that there was no change in cell viability, indicated by trypan blue staining (**Figure 6.2B**).

6.3.2 M344 treatment decreases pancreatic cancer cell migration

To date, no published data has reported the effects of M344 on cell migration. Pancreatic cancer is known for its ability to metastasize early and have high migratory capabilities (Das and Batra 2015). Furthermore, APLP2 has been extensively implicated in the regulation of cell migration and this extends to both APLP2-CT and GAG-APLP2 (Pandey et al., 2015). When S2-013 cells were treated with 10 μM M344, it was found that the ability of these cells to migrate was significantly reduced (**Figure 6.3A**). This

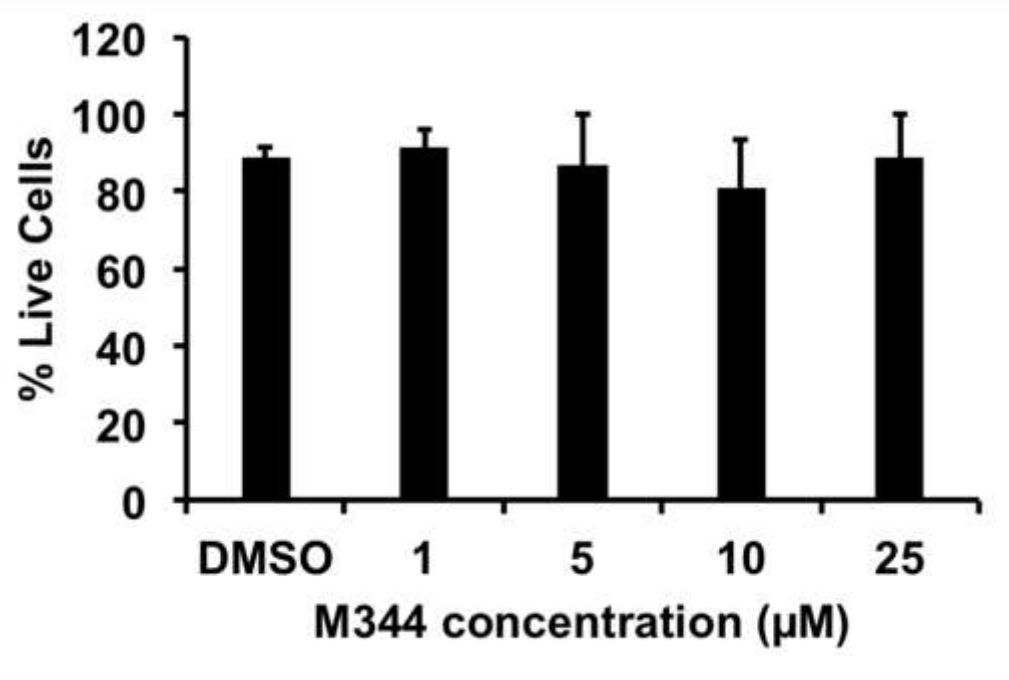
Figure 6.2. *S2-013 pancreatic cancer cell proliferation is decreased upon M344 treatment but no change in cell viability is observed.* **(A)** S2-013 cell proliferation after treatment with various concentrations of M344 was assessed by MTT assay at the indicated time points. Graphs are representative of optical density. The results are representative from 2 separate experiments. Error bars represent the standard error of the mean. Significance is set at *p<0.05, **p<0.01, ***p<0.001. **(B)** Viability of S2-013 cells was assessed by the trypan blue exclusion assay at 72 h post-treatment with M344. Cells were treated with various concentrations of M344 and viability was graphed as percentage of live cells. The results are representative from 4 separate experiments. Error bars represent standard error of the mean. Significance is set at *p<0.05, **p<0.01, ***p<0.001.

A.



Time Post-Treatment

B.



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was further supported by a decrease in the ability of the S2-013 cells to fully close the scratch in a wound-healing assay (**Figure 6.3B**).

6.5 Discussion

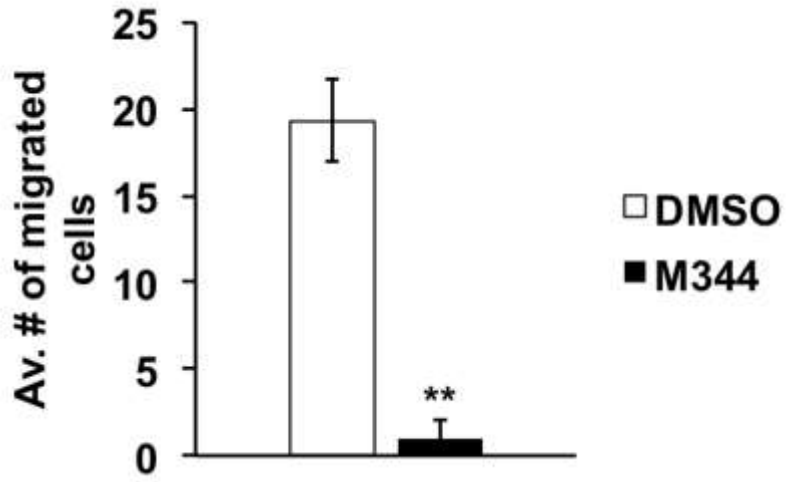
HDAC inhibitors have been found to decrease cell proliferation and induce apoptosis in a variety of different cancer types (Yeung et al., 2012). A recent study in a model of Alzheimer's disease indicated that an HDAC inhibitor, M344, decreases the mRNA and protein expression of BACE1 (Volmar et al., 2017). BACE1 has been found to be expressed in pancreatic cancer cells along with BACE2 (Figueroa et al., 2001). To date, however, no one has investigated if alterations in BACE1 expression are seen following M344 treatment of pancreatic cancer and if so, what happens to the processing and subsequent cleavage of APLP2.

We found through these studies that in pancreatic cancer cells, M344 treatment reduced proliferation, but has no overall effect on cell viability. Additionally, cell migration and invasion were decreased as compared to the vehicle control (DMSO) after M344 treatment. We further showed that M344 treatment at various time points induces a reduction in APLP2-CT. Additionally, we unexpectedly observed a reduction in the molecular weight of GAG-APLP2 upon treatment. As this modified form of APLP2 has been implicated in affecting cell migration in CHO cells (Li et al., 1999), we expect that some of the phenotypes that are observed have the potential to be caused by this alteration as well.

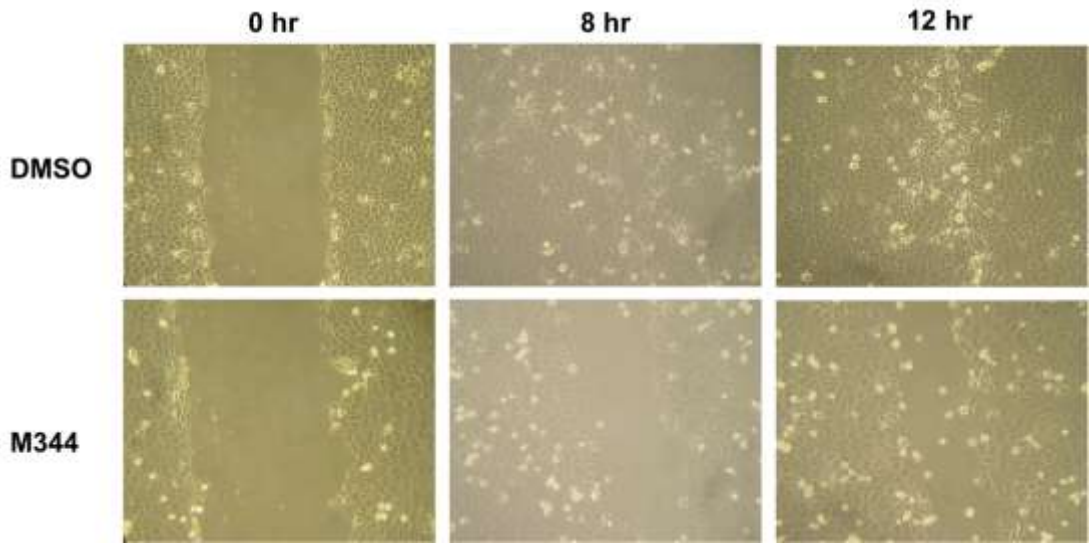
BACE1 is also able to cleave APP, which is a family member of APLP2 (Walsh et al., 2007). APP has been found to be expressed in pancreatic cancer cells, including S2-013 cells, and has been previously indicated to affect cell proliferation in this disease (Hansel et al., 2003; Peters et al., 2012; Venkataramani et al., 2010). Comprehension of

Figure 6.3. *Migration of pancreatic cancer cells is reduced upon treatment with the HDAC inhibitor M344.* **(A)** Transwell assay was performed to assess migration of S2-013 cells. Cells were pre-treated with 10 μM of M344 or DMSO for 24 h. After treatment, cells were plated into 8-μm inserts and incubated for 24 h. Inserts were fixed and stained and photographs of 3 random fields were taken and the results were averaged. Graphs show average numbers of cells that migrated. The results are representative of 3 separate experiments. Error bars represent standard error of the mean. Significance is set at *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(B)** Scratch assays were also performed to assess cell migration. S2-013 cells were pre-treated with 10 μM M344 or DMSO for 24 h. Using a 200 ul pipette tip, a scratch was made in the layer of cells and non-drug containing media was replaced. Pictures were taken of the area at 8, 12, and 26 hours after the scratch was made. The results are representative of 2 separate experiments.

A.



B.



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the role that processing of APP may play in the observed phenotypes remains incomplete. Additionally, BACE1 has the ability to cleave other targets (Kuhn et al., 2007; Li and Sudhof 2004; Sugimoto et al., 2007). The role that these other targets of BACE1 enzymatic activity may be playing in the changes observed in cell proliferation and migration have not been investigated to date. Furthermore, the effects that the additional proteins targeted by reduction in BACE1 enzymatic activity have on APLP2 processing (both C-terminal fragment formation and glycosaminoglycan modification) remains unknown.

