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The Role of Metastasis Suppressor CD82 in the Deactivation of the c-Met Signaling Pathway in Prostate Cancer

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The Role of Metastasis Suppressor CD82 in the Deactivation of the c-Met Signaling
Pathway in Prostate Cancer

Katie Lynn Uhl

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

Partial Fulfillment of the Requirements

For the Degree of

Cell and Molecular Biology

Department of Cell and Molecular Biology

April 2017

Dedication Page

This thesis is dedicated to my family and friends. They provided me with invaluable confidence and encouragement during my entire graduate career. I am eternally grateful to my heavenly Father for giving me life, hope, and grace. I also dedicate this thesis to Cassandra Hogan and Terri Fiebig, who inspired me to be the person I am today, and their families.

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Abstract

BACKGROUND AND PURPOSE: Prostate cancer is the second leading cause of cancer deaths among men in the United States. Metastasis plays a major role in patient prognosis and treatment options. One of the factors that influences metastasis is the activation of the c-Met signaling pathway. Activation of the growth factor c-Met results in cytoskeletal rearrangement, migration, and invasion. Previous studies have shown CD82 to act as an active metastatic suppressor in normal, healthy cells and is downregulated in various forms of cancer. When CD82 is re-expressed in cancer cells, the cells no longer express metastatic characteristics. The purpose of this study was to determine what effect, if any, re-expression of CD82 in prostate cancer cells had on c-Met mediated migratory characteristics. **METHODS AND MATERIALS:** Two prostate cancer cell lines were utilized for this project. PC3-29 cells have been engineered with a vector that expresses CD82 and PC3-5V carry an empty vector and were used as a control in this study. **ANALYSES:** Expression levels of CD82, c-Met, related migratory proteins, and the activation state of c-Met were determined via western blot analysis. Visualization of cytoskeletal changes was done by staining F-actin fibers and focal adhesions. **RESULTS:** Re-expression of CD82 in PC3-29 cells led to a decrease in c-Met activation. CD82 prevented the formation of F-actin fibers and focal adhesions needed for metastasis. **CONCLUSIONS:** CD82 directly impacts the activation of c-Met by preventing the phosphorylation of Rac1, inhibiting the cytoskeletal changes needed for metastasis to occur.

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Abbreviations

Abbreviation	Complete Term
PSA	Prostate specific antigen
c-Met	Tyrosine-protein kinase Met
HGF	Hepatocyte growth factor
CAM	Cell adhesion molecule
EMT	Epithelial to mesenchymal transition
FAK	Focal adhesion kinase
Src	Sarcoma family kinase
ECM	Extra cellular matrix
P13K	Phosphoinositide 3-kinase
GAB1	GRB2-associated-bindign protein 1
Grb2	Growth factor receptor-bound protein 2
SHP2	Small heterodimer partner
STAT3	Signal transducer and activator of transcription 3
ECM	Extra cellular matrix
MMP	Matrix metalloprotein
TIMP-1	Tissue inhibitor of metalloprotein-1
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
TMPRSS2	Transmembrane protease, serine 2
TET2	Tet methylcytosine dioxygenase 2
PBRM1	Protein polybromo-1
AP2	Activator protein 2
p130Cas	P130 Crk associated substrate
ERK	Extracellular signal-regulated kinase
AKT	RAC-alpha serine/threonine-protein kinase
Grb2	Growth factor receptor-bound protein 2
SOS	Son of sevenless
BSA	Bovine serum albumin
PBS	Phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
STI	Soybean trypsin inhibitor
BCA	Bicinchoninic acid
SDS	Sodium dodecyl sulphate
PVDF	polyvinylidene difluoride
TBST	Tris-buffered saline/Tween 20
PTYR	Phosphorylated tyrosine
ROS	Reactive oxygen species
PDGF	Platelet-derived growth factor

Chapter One: Introduction

Prostate cancer is the second leading cause of cancer deaths among men in the United States (1). The traditional treatments for prostate cancer are castration and androgen-ablative therapies (2). Prostate cancer cases can be divided into two categories: typical and non-typical. Typical prostate cancer over-expresses androgen receptors and prostate specific antigen (PSA).

Therapeutics for prostate cancer have taken advantage of these two targets, but, recently research has found that the use of androgen-ablative therapies and related chemotherapies can lead to resistance in recurrent prostate cancer cases (2). Non-typical prostate cancer cases do not express androgen receptors nor PSA, significantly limiting treatment options. This expanding need for new drug targets for prostate cancer treatment has spawned hundreds of research studies into this disease.

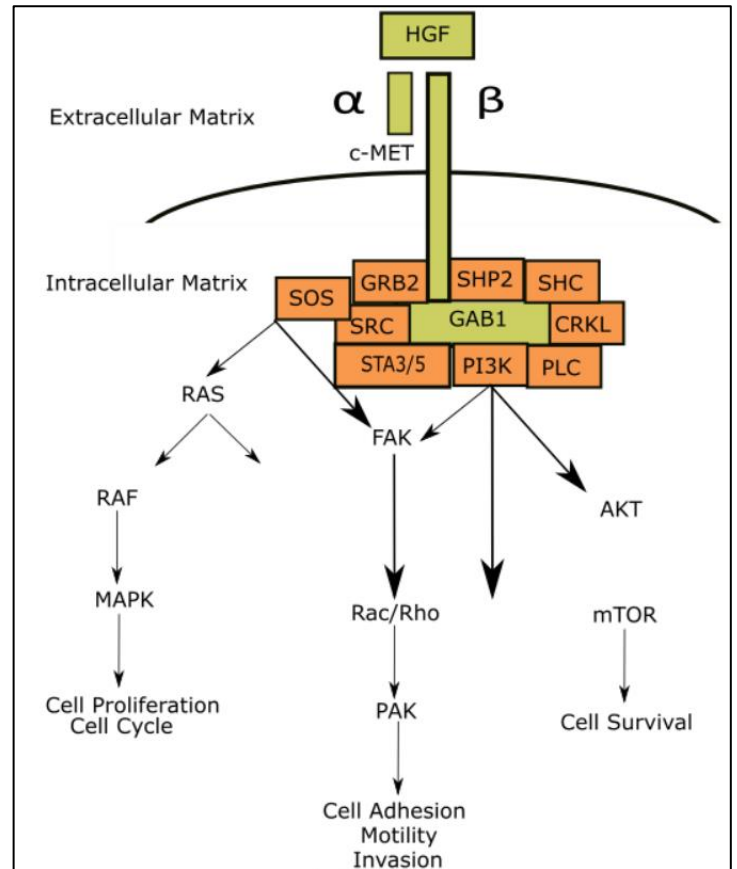


Figure 1: Outline of the c-Met signaling pathway. c-Met has been shown to have one endogenous ligand, HGF. The docking of HGF to the c-Met receptor on the cell surface signals a multitude of cellular activities that aid in the development of metastatic characteristics. Modified from Sridhar and Miranti, 2006.

The rate at which a malignant tumor begins to spread, or metastasize, has a direct impact on patient prognosis. A localized tumor has better treatment options than one that

has metastasized to other areas of the body. One of the factors that has been shown to lead to metastasis is the activation of the c-Met signaling pathway. The c-Met pathway has been implicated in many cellular activities, including metastasis (Figure 1) (3). Hepatocyte growth factor (HGF) is the only known ligand of the c-Met receptor. HGF has been shown to promote the invasiveness of cancer cells by increasing matrix adhesion and encouraging the migration of tumor cells (4). The basement membrane is a thin, fibrous barrier separating the endothelium from the connective tissue underneath. Once cancer cells have broken through this membrane, they are able to spread to the nearest lymph node. The lymphatic system will give the cells direct access to the bloodstream. c-Met signaling can also be activated by engaging integrins. Integrins allow the cell to respond to rapidly changing conditions in the cellular environment. An elevation in integrin levels has been associated with tumorigenesis and metastasis (5). Activation of c-Met results in the phosphorylation of four separate sites within the cell.