In total, these discoveries have elucidated that M344 has the ability to decrease pancreatic cancer cell proliferation and migration, and our findings are consistent with the mediation of these effects through M344 altering the processing of APLP2. As alterations in APLP2 processing, we have noted reduction in the level of APLP2-CT as well as diminution of the molecular weight of GAG-APLP2. Since pancreatic cancer still has a bleak 5-year survival rate that is the lowest among major solid tumors (Siegel et al., 2018), better treatments of this disease remain urgently needed. Studies such as this one suggest a potential new use for M344 in the treatment of this lethal disease.

CHAPTER 7: Summary and future directions

7.1 Summary of Research

Human leukocyte antigen (HLA) class I molecules are the human form of major histocompatibility complex (MHC) class I molecules. These molecules are composed of three main components, a light chain (also termed β_2m), heavy chain, and antigenic peptide. The heavy chains of these molecules are encoded by three genetic loci that encode for three different isotypes, an –A, -B, and –C isotype. A unique aspect of these molecules is their high rate of polymorphism. This has led to thousands of individual sequence variations within the heavy chain isotypes and these variations are termed allotypes. The best-known role for these molecules is within the immune system where they present endogenous antigenic peptides to cytotoxic $CD8^+$ T cells. However, recent research has indicated that these molecules and their components could be playing a role outside of this known immune function. Studies in transplantation biology models have indicated that HLA class I molecules can contribute to cell proliferation and migration in smooth muscle and endothelial cells. Additionally, β_2m has been found to be highly expressed in some cancer types including breast, prostate, and renal cell carcinoma and has been found to play a role in promoting migration of renal cell and oral squamous cell carcinomas. However, no studies have been published thus far on investigation of the roles that these molecules may be playing outside of their known immune function in pancreatic cancer. Additionally, no research has been carried out on the molecular activities that specific isotypes or allotypes of these molecules may have in these non-immune functions. Therefore, based on the findings from these previous studies, I sought to investigate the role of components of the HLA class I molecule in pancreatic cancer cell migration. I *hypothesized* that the components of the HLA class I molecule (β_2m and the HLA heavy chains) will affect pancreatic cancer cell migration and that this will be heavy chain isotype and allotype specific.

Pancreatic cancer is known to metastasize early, and a majority of patients fall within the category of having distant disease. Pancreatic cancer has the worst prognosis of any major solid tumor, and those individuals with distant disease have an even more dismal prognosis. Therefore, we first sought to investigate the role of the light chain, β_2m, in pancreatic cancer migration. Studies using a Peggy Sue (ProteinSimple) Simple Western instrument showed that β_2m is amply expressed in an immortalized but not fully transformed cell line (hTERT-HPNE cells) as well as in a panel of pancreatic cancer cell lines. When β_2m was knocked down using β_2m-specific siRNA in this panel of cells, migration was assessed using transwell assay. It was found that the hTERT-HPNE cells did not have any change in migration upon knockdown of β_2m. In the S2-013 and PANC-1 pancreatic cancer cell lines, when β_2m expression was knocked down, decreased migration was observed. Conversely, the MIA PaCa-2 cell line increased migration when β_2m expression was lost. To further investigate the mechanism behind this, amyloid precursor like protein 2 (APLP2), a protein that our lab has shown as pro-migratory in this disease, was investigated. Interactions between this protein and HLA class I molecules in complex with β_2m were investigated, and it was found that in the S2-013 and PANC-1 cells there was an interaction, but this interaction did not exist in the MIA PaCa-2 cells. Furthermore, there was no change in overall APLP2 protein expression in the hTERT-HPNE cells upon β_2m knockdown while there was a decrease in APLP2 expression in both the S2-013 and PANC-1 cells. In the MIA PaCa-2 cells, an increase in APLP2 protein expression was observed. The presence of an interaction between HLA class I molecules and APLP2, as well as corresponding changes to APLP2 protein expression upon β_2m knockdown, all align with the observed changes in cell migration, thus leading us to the conclusion that the effects β_2m has on pancreatic cancer cell migration are potentially mediated through APLP2.

The influence of the HLA class I heavy chains on pancreatic cancer cell migration was also investigated. Initial studies investigated the effects of HLA-B on pancreatic cancer. HLA-B, similarly to β_2m, was expressed in all pancreatic cancer cell lines tested, as compared to the hTERT-HPNE cells. Using a pan-HLA-B specific siRNA, knockdown of HLA-B was accomplished in S2-013, PANC-1, and MIA PaCa-2 cells and migration was investigated. An increase in migration occurred in the S2-013 cells upon knockdown of HLA-B, while a reduction in migration was observed in both the PANC-1 and MIA PaCa-2 cells there. Integrins have been found to interact with HLA class I molecules in transplantation biology models and are also crucial to pancreatic cancer cell migration. Therefore, upon knockdown of HLA-B, the expression of the components of the heterodimer of ITGA2/ITGB1 were investigated. In the S2-013 cell line, there was an increase in total protein expression of ITGB1 but no change in ITGA2. Surface expression of both of these integrins increased in this cell line upon HLA-B knockdown. Conversely, in the PANC-1 and MIA PaCa-2 cell line, total ITGB1 expression was reduced following HLA-B knockdown. After HLA-B knockdown, ITGA2 in the PANC-1 cell line followed the same trend as S2-013 cells for total expression; however, MIA PaCa-2 cells do not express ITGA2. Furthermore, the level of the downstream effector FAK decreased (both total FAK and FAK phosphorylated at Y397) indicating that focal adhesions are also being decreased upon HLA-B knockdown. Thus, both integrins and FAK may be potential intermediates of HLA-B's effect on pancreatic cancer cell migration.

Effects of the HLA-A heavy chain and its allotypes were also investigated. Initially, the expression of HLA-A in a panel of pancreatic cancer cell lines in comparison to the hTERT-HPNE cells was confirmed. HLA class I molecules are sometimes down-regulated in cancer as a form of immune escape; however, our results, as well as other studies in breast, lung, and gastric cancer, show that HLA class I downregulation on

cancer cells is often not evident. S2-013 cells express both the HLA-A2 and HLA-A24 allotypes, while PANC-1 cells have HLA-A2 and MIA PaCa-2 cells have HLA-A24. Each of these specific allotypes was knocked down using siRNA specifically designed by our lab to target these individual heavy chain allotypes, and knockdown efficiency ranged from 50-99%. In the S2-013 cells, when HLA-A2 was knocked down, cell migration was reduced, while knockdown of HLA-A24 in this same cell line greatly induced cell migration. Conversely, however, the same allotypes in the other cell lines had the opposite effects on migration. In the PANC-1 cell line, HLA-A2 knockdown led to a corresponding boost in cell migration, whereas in the MIA PaCa-2 cell line HLA-A24 knockdown led to a decrease in cell migration. Mechanistic studies indicated that HLA-A2 interacts with EGFR in PANC-1 cells and HLA-A2 knockdown induces an increase in EGFR in this same cell line. Conversely, HLA-A2 in S2-013 cells did not interact with EGFR and HLA-A2 knockdown reduced EGFR protein expression in this cell line. Studies have determined that APLP2 knockout leads to an increase in EGFR expression and current studies are underway to investigate a role for APLP2, EGFR, and the HLA-A allotypes in affecting pancreatic cancer cell migration. Thus, it is speculated that EGFR acts as a potential intermediate in the effects of HLA-A allotypes on pancreatic cancer cell migration. Further studies delving deeper into the mechanisms mediating the effects of HLA-A24 on pancreatic cancer cell migration are still ongoing.

The final studies that were conducted as part of this dissertation research include investigation of the effects of the HDAC inhibitor M344 on APLP2 processing in pancreatic cancer cells and on pancreatic cancer cell proliferation and migration. Previous studies using M344 found that, in a model of Alzheimer's disease, treatment with this inhibitor reduced levels of the proteolytic enzyme BACE1. BACE1 is involved in cleavage of the APP family of proteins, including APLP2, which is a protein found by our lab to be highly expressed in pancreatic cancer cells. Previous studies from our lab

have implicated a proteolytic fragment of APLP2 (APLP2-CT) in pancreatic cancer cell migration and proliferation. Therefore, we sought to study the effects of M344 on pancreatic cancer cell proliferation and migration through its influence on APLP2 processing. We found that M344 treatment led to a significant reduction in cell proliferation, especially at 48 and 72 hours post-treatment. Similar results were seen with cell invasion and migration, where treatment with M344 led to a reduction in the ability of cells to migrate and invade, as demonstrated by the wound scratch assay and transwell assay. Interestingly, there was no significant effect on cell viability in the S2-013 cells after M344 treatment. Upon investigation into the effects of M344 on APLP2 expression, we detected no changes in the full-length form of APLP2, but we observed a reduction in APLP2-CTF upon treatment with increasing concentrations of M344. Unexpectedly, we also observed a downward shift in the molecular weight of the chondroitin sulfate glycosaminoglycan modified form of APLP2 (GAG-APLP2), which suggests that the structure of carbohydrate moiety. This modified form of APLP2 has been previously implicated in cell migration. Mouse orthotopic xenograft transplantation studies are currently underway to test the effects of M344 in comparison with a standard of care treatment for pancreatic cancer (gemcitabine). Preliminary results indicate that primary tumor growth is not affected much by this drug, but that the rates of metastatic disease in the tumor-bearing mice is reduced compared with PBS control or gemcitabine treatment. The overall results of this study point to M344 as a potential treatment for this lethal disease, acting via effects on BACE1 as well as APLP2, and may lead to other treatments targeting this axis.

In conclusion, this dissertation sought to investigate the overall role that components of the HLA class I molecule have in the promotion of pancreatic cancer cell migration, in conjunction with APLP2 and other proteins. We observed that β_2m promotes pancreatic cancer cell migration with APLP2 as a potential intermediary in this

process. Additionally, we identified a novel role for the HLA class I molecules in pancreatic cancer cell migration that was discovered to be allotype-specific. Overall, these studies implicate HLA class I molecules, depending on the specific allotype, in the promotion or reduction of migration (and therefore possibly metastasis) of pancreatic cancer cells. This suggests that HLA class I molecules may be useful as prognostic factors for this lethal disease in the future.