In addition to integrins, the absence or reduction of tumor suppressor proteins also play a major role in the promotion of tumorigenesis. Previous studies have shown the tumor suppressor protein CD82 to be an active metastasis suppressor in normal, healthy cells (5). The downregulation of CD82 has been confirmed in metastatic tumors, indicating that the protein may play a role in the suppression of metastatic signaling (5). Cells that express physiological levels of CD82 show an overall decrease in migratory behavior (5). CD82's ability to interact with c-Met opens an entirely new area of research into c-Met and its metastatic signaling cascade. A better understanding of the mechanisms behind prostate cancer metastasis would be invaluable to developing new treatments for castration-resistant and non-typical prostate cancer.

Incidence, Detection, and Treatment of Prostate Cancer

In the year 2013, the most recent data available from the Centers for Disease Control and Prevention, a total of 176,450 men were diagnosed with prostate cancer in the United States (6). Of those men diagnosed, 27,681 died from prostate cancer in that same year (6). The incidence of prostate cancer is steadily rising worldwide, necessitating the need for more research into this particular form of malignancy (7). A better understanding of the risk factors and pathology of this disease is important for developing better treatments.

The risk factors that can lead to an increased chance of developing prostate cancer are varied. One of the most important risk factors is age. It is estimated that only 25% of prostate cancer cases will be diagnosed before the age of 75. Of all the different types of cancer, prostate cancer has been shown to have the steepest age-incidence curve (7). There also appears to be a genetic component in prostate cancer cases, as individuals who have a first-degree relative diagnosed with the disease have a higher risk of developing prostate cancer (7). Other risk factors include exposure to radiation, recurring urinary tract infections, and a history of smoking (7).

Currently, there are two methods of detecting prostate cancer that have gained clinical acceptance: PSA screening and rectal examinations. The 1980's saw a rise in prostate cancer diagnosis as the clinical world was introduced to the prostate-specific antigen screening (PSA) (8). PSA is a protein produced in the prostate glands and elevated levels of this protein have been linked to the development of prostate cancer. PSA has been used to aid in the diagnosis of prostate cancer and patient prognosis because of this relationship. Recently, the value of using prostate specific antigen as a

method for detecting the presence of cancer has been called into question. The development of the PSA screening test in the mid-1980's led to an increase in the reported number of prostate cancer cases; better diagnostic tools generally lead to more cases of a disease being discovered. A study conducted in 2002 revealed that, per a computer model of incidence rates, approximately 29% of prostate cancer diagnosis among white males and 44% among black males were not clinically significant (9). Treating low grade prostate cancer with high grade chemotherapy can lead to resistance over time. Research studies conducted among original tissue samples taken from patients in the 1980's revealed that most of the cases diagnosed were not clinically significant, highlighting the lack of specificity in PSA screening (9).

The sensitivity of the PSA screening test can be improved. PSA levels can predict patient prognosis in 25% of all differentiated cancers when used in combination with the Gleason histological diagnosis method (10). Approximately 50% of the men diagnosed with prostate cancer will find that the cancer has metastasized beyond its tissue of origin (10). These statistics demonstrate the need for more specific detection methods of prostate cancer, which can only be achieved through more intensive research.

Cellular and Molecular Pathology of Prostate Cancer

Carcinogenesis is the transition of a benign tumor to a malignant one. It is an important step in the progression of prostate cancer that has yet to be fully understood. Research has provided significant evidence to suggest that carcinogenesis involves major genetic changes within the once-healthy tissue, including the activation of oncogenes and the loss of tumor suppressor genes (11).

There are three distinct phenotypes currently associated with the transition of normal prostate tissue to prostate cancer (11). The first is the secretory luminal cells in the prostate epithelial tissue beginning to express prostate specific antigen (PSA), as well as cytokeratins 8 and 18 (12, 13, 14, 15). PSA is a serine protease produced by prostate epithelial cells and its production is regulated by the male hormone androgen (8). The second most important phenotype is that the basal cells, which function to connect the epithelial tissue to the stroma, begin to express cytokeratins with high molecular weights (12) (8). Cytokeratins are proteins made up of keratin filaments found in the cytoskeletons of epithelial cells (13). The final phenotype is when the neuroendocrine cells, responsible for secreting products that may promote cell growth, begin to differentiate (8). The development of these phenotypes result in a decline in cellular division in the basal cell layer and an increase in the proliferation of the luminal secretory cells (8). These changes that occur prior to a cell acquiring a diseased phenotype are referred to as “pre-malignant proliferation disorders” and have been associated with the abnormal expression of both tumor suppressor genes and oncogenes (8).

Genetic Factors Influencing Metastatic Characteristics

Metastasis is possibly the deadliest characteristic that a malignant tumor can acquire. Once a tumor has penetrated the barrier that separates the tissue from the blood supply, the cancer is free to travel throughout the entire body. It is imperative that more research efforts into better understanding the mechanisms behind the transition to cancer cells and the conversion of a primary tumor to a metastatic one. As mentioned before, the metastatic status of prostate cancer plays a major role in patient prognosis, as well as in

the selection of an appropriate treatment method. The metastatic status of the cancer hinders treatment efficacy.

Cancer is a condition in which cell populations disregard mitotic signaling and rapidly begin to divide. Within a cell's genetic code are the instructions for cellular division, for which there are checkpoints a cell must meet before it divides by a process known as mitosis. These checkpoints ensure that the cell has doubled all genetic material, organelles, and other cellular resources so the process of mitosis results in two complete, healthy cells. When a cell transitions into a cancerous cell it begins to reproduce at such a rate as to outcompete all the normal cells in the surrounding tissue. The accumulation of these cancerous cells in one place leads to the development of a malignant tumor. The scientific community now recognizes that cancer arises when there are mutations in one of two sets of genes: proto-oncogenes and tumor suppressors. Proto-oncogenes are genes that have the potential to protect cells from apoptosis, or controlled cell death. Alterations in proto-oncogenes that lead to their activation include gene amplification, overexpression, and other types of mutations (14). Tumor suppressors genes prevent tumor formation. These genes become inactive when a mutation occurs in both alleles (14). CD82 is a tumor suppressor that has been demonstrated to be downregulated in prostate cancer (5).

Growth factor receptors also aid in the development of metastatic characteristics in advanced cancers. Similar to proto-oncogenes, these receptors are responsible for regulating cell growth and division. The overexpression of growth factor receptors has been observed in multiple types of cancer, including prostate cancer. c-Met is a growth factor receptor that has been demonstrated to have an impact in the transition from a

localized tumor cell to a metastatic tumor. The study described in this manuscript had the goal of determining the way in which the tumor suppressor CD82 interacts with the cell signaling mediated cascade by the growth factor receptor known as c-Met.

Progression from Localized Tumor to Bone Metastasis in Prostate Cancer

The degree to which a prostate cancer tumor has metastasized is directly proportional to the expected survival rate of that patient. Patients with a localized tumor have a five-year survival rate of approximately 100%; for those with a metastatic phenotype, the five-year survival rate can drop to as low as 31% (15). While prostate cancer is able to metastasize to any other tissue type in the body, it demonstrates a proclivity towards invading the bone (15). The location of the prostate gland in the male pelvic region provides an environment rich in bone to metastasize to. One of the hallmarks of prostate cancer development is its dependence on stimulation via the secretion of androgen. Androgen binds to the receptor found in the cytoplasm; upon activation, the receptor will then translocate into the nucleus (15). Once the complex has entered the nucleus, it will go on to influence the transcription of androgen-related genes and the proliferation of the prostate cancer cells (15).