7.2 Investigate the effects of HLA class I molecules on pancreatic cancer cell proliferation and viability

The aim of this section is to propose experiments to investigate further functional roles that the HLA class I molecules may be playing in pancreatic cancer. Dysregulated cell proliferation is also a hallmark of cancer, so investigating the role that HLA class I molecules in regulating this process in pancreatic cancer is still needed. This information has the potential to yield a greater understanding of the roles of these molecules in cancer outside of their well-known immune function.

7.2.1 Previous studies on the effects of HLA class I molecules on cell proliferation and viability

In addition to cell migration, HLA class I molecule components have been implicated in affecting cell proliferation. Studies in transplantation biology models showed that addition of a pan-HLA class I antibody induced cell proliferation (Jin et al., 2002, Jin et al., 2005). In breast, prostate, and renal cell carcinoma models, overexpression of the HLA class I light chain β_2m also increased cancer cell proliferation (Josson et al., 2011).

7.2.2 Addition of a pan-HLA class I antibody reduces cell proliferation

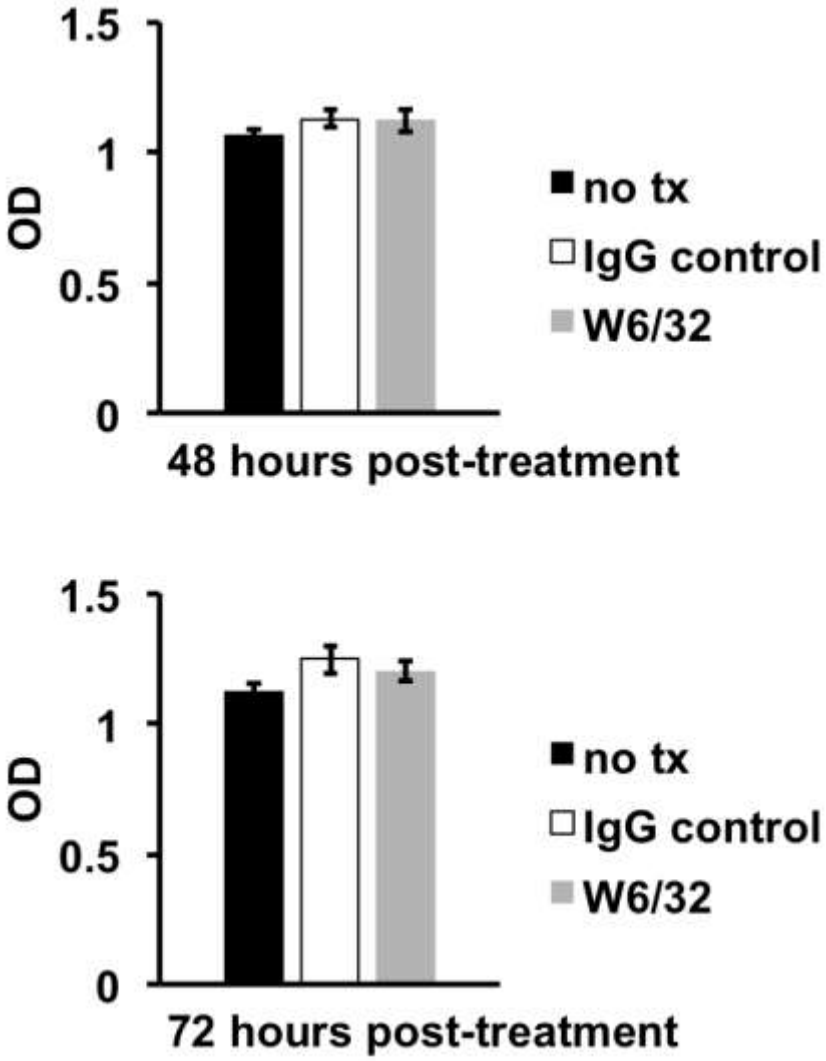
To date, no studies have been published indicating if either β_2m or specific isotypes or allotypes of the HLA class I heavy chain affect pancreatic cancer cell proliferation. Studies in transplantation biology have shown that treatment of endothelial and smooth muscle cells with the W6/32 antibody (an antibody recognizing all HLA class I heavy chains when in complex with β_2m and peptide) induced cell proliferation, and suggested that this was likely due to complex formation with growth factor receptors such as FGFR on the cell surface (Jin et al., 2002; Jin et al., 2005). Additionally, studies done in hematological malignancies indicated that treatment of these cells with a β_2m specific antibody led to a reduction in cell viability that only affected cancerous lesions (Yang et al., 2006).

In our studies, S2-013 cells were treated were 10 ng/ml of azide-free W6/32 antibody. Azide has been previously described to affect cell viability (Ishikawa et al., 2006; Slamenova and Gabelova 1980) so removal of this additional variable allowed for proper investigation of HLA antibodies on cell proliferation. Contrary to what was observed in transplantation biology models and hematological malignancy models, antibody addition did not affect pancreatic cancer cell proliferation at any timepoint tested (**Figure 7.1**)

7.2.3 Knockdown of the HLA-A heavy chain reduces cell proliferation but knockdown of the HLA-B heavy chain has no effect on cell proliferation in a pancreatic cancer model

Figure 7.1. *No change in pancreatic cancer cell proliferation is observed upon treatment with W6/32 antibody for 48 or 72 hours.* S2-013 cells were treated with 10 ng/ml of W6/32 or control IgG antibody for 48 or 72 hours. The control ("no treatment") wells only received an equivalent volume of 10% RPMI. Proliferation was evaluated by MTT assay. Error bars indicate the standard error of the mean. The results are representative of 3 separate MTT assays performed.

Figure 7.1.


1.5
1
0.5
0
OD
no tx
IgG control
W6/32
48 hours post-treatment
1.5
1
0.5
0
OD
no tx
IgG control
W6/32
72 hours post-treatment

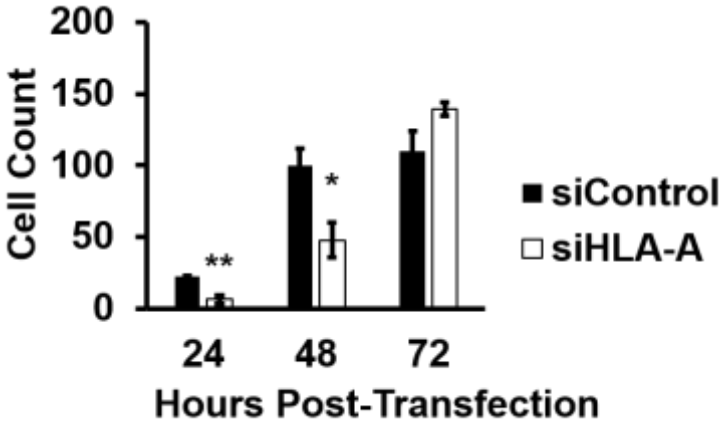
Some studies have indicated that HLA-A affects PBMC cell proliferation and that HLA-B knockdown leads to a decrease in cell proliferation in laryngeal carcinogenesis (Guo et al., 2008; Taylor et al., 1986). To examine the influence of HLA-A on pancreatic cancer cell proliferation, HLA-A heavy chain was knocked down in the S2-013 pancreatic cancer cell line using pan-HLA-A specific siRNA and a reduction in cell proliferation (as indicated by total cell count) was observed at nearly all timepoints. The one exception to this was at 72 hours, which could be due to experimental error (**Figure 7.2A**). This trend, however, was not observed when the HLA-B isotype was knocked down in S2-013 pancreatic cancer cells (**Figure 7.2B**). Under these conditions, no change in cell proliferation (as indicated by total cell count) was observed at any timepoint tested. Knockdown of β_2m (using β_2m-specific siRNA) in the S2-013 cell line showed the same trend as HLA-B knockdown, i.e. no change in cell proliferation (as indicated by total cell count) was observed at any timepoint tested (**Figure 7.2C**).

7.2.4 Knockdown of β_2m, HLA-A, or HLA-B has no effect on pancreatic cancer cell viability

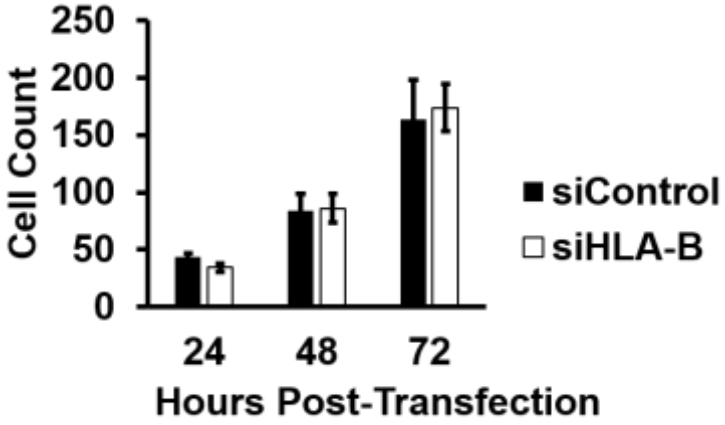
Previous studies in various cell types have indicated that, in addition to changes in cell proliferation, reductions in cell viability occur upon knockdown of β_2m, HLA-B heavy chain, or HLA-A heavy chain (Jin et al., 2005). However, knockdown of any of the above components in pancreatic cancer cells using siRNA specific to these proteins failed to show changes in cell viability at any timepoint tested (**Figure 7.3A-C**). This was seem also seen in the S2-013 pancreatic cancer cell line and in a panel of other pancreatic cancer cell lines.