The dependence of prostate cancer on the secretion of androgen has led to castration serving as the most common treatment option (15). While surgical castration can be performed, chemical castration is a temporary alternative; patients are treated with antiandrogen drugs that function to either prevent androgen from being secreted or prevent androgen from binding to its receptor. The effectiveness of castration on early metastatic prostate cancer is limited, as the disease will eventually adapt to the new

chemical environment and become “castration resistant” (15). One hypothesis behind this phenomenon is that the tumor gradually becomes hypersensitive to androgen stimulation, lowering the stimulation threshold so that it will take less androgen to elicit the same initial response (15). Another possibility is that the tumor adapts in such a way that both the autocrine and paracrine production of androgen is upregulated in order to compensate for the effects of castration (15). The androgen receptor could also be adapting to the lack of androgen by becoming activated via other steroids, such as estrogens, and ligand-independent activation by tyrosine kinases (15).

The metastasis of prostate cancer is a complex, multistep process in which a tumor cell will leave its tissue of origin and spread to a secondary tumor site (15). The first step of the metastatic process is decreased cell adhesion, which is characterized by a shift from epithelial to mesenchymal tissue (15). This phenomenon is known as the epithelial to mesenchymal transition (EMT), and is when epithelial cells begin to take on the properties of mesenchymal stem cells. While EMT has yet to be fully understood, one of the hallmark traits is cadherin switching (15). Normal epithelial cells express a large amount of E-cadherin. During cadherin switching, the production of E-cadherin is downregulated, and expression of N-cadherin is upregulated to levels normally associated with mesenchymal stem cells (15). There is also an association between decreased E-cadherin expression and decreased β -cadherin expression, both of which have been linked to more aggressive cases of prostate cancer (15). In vitro model system studies have proven that the levels of E-cadherin are significantly reduced in more invasive human prostate cancer cell lines (15).

When a cell has undergone EMT, there is a noticeable decrease in cell adhesion that is observed. The second step in metastasis is the cells demonstrating mesenchymal properties begin to detach from the primary tumor site. It is critical that normal cells become anchored to their environment, as forming cell junctions with adjacent cells is needed for adequate cell-to-cell communication. For cancer cells, the dependence on physical stability is not as crucial. Normal prostate epithelial cells are limited in their ability to migrate, as the basal layer of cells is attached to the lumen, which in turn is attached to the basement membrane (15). Metastasis is characterized by decreased cell adhesion; early metastatic prostate cancer cells have been shown to have altered expression of molecules that are critical to cell adhesion, such as E-cadherin (15). E-cadherin is a cell adhesion molecule (CAM) that serves to form an anchor between cells and also works with β -cadherin to hold the actin cytoskeleton in place (15).

Integrins also play a role in the decreased cell attachment observed in the initial stages of metastatic prostate cancer. These signaling molecules are responsible for regulating cellular processes such as cell survival, cell motility, and cell migration by associating with focal adhesions (15). The principle integrins found in normal prostate epithelial cells are $\alpha 5\beta 1$, $\alpha 6\beta 1$, and $\alpha 6\beta 4$, along with slightly lower expression of $\alpha v\beta 3$ and $\alpha 3\beta 1$ (15). There is a correlation between the reduced expression of $\alpha 6$ and the invasiveness of prostate tumor biopsy samples that has been experimentally demonstrated (15). The reduced expression of these critical integrins results in a decrease in cell adhesion which further stimulates metastasis in malignant tumors (15).

Signaling through focal adhesions, (macromolecular assemblies that transmit regulatory signals), can also be accomplished by nonreceptor tyrosine kinase focal

adhesion kinase (FAK) and the Src family of kinases (15). When a cell is preparing to migrate, focal adhesions are assembled at the edges of the cell and form structures that are known as lamellipodia, allowing the cell to anchor itself to the extracellular matrix (ECM) to pull itself along the tissue. Once the cell has finished contacting, the focal adhesions will disassemble and then re-form for the next movement (15). Studies have shown that the inhibition of FAK has a significant effect on a cell's ability to migrate, including changes in integrin concentrations (15). PC3 cells a correlation between high levels of FAK expression and an increase in the development of metastatic characteristics (15). After integrins associate with FAK, the phosphorylation of FAK leads to the phosphorylation of Src (15). The Src family of kinases stimulates cell migration via the phosphorylation of the E-cadherin/ β -cadherin complex (Figure 2). The Src family of kinases has been shown to be activated by several different mechanisms, including the growth factor receptors. Phosphoinositide 3-kinase (P13K) is part of a family of signal transduction enzymes that is responsible for activating β -catenin.

Prostate cancer cells can migrate in part due to the partial degradation of the ECM by molecules known as proteinases (15). The major proteinases that are involved in the degradation process associated with the migration of prostate cancer cells are matrix metalloproteinases (MMPs) and serine proteinases (15). MMPs are a family of proenzymes that bind to zinc and remain activated until proteolytic cleavage occurs (15). ProMMP-2 is activated by Type 1 MMP, which then activates MMP-2 and MMP-9 (15). Compared to normal levels found in prostate epithelial cells, metastatic prostate cancer cells have a higher ratio of MMP-2/-9 to tissue inhibitor of metalloproteinases-1 (TIMP-

1) (15). Similar elevated ratios have been correlated with higher scoring tumors on the Gleason scale, and thus is also associated with a higher patient fatality rate (15).

When the ECM has finally degraded sufficiently to allow the malignant cells to infiltrate the circulatory system, they must remain in circulation long enough to attach to the vascular endothelium (15). The prostate cancer cells must then interact with the surrounding tissue to leave the blood vessel and enter the bone, which is one of the most poorly understood aspects of prostate cancer metastasis (15). As mentioned before, prostate cancer displays a high affinity for metastasizing to bone tissue (15). One aspect of the binding of metastatic prostate cancer to the bone is that the endothelial tissue within the bone is constitutively expressing adhesion molecules, such as P-selectin (15). P-selectin is a transmembrane protein that is commonly expressed in both endothelial tissue and platelets. Specifically, it is found in Weibel-Palade bodies in endothelial tissue and in the alpha granules in platelets (16). When the appropriate cells are stimulated, P-selectin is translocated to the plasma membrane (16).