Figure 7.2. *HLA-A knockdown led to a reduction in cell proliferation while neither HLA-B knockdown nor β2m had an effect.* At the indicated time points post-transfection, cell proliferation following HLA-A or HLA-B knockdown was assessed by tryan blue staining. Live cells were counted and the outcomes were graphed. S2-013 cell proliferation post-β_2m knockdown was assessed by MTT at the indicated time points post-transfection of siRNA. The results are from one experiment each for HLA-A knockdown, HLA-B knockdown, and β_2m knockdown. Errors bars represent the standard error of the mean. Significance is set at $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 7.2. (A)



(B)



(C)

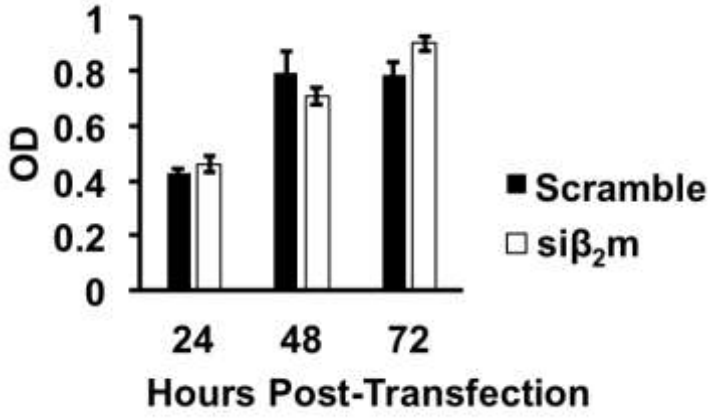
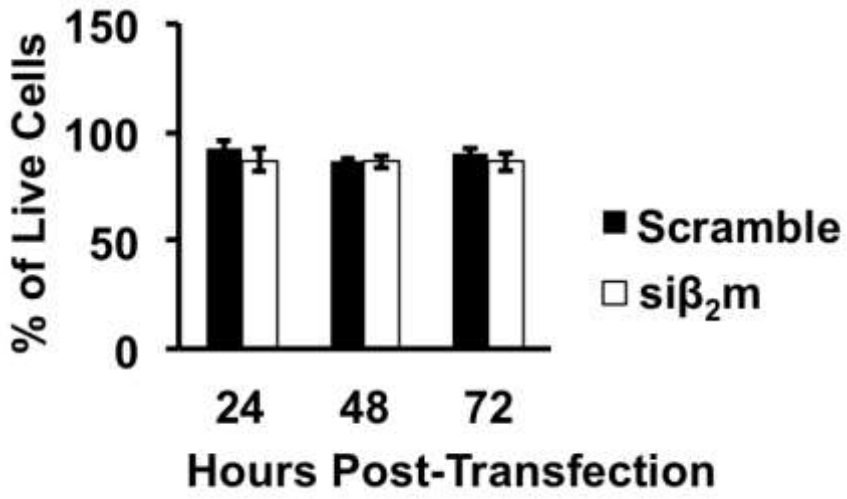
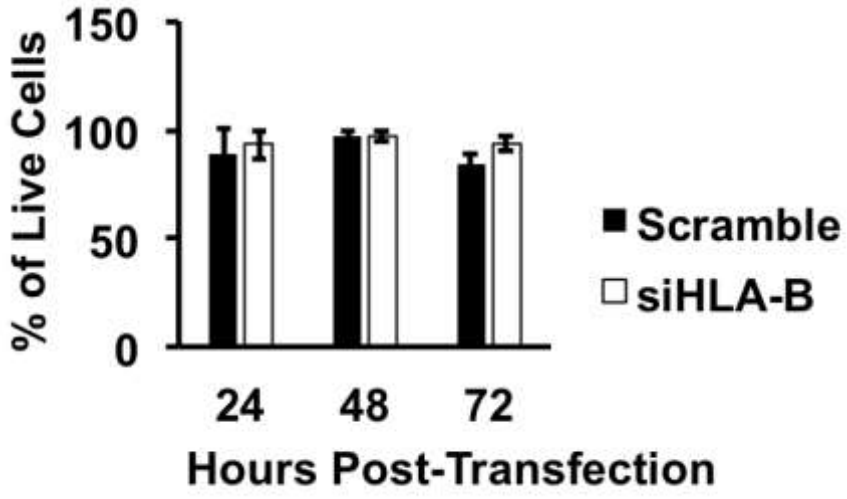


Figure 7.3. *Knockdown of any component of the HLA class I molecule has no effect on cell viability.* Cell viability is assessed by trypan blue exclusion assay. Viability is graphed as percentage of live cells in the total cell number. All of the results are representative of 3 separate experiments. Errors bars represent standard error of the mean. Significance is set at $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

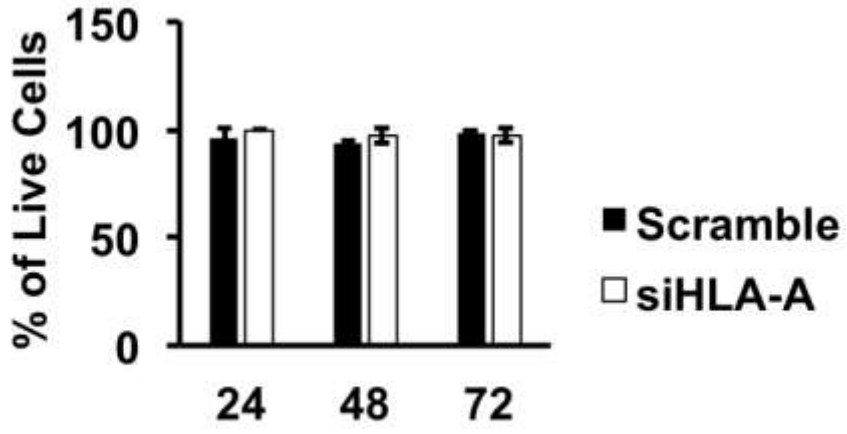
Figure 7.3. (A)



(B)



(C)



7.2.5 Discussion

S2-013 cells treated with W6/32 antibody (recognizing all HLA class I heavy chains in complex with β_2m) showed no alterations in cell proliferation, as was also the case when the HLA-B heavy chain or the HLA light chain β_2m was knocked down in this cell line. Conversely, in cells with transient knockdown of HLA-A, there was a significant reduction in S2-013 cell proliferation, at nearly all timepoints tested. Conclusions that can be drawn from these studies are that the HLA-A isotype plays a role in pancreatic cancer cell proliferation and that this is a function independent from HLA-B and β_2m. Future endeavors investigating the role of specific HLA-A allotypes in control of this process should be considered. Additionally, the mechanism behind the effects of HLA-A on S2-013 proliferation should also be analyzed.

7.3 Elucidate the roles of the HLA-B specific allotypes in pancreatic cancer cell migration

7.3.1 Previous studies of HLA-B allotypes in cancer and cell migration

As shown in a previous chapter of this dissertation, specific allotypes of the HLA-A heavy chain have differential effects on cell migration. The goal of the studies described in this section was to determine how allotypes of the HLA-B heavy chain contribute to pancreatic cancer cell migration. These studies ultimately have the goal of further characterizing the role of specific allotypes of the HLA class I molecule heavy chain play in promoting the migration of pancreatic cancer cells and potentially metastasis in patients.

7.3.2 Knockdown of HLA-B7 or HLA-B59 in S2-013 cells leads to a reduction in cell migration

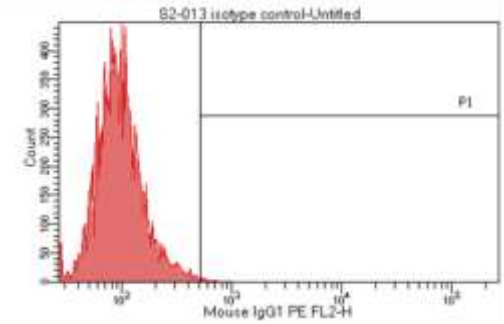
In S2-013 cells, specific siRNAs targeting the allotypes that exist in this cell line (HLA-B7 and HLA-B59) were used to knock down each individual allotype. Knockdown of HLA-B7 was confirmed using flow cytometry (**Figure 7.4**). HLA-B59 protein knockdown confirmation is not currently technically feasible, due to the lack of a commercially available antibody with the appropriate specificity. Knockdown of either allotype resulted in a significant reduction in cell migration (**Figure 7.5A-B**). This suggests, in context with the data on the pan-HLA-B siRNA knockdowns described in an earlier chapter of this dissertation, that knockdown of each allotype alone reduces migration, but reduction of both allotypes together could lead to the opposite phenotype.

7.3.3. Discussion

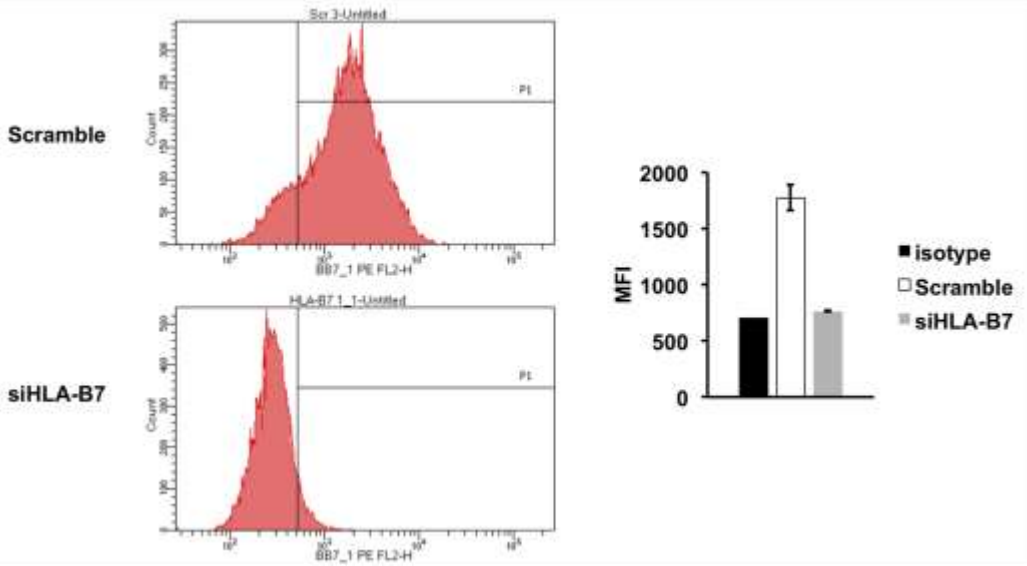
As indicated in a preceding chapter, S2-013 cells transfected with a pan-HLA-B siRNA demonstrate faster migration. However, when each individual allotype is knocked down separately, a reduction in migration rate is observed in both. These studies have further illuminated the role of HLA class I allotypes, specifically HLA-B allotypes, in pancreatic cancer cell migration. Additionally, these findings demonstrate that the migratory phenotype can be changed if only one specific allotype versus both are knocked down. Further confirmation of these trends in other pancreatic cancer cell lines that express these particular allotypes is needed. Additionally, investigation of the mechanisms that control these differences should also be conducted.