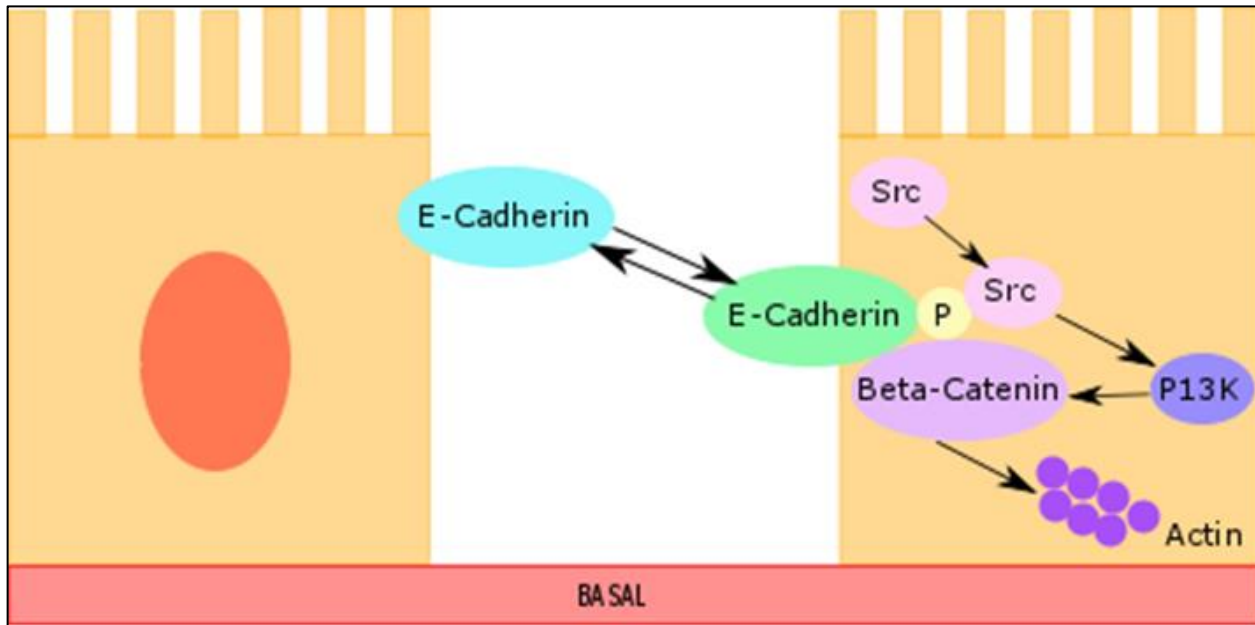


Figure 2: Graphical representation of the Src stimulation of metastatic invasion via the phosphorylation of the E-cadherin/ β -catenin complex. When the E-cadherin molecule is phosphorylated, it forms a complex with β -catenin. The Src protein is then activated, and the complex is able to interact with the actin cytoskeleton. Adapted from Jin, et al. (2011) (15)

Somatic Mutations Contributing to the Metastatic Phenotype in Castration

Resistant Prostate Cancer

Molecular biomarkers are objective characteristics that indicate a diseased phenotype, even if the patient is not yet presenting symptoms. In terms of prostate cancer, molecular biomarkers would improve patient prognosis by providing physicians with the means of distinguishing between aggressive subtypes and identifying metastasis early on. A twin study published in 2009 estimates that approximately 42% of disease disparity can be linked to genetic variation, some of which are linked to somatic alterations (17). Insufficient data regarding a metastatic phenotype is partially due to the heterogeneity of primary prostate tumors (18). Prostate cancer tumors are often made up of many

genetically distinct layers that make it difficult to identify the actual mutations that led to the cancer cells migrating from their tissue of origin.

There is also substantial evidence to support the association between somatic mutations and the development of hormone-related cancers. One of the most well-known associations is between specific mutations in the BRCA1 and BRCA2 genes and the development of breast cancer. A study published in 2013 found that exomes of metastatic prostate cancer tumors contained an Ashkenazi Jewish germline mutation del185AG in BRCA1 (18). The BRCA1 gene is located on the seventeenth chromosome; the germline mutation del185AG indicates a 185 deletion of AG (19). Specifically the BRCA1 gene is responsible for the regulation of the cell cycle and DNA repair (19). In addition to the del185AG germline mutation, researchers found sixty-two somatic alterations in proteins, some of which were products of cancer related genes (18). The cancer related genes found to have genetic variants amongst the prostate cancer tumor samples were TMPRSS2-ERG, TET2, and PBRM1 (18). The majority of the alterations were present in only the metastatic prostate cancer tumor samples, with a smaller group of alterations being specific to primary tumors only (18).

CD82/KAI1 Protein as a Metastasis/Tumor Suppressor

The metastatic suppressor CD82 was first characterized by Dong, et al. in 1995 (20). Microcell-mediated chromosome transfer revealed the location of the gene to be 11p11.2-13 and was later confirmed using Southern blot analysis (20). The four-domain structure of CD82 places it in the tetraspanin superfamily (21). Tetraspanins have been found only in multi-cellular organisms and are expressed at relatively similar levels in all

organisms in which they are present (21). While CD82 does not appear to have any function by itself, it has been found to associate with other proteins and regulate their functions. The down-regulation of CD82 has been linked to tumor metastasis and poor patient prognosis. Studies have shown that the loss of CD82 expression has been implicated in approximately 70% of all prostate cancer cases (7).