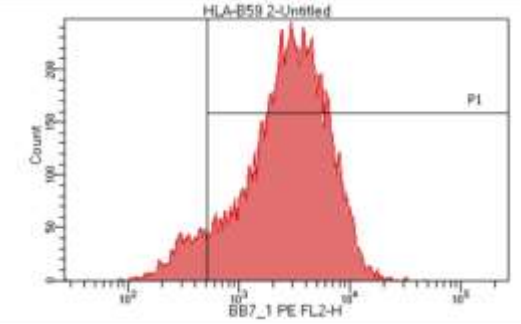
Figure 7.4. *HLA-B7 knockdown confirmation for S2-013 pancreatic cancer cells.* Flow cytometry was performed using an HLA-B7 antibody 72 h post-transfection with HLA-B7 or HLA-B59 specific siRNA. Scramble is control non-targeting siRNA. HLA-B7 was robustly expressed in S2-013 cells as evidenced by staining in the scramble siRNA-transfected cells. HLA-B7 knockdown was confirmed in cells transfected with HLA-B7 siRNA. HLA-B59 siRNA has no observable effect on HLA-B7 expression and was included as a control. The results are shown are from one experiment.

Figure 7.4.



Isotype control

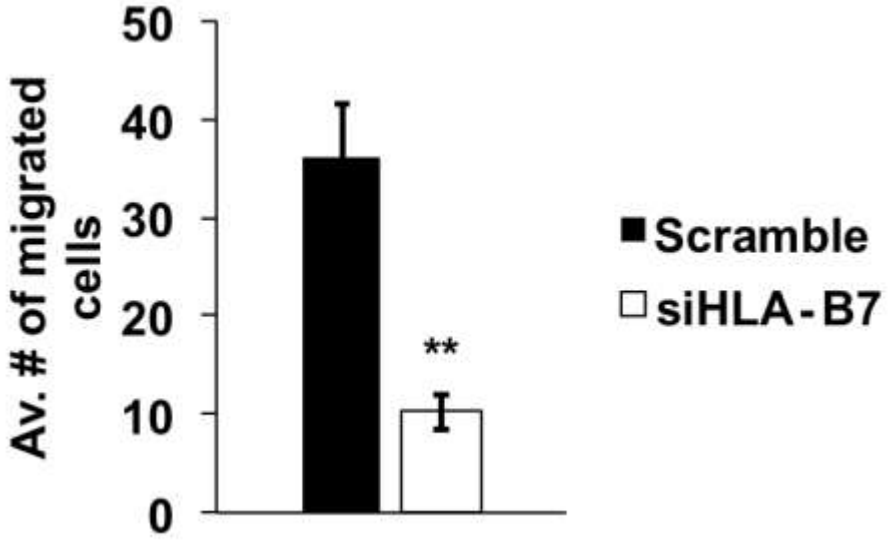




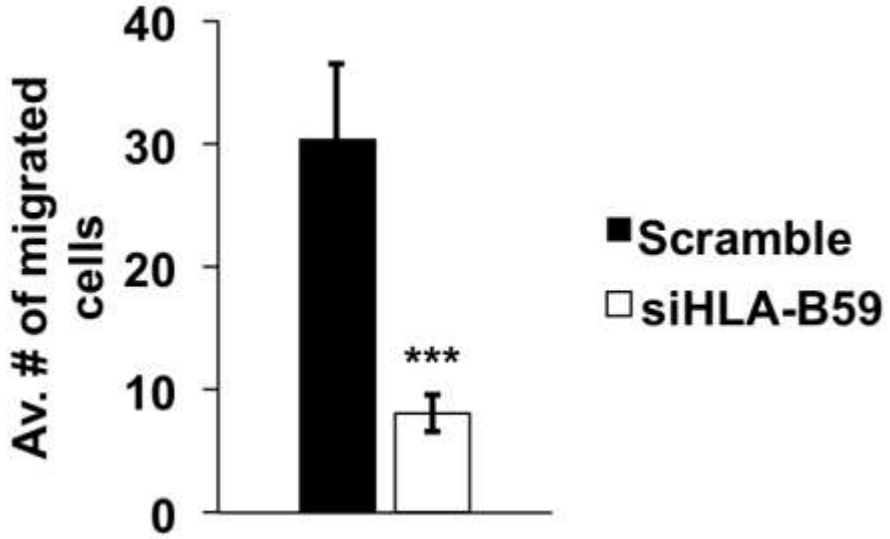
HLA-B59 siRNA transfection stained for HLA-B7.

Figure 7.5. *Knockdown of either allotype of HLA-B in S2-013 cells leads to a reduction in cell migration.* **(A)** Transwell assays were used to determine cell migration 72 h post-transfection with HLA-B7-specific siRNA. S2-013 cells, at 72 h post-transfection, were replated into 8-μm inserts and incubated for 24 h. Cells were stained and pictures were taken of 3 random fields. Counts were averaged and graphed with error bars representing the standard error of the mean. The results are representative of 5 separate experiments. Significance was set at $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$. **(B)** Transwell assays were used to determine cell migration at 72 h post-transfection with HLA-B7-specific siRNA. S2-013 cells, at 72 h post-transfection, were replated into 8-μm inserts and incubated for 24 h. Cells were stained and pictures were taken of 3 random fields. Counts were averaged and graphed with error bars representing the standard error of the mean. The results are representative of 5 separate experiments. Significance was set at $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 7.5. (A)


Av. # of migrated cells
50
40
30
20
10
0
**
Scramble
siHLA-B7

(B)


Av. # of migrated cells
40
30
20
10
0
***
Scramble
siHLA-B59

7.4 Identify the functional relevance of the effects of TGF-β treatment on the regulation of cell migration by HLA class I molecules

The goal of these studies was to investigate if TGF-β alters pancreatic cancer cell migration when HLA class I heavy chain isotypes or allotypes are knocked down by siRNA transfection. The results of these studies can provide further insight into crosstalk between certain growth factor ligands and HLA class I molecules on pancreatic cancer cell migration, and could ultimately provide additional targets for treatment of this lethal disease.

7.4.1 Previous studies on TGF-β and HLA class I molecules

A previous report showed that treatment of prostate cancer cells with TGF-β can induce changes in expression of HLA class I molecules. Specifically, HLA-B mRNA was down-regulated, and the surface expression of this particular isotype was reduced (Chen et al., 2015). However, no studies have thus far investigated the effects of TGF-β on migration or proliferation in the context of knockdown of HLA class I molecules.

7.4.2 S2-013 cells in which HLA-B is knocked down and that have been treated with TGF-β have a significant induction in migration

As mentioned in an earlier chapter of this dissertation, knockdown of the HLA-B isotype in S2-013 cells leads to an increase in pancreatic cancer cell migration. Interestingly, when S2-013 cells deficient in HLA-B are treated with TGF-β, the increased migration phenotype is further enhanced. Upon TGF-β treatment, S2-013 cells transfected with control siRNA, showed a modest yet significant increase in cell migration. However, when S2-013 cells were transfected with HLA-B siRNA, the

increase in cell migration that was observed without treatment of TGF-β was elevated even further when TGF-β was introduced into the medium (**Figure 7.6**).

7.4.3 Treatment with TGF-β alters expression of HLA-B in pancreatic cancer cells in which HLA-A has been knocked down

The effect of TGF-β on heavy chain expression compensation was also investigated in the S2-013 cells. Compensation between heavy chains does not usually occur in the S2-013 cell line upon knockdown of one HLA class I isotype; however, when S2-013 cells were treated with TGF-β after HLA-A knockdown, there was a robust increase in the expression of HLA-B (as indicated by HC-10) (**Figure 7.7**).

7.4.4 Discussion

These data indicate that the expression of HLA class I molecules, specifically HLA-B molecules in S2-013 pancreatic cancer cells, is highly responsive to TGF-β treatment. The phenotype in S2-013 cells that was observed when HLA-B was knocked down, i.e., that migration was facilitated, was further accentuated by TGF-β treatment. Moreover, these data indicate that the presence of TGF-β in the tumor microenvironment could lead to greater migration of pancreatic cancer cells, consistent with findings by others that TGF-β increases pancreatic cancer cell migration (Witte et al., Oncology Reports 2017). Therefore, these studies are relevant to a signaling pathway that may be contributing to metastasis in this deadly disease.

7.5 Further determine the intersection of HLA class I molecules and integrins in pancreatic cancer cell migration and proliferation

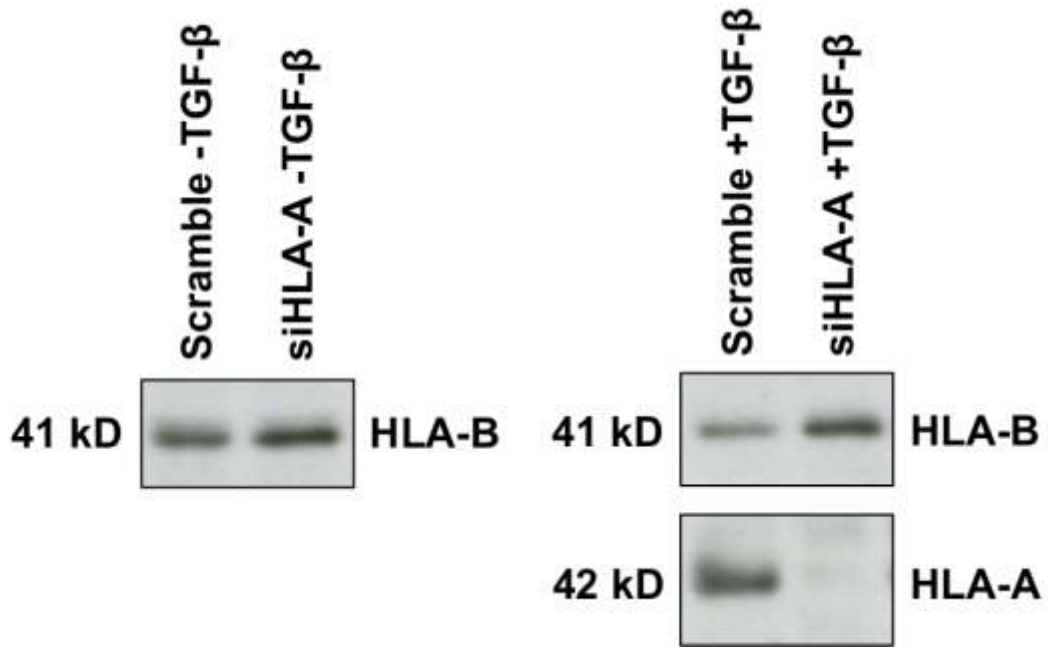
Figure 7.6. *TGF-β treatment further enhances the increase in pancreatic cancer cell migration.* S2-013 cells were treated with either maintenance media or with 10 ng/ml of TGF-β at 24 h post-transfection. Treatment lasted for 48 h and then cells were plated for transwell assay in 8-μm inserts and incubated for 24 h. Cells were fixed and stained and photographed, taking 3 random fields. Averages were taken and graphed with error bars indicating the standard error of the mean. The results are representative of 2 separate experiments. Significance is set at $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 7.6.


Av. # of migrated cells
400
350
300
250
200
150
100
50
0
Scramble no tx
HLA-B no tx
Scramble +TGFb
HLA-B +TGFb

Figure 7.7. *HLA-B expression was induced upon HLA-A knockdown when cells were treated with TGF-β.* S2-013 cells were treated with either maintenance media or maintenance media with 10 ng/ml of TGF-β 24 h post-transfection with HLA-A-specific siRNA. Treatment lasted for 48 h and then cell lysates were collected and immunoblotted for HLA-B (with the HC-10 antibody). Hsc70 was used as a loading control. The results shown are from one experiment.

Figure 7.7.


Scramble -TGF-β
siHLA-A -TGF-β
41 kD
HLA-B
Scramble +TGF-β
siHLA-A +TGF-β
41 kD
HLA-B
42 kD
HLA-A

The aim of this section is to propose experiments to identify additional cancer-related integrins with which HLA class I molecules interact in pancreatic cancer cells, as well as to investigate if integrins have effects on HLA class I molecule expression. Integrins contribute to many cancer-related processes such as proliferation and migration (Bianconi et al., 2016). Thus, investigating the intersection between HLA class I molecules and integrins in regulating these processes in pancreatic cancer thus is relevant. This information has the potential to allow a greater understanding of the roles of HLA class I molecules in cancer outside of immunological functions.

7.5.1 Previous studies on HLA class I and integrins

In addition to the integrin α2β1 heterodimer and EGFR, the cancer-associated integrin beta 4 (ITGB4), was also previously investigated as interacting with HLA class I molecules (Zhang et al., 2010). These studies conducted in transplantation biology models indicated that HLA class I molecules and ITGB4 interact and that this interaction can affect cell migration and proliferation (Zhang et al., 2010). No studies, however, have investigated if this interaction exists in pancreatic cancer. Furthermore, even though it was shown in an earlier chapter of this dissertation that HLA class I molecules can interact with the α2β1 heterodimer, no studies have yet been reported that have investigated if integrins affect HLA class I expression and if this alters pancreatic cancer cell migration.

7.5.2 Certain isotypes of the HLA class I molecule heavy chains interact with ITGB4

ITGB4 is an integrin molecule that is over-expressed in a variety of cancers, including pancreatic cancer, and that is involved in the motility of various types of cancer cells (Cruz-Monserrate and O'Connor 2008; Masugi et al., 2015; Mercurio and

Rabinovitz 2001). Our analysis of a panel of pancreatic cancer cell lines determined that only S2-013 and BxPC3 cells express this integrin (data not shown). Co-immunopreciptation experiments for ITGB4 in these two cell lines identified an association of ITGB4 with both HLA-A and HLA-B heavy chains in S2-013 cells (**Figure 7.8**). However, in the BxPC3 cells, only an association of ITGB4 with HLA-A was observed, although this could be due to the relatively low expression of HLA-B in this cell line (**Figure 7.8**).

7.5.3 Knockdown of certain integrins affects pancreatic cancer cell migration

Additionally, in a panel of pancreatic cancer cells, knockdown of various integrin molecules was performed and the effects on cell migration were assessed by transwell assay. In S2-013 cells, migration was found to be significantly increased when either ITGB1 or ITGA2 was knocked down (**Figure 7.9 and 7.10**). In the PANC-1 pancreatic cancer cell line, knockdown of ITGA2 had no effect on cell migration, while ITGB1 knockdown significantly reduced the migration rate (**Figure 7.9 and 7.10**). ITGB1 knockdown decreased cell migration to a similar extent In the MIA PaCa-2 cell line (**Figure 7.9**). The MIA PaCa-2 cell line does not have ITGA2, as mentioned earlier in this dissertation, so knockdown of ITGA2 could not be pursued in this cell line.

7.5.4 Knockdown of certain integrins affects HLA class I isotype overall protein expression

Due to the interactions that exist between ITGA2 and ITGB1 with HLA class I molecules (as mentioned earlier in this dissertation), as well as the effects that these integrins have on cell migration, the changes in the expression levels of the HLA class I heavy chain isotypes upon knockdown of ITGA2 and ITGB1 were also assessed. In

Figure 7.8. *ITGB4 associates with HLA class I molecules in pancreatic cancer cells.* Cell lysates were collected and an ITGB4 antibody was used for immunoprecipitations in S2-013 and BxPC3 pancreatic cancer cell lines. Negative controls (an anti-rabbit IgG antibody as well as an ITGB4 antibody with no cells) were also included. Immunoblotting was then performed on the immunopreciptations, probing with antibodies specific to HLA-A or HLA-B. Probing for ITGB4 was also done to confirm the success of the ITGB4 immunopreciptation. The results shown are from one experiment for both S2-013 and BxPC3.

Figure 7.8.

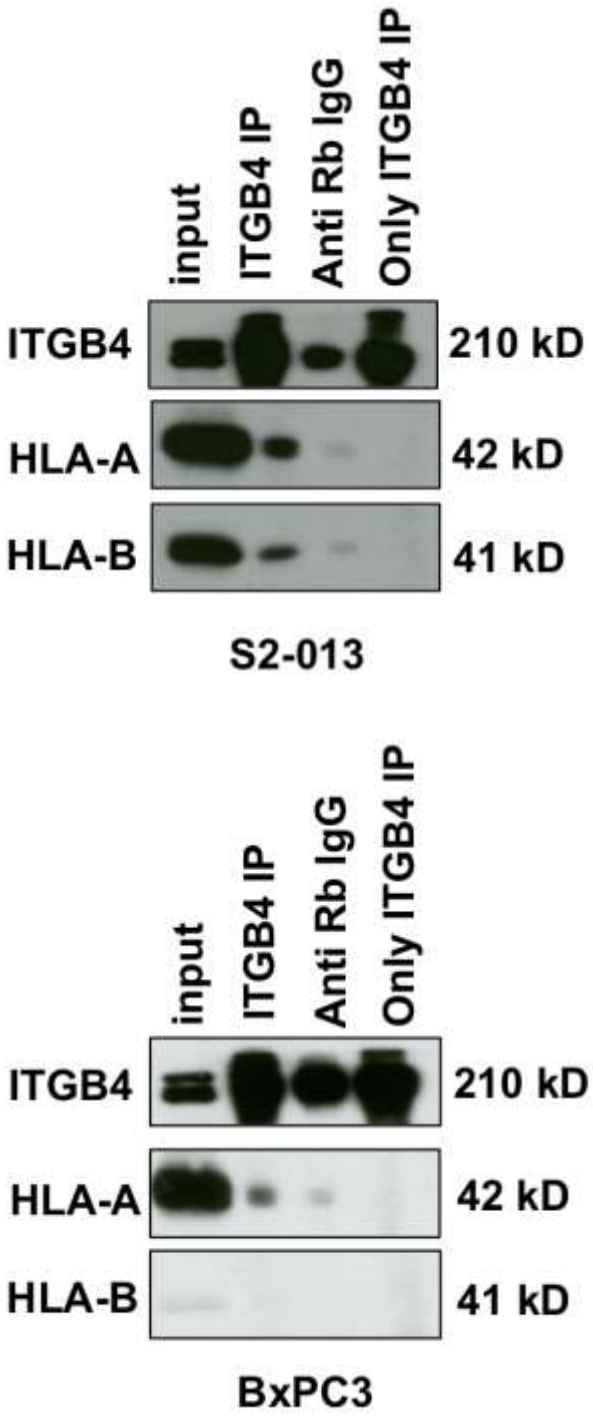
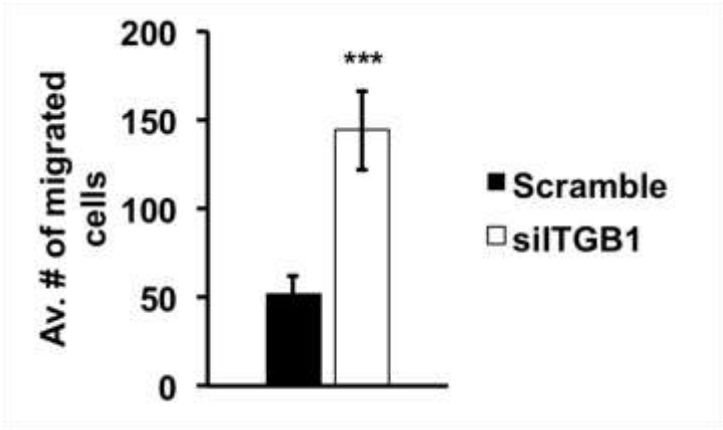
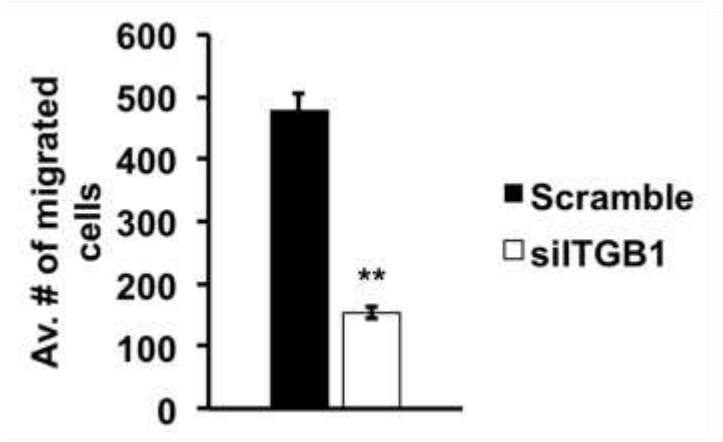

input
ITGB4 IP
Anti Rb IgG
Only ITGB4 IP
ITGB4
210 kD
HLA-A
42 kD
HLA-B
41 kD
S2-013
input
ITGB4 IP
Anti Rb IgG
Only ITGB4 IP
ITGB4
210 kD
HLA-A
42 kD
HLA-B
41 kD
BxPC3

Figure 7.9. *Knockdown of ITGB1 in pancreatic cancer cell lines affects cell migration.* Transwell assays were used to determine the migration at 72 h post-transfection of ITGB1-specific siRNA-transfected pancreatic cancer cells. The cells, at 72 h post-transfection, were replated into 8-μm inserts and incubated for 24 h. Cells were stained and pictures were taken of 3 random fields. The counts were averaged and graphed with error bars indicating the standard error of the mean. The results are representative of 2 separate experiments for S2-013 cells, 3 experiments for PANC-1 cells, and 2 experiments for MIA PaCa-2 cells. Significance is set at $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

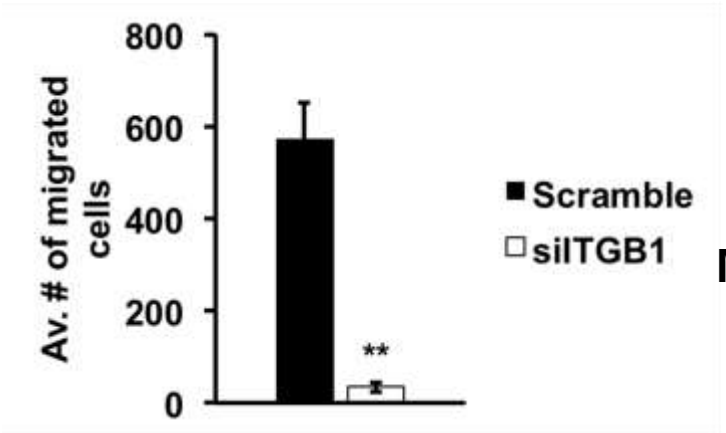
Figure 7.9.



S2-013



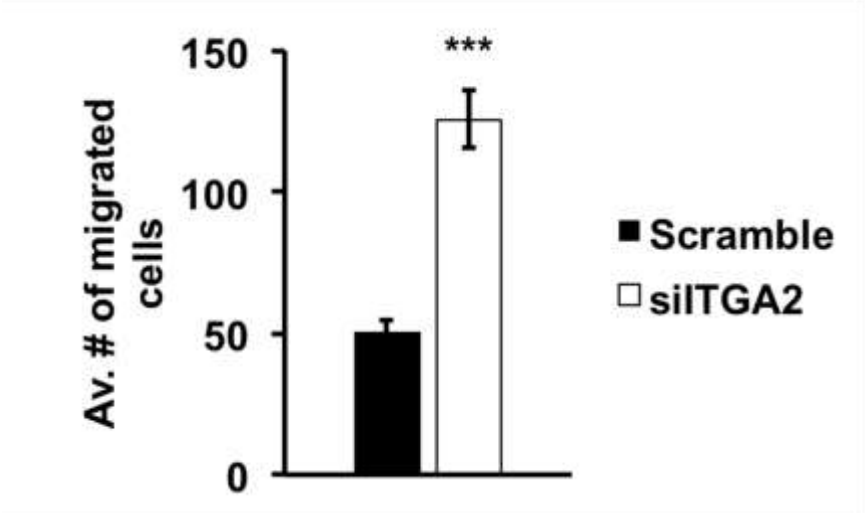
PANC-1



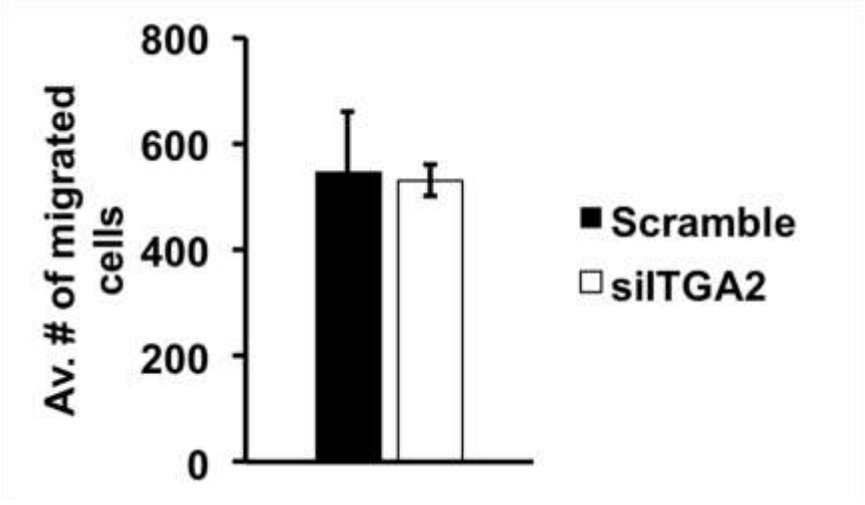
MIA PaCa-2

Figure 7.10. *Knockdown of ITGA2 in pancreatic cancer cell lines alters cell migration.* Transwell assays were used to determine cell migration 72 h post-transfection with HLA-B7-specific siRNA. The cells, at 72 h post-transfection, were replated into 8-µm inserts and incubated for 24 h. Cells were stained and pictures were taken of 3 random fields. The counts were averaged and graphed with error bars indicating the standard error of the mean. The results are representative of 2 separate experiments for S2-013 cells and 4 separate experiments for PANC-1 cells. Significance is set at $p<0.05$; *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Figure 7.10.



S2-013



PANC-1

S2-013 cells, HLA-A and HLA-B protein expression levels were decreased if ITGB1 was knocked down, but this did not occur in the case of ITGA2 knockdown (**Figures 7.11A and 7.12**). Interestingly, no change in the expression level of either HLA class I heavy chain has been observed thus far upon knockdown of either integrin molecule (ITGA2 or ITGB1) in the PANC-1 cell line (**Figures 7.11B and 7.12**). Similarly, no change in either HLA heavy chain isotype was observed in the MIA PaCa-2 cells upon ITGB1 knockdown (**Figure 7.11B**).

7.5.5 Discussion

Integrins are important cell-surface molecules in many cancer-related processes including proliferation, migration and angiogenesis (Bianconi et al., 2016). HLA class I molecules have previously been found to interact with ITGB4 in transplantation biology models and this interaction was found to contribute to cell proliferation and migration (Zhang et al., 2010). However, whether a corresponding HLA class I/integrin association, with similar effects, exists in pancreatic cancer has yet to be investigated. These data encourage further studies into the role of HLA class I molecules in conjunction with ITGB4 in pancreatic cancer. Additionally, these data support further studies into how integrins may regulate pancreatic cancer cell migration through effects on the expression of HLA class I molecules. This has implications not only for cell migration, but also for normal immune recognition and for the efficacy of immunotherapy approaches for pancreatic cancer. Further investigations into other integrins important in pancreatic cancer such as ITGA3 and ITGA6 (Cruz-Monserrate and O'Connor 2008; Lee et al., 2019; Masugi et al., 2015) would also be crucial to further this research.

Figure 7.11. *HLA class I heavy chain expression is altered in ITGB1-knockdown S2-013 cells but not in ITGB1-knockdown PANC-1 or MIA PaCa-2 cells.* The pancreatic cancer cell lines **(A)** S2-013 and **(B)** PANC-1 and MIA PaCa-2 were transfected with ITGB1-specific siRNA or scramble control non-targeting siRNA. At 72 h post-transfection, cell lysates were collected. Immunoblots were performed on the cell lysates investigating the effects of ITGB1 knockdown on HLA-A and HLA-B protein expression. Hsc70 was used as a loading control and ITGB1 knockdown was confirmed in these lysates by probing with an anti-ITGB1 antibody. The results are representative of 2 separate experiments for S2-013. For both PANC-1 and MIA PaCa-2 cells, results shown are from one experiment.

Figure 7.11. (A)

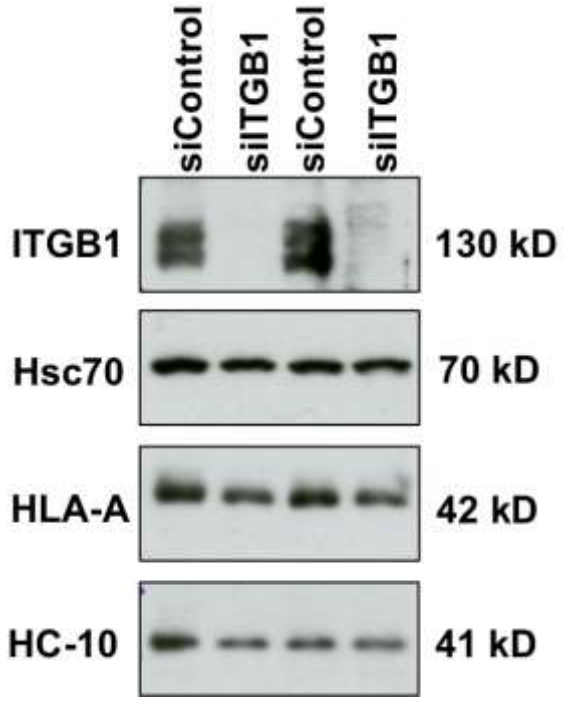

siControl
siITGB1
siControl
siITGB1
ITGB1
130 kD
Hsc70
70 kD
HLA-A
42 kD
HC-10
41 kD

Figure 7.11. (B)

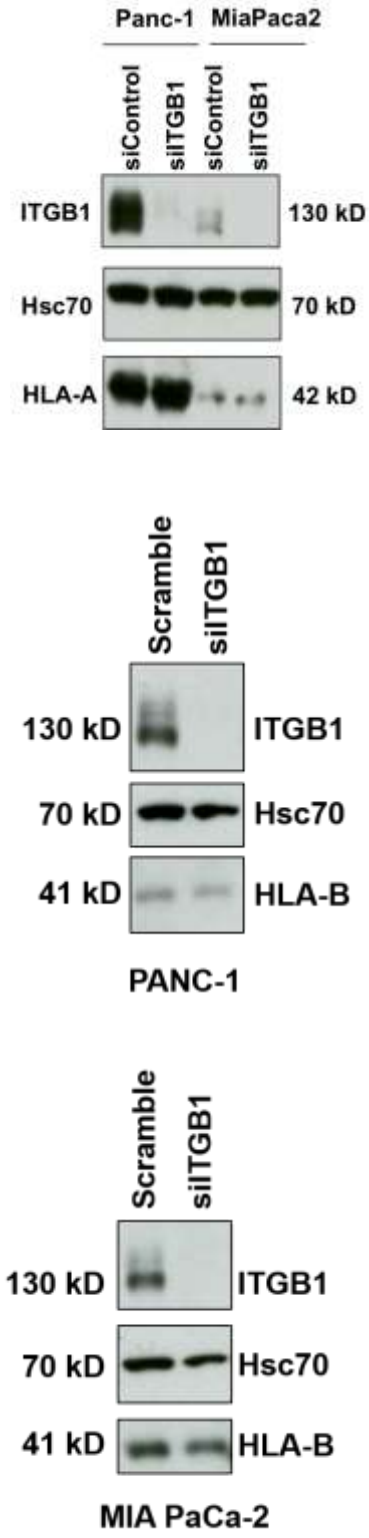
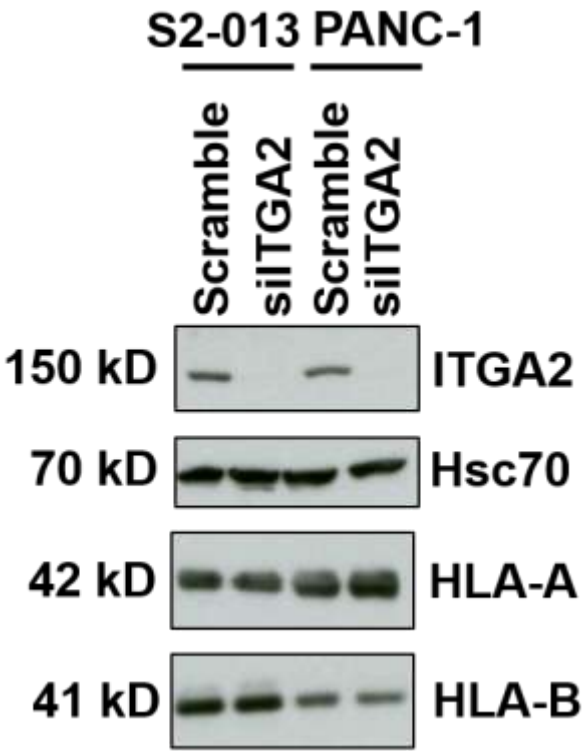

Panc-1
MiaPaca2
siControl
siITGB1
siControl
siITGB1
ITGB1
130 kD
Hsc70
70 kD
HLA-A
42 kD
Scramble
siITGB1
130 kD
ITGB1
70 kD
Hsc70
41 kD
HLA-B
PANC-1
Scramble
siITGB1
130 kD
ITGB1
70 kD
Hsc70
41 kD
HLA-B
MIA PaCa-2

Figure 7.12. *HLA class I heavy chain expression is altered in ITGA2-knockdown S2-013 cells but not in ITGA2-knockdown PANC-1 cell.* Cells were transfected with ITGA2-specific siRNA or scramble control non-targeting siRNA. At 72 h post-transfection, cell lysates were collected. Immunoblots were performed on cell lysates to investigate the effects of ITGA2 knockdown on HLA-A and HLA-B expression. Hsc70 is used as a loading control and ITGA2 knockdown was confirmed in these lysates by probing with an ITGA2-specific antibody. The results are representative of 2 separate experiments for S2-013. For PANC-1 cells, results shown are from one experiment.

Figure 7.12.


S2-013
PANC-1
Scramble
siITGA2
Scramble
siITGA2
150 kD
ITGA2
70 kD
Hsc70
42 kD
HLA-A
41 kD
HLA-B

7.6 Investigate role of the Hippo pathway downstream of HLA class I molecules

Previous studies have reported an intersection between members of the Hippo signaling pathway and integrins as well as TGF-β (Margadant and Sonnenberg 2010; Xiang et al., 2018). Therefore, studies were initiated to investigate the role that the Hippo signaling pathway plays downstream of HLA class I molecules, as other work described in this dissertation has indicated that intersecting pathways involving integrins and TGF-β are affected by HLA class I molecules. These studies could lead to further knowledge about downstream signaling pathways that are affected by HLA class I molecules and thus could lead to a greater repertoire of targets for treatment of this devastating disease.

7.6.1 pYap and pLATS1 are differentially affected by HLA-A and HLA-B knockdown

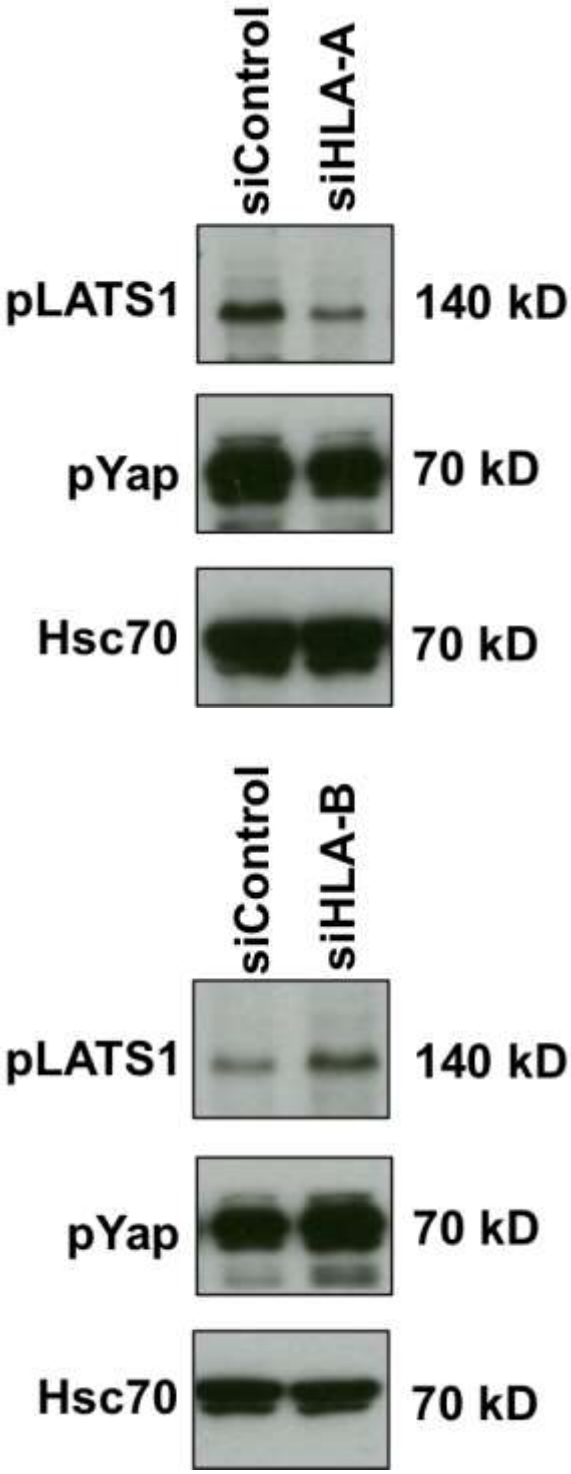
In S2-013 cells, knockdown of HLA-A and HLA-B was achieved by transient siRNA knockdown (data not shown). The expression of pYap S125 (i.e., the Hippo pathway protein Yap phosphorylated at a common activation site of this molecule) was assessed post-HLA heavy chain isotype knockdown. The pYap S125 level was found to decrease when HLA-A was knocked down but increase when HLA-B was knocked down (**Figure 7.13**). The effect of HLA-A and –B on the expression of the upstream Hippo pathway molecule LATS1 was also investigated. The phosphorylation of LATS1 is mediated by Mst1/2, which leads to activation of LATS1. Once activated, LATS1 can phosphorylate Yap, which leads to its subsequent inactivation and degradation (Yu and Guan 2013). Similarly, pLATS1 S909 was decreased in HLA-A knockdown and increased in HLA-B knockdown cells (**Figure 7.13**).

7.6.2 Discussion

The Hippo pathway is dysregulated in a variety of different cancers, including pancreatic cancer (Rozengurt et al., 2018; Zanconato et al., 2016). The pathway controls a variety of different cancer-related phenotypes such as cell proliferation, progression through the cell cycle, and cell migration (Yang et al., 2015). To date, no studies have implicated HLA class I molecules in modulation of the Hippo pathway in any model, including cancer. The role of the Hippo pathway in immunology is an active area of research, however, and further investigation into the role of HLA class I molecules in the Hippo pathway, as well as its potential influence on HLA class I expression, is warranted, and may contribute to the development of new molecular therapeutics and immunotherapeutics for pancreatic cancer and other cancer types.

Figure 7.13. *Components of the Hippo pathway are differentially regulated by HLA-A and HLA-B knockdown in S2-013 pancreatic cancer cells.* S2-013 cells were transfected with HLA-A, HLA-B, or control siRNA. At 72 h post-transfection cell lysates were collected and immunoblotted for either pLATS1 S909, pYap S125, or Hsc70 (loading control). The results are representative of 4 separate experiments for HLA-A knockdown and 3 separate experiments for HLA-B knockdown.

Figure 7.13.


siControl
siHLA-A
pLATS1
140 kD
pYap
70 kD
Hsc70
70 kD
siControl
siHLA-B
pLATS1
140 kD
pYap
70 kD
Hsc70
70 kD

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