One of the mechanisms by which CD82 controls metastasis is by influencing cellular adhesion. A study published in 2015 demonstrated that the expression levels of CD82 play a role in inhibiting both metastasis and invasion in primary tumor cells (22). When malignant tumor cells were modified to over-express CD82, researchers found that the cells over-expressing the CD82 protein adhered more strongly to the endogenous matrix, and the ability of the cells to adhere to the endothelium tissue of blood vessels was greatly diminished (22). The over-expression of CD82 inhibits a tumor cell's ability to invade other tissues by influencing cellular adherence to the tissue of origin (22). This means that when CD82 is over-expressed in tumor cells they are firmly adhered to the surrounding tissue, and the probability of the tumor metastasizing is very low. CD82 expression is up-regulated by the cellular tumor antigen p53, the proto-oncogene junB, and the transcription factor known as AP2 (5). The over-expression of CD82 also prevents tumor cells from adhering to the walls of blood vessels. It should be noted here that while CD82 does inhibit tumor metastasis, it does not interfere with primary tumor formation (5). CD82 interferes with a primary tumor's ability to invade and migrate to non-native tissues (5). What is currently not known is the mechanism by which CD82 can suppress tumor cell invasion and metastasis.

Regulation of Growth Factor Receptor c-Met by CD82

CD82 regulates cellular activities by associating with proteins from its location in the cellular membrane. One of the proteins that CD82 has been found to associate with the growth factor receptor known as c-Met (23). Growth factors are proteins that are responsible for maintaining the growth and development of cells by regulating intracellular activity via a biological phenomenon known as a signaling cascade. c-Met is a growth factor receptor that is found within the cellular membrane in all cells, whether it be cancerous or healthy. The only known ligand that binds to c-Met is hepatocyte growth factor (HGF). When HGF docks at the binding site located on the α subunit of the c-Met protein, the protein is activated. Activation of the c-Met protein results in the phosphorylation of four key sites: pY1003, pY1234/1235, pY1349, and pY1356. The phosphorylation of these sites recruits specific downstream molecules that lead to downstream signaling events, such as cell survival, cell proliferation, cytoskeletal rearrangement, cell adhesion, invasion, and migration (Figure 1).

The c-Met receptor can also be activated by integrin crosstalk within the cytoplasm. Integrins are small signaling molecules that facilitate a connection between the extracellular matrix and intra-cellular structures, such as the cytoskeleton (23). The interaction between integrins results in the phosphorylation of the c-Met phosphorylation sites and the signaling cascade begins. Several studies have reported a change in integrin levels within cancerous tissues, with prostate cancer cells showing elevated levels of $\alpha 3\beta 1$ and $\alpha 6\beta 1$ (23). These integrins have been shown to regulate the same cellular activities as c-Met, further implicating them in regulating cell migration and metastasis (5).

Research into c-Met has yet to reveal the specific ways in which CD82 interferes with the c-Met signaling cascade and its related downstream molecules. An experiment conducted by Sridhar and Miranti in 2006 provided significant insight into the regulatory action of CD82 on the c-Met signaling pathway. Using the prostate cancer cell line PC3, researchers performed a series of experiments to test the hypothesis that CD82 regulates the integrin-mediated activation of the c-Met pathway (5). They chose a metastatic prostate cancer model, as c-Met has been shown to be highly over-expressed in prostate cancer and has been linked to chemotherapy resistance. One of the study's major findings was that CD82 does not regulate the actual expression of c-Met. After CD82 was re-introduced into the metastatic prostate cancer cells, western blots showed that there was the same concentration of c-Met protein in both the experimental cells and the controls (5). However, when the activation levels of c-Met were tested, there was a definite difference between the two cell lines. The cells that were expressing CD82 showed markedly reduced levels of activated c-Met, while the levels of activated c-Met in control cells remained high (5). Even though c-Met is still being produced at the same rate within the cancerous tissues, the re-expression of CD82 appears to prevent c-Met from being activated (5).

Having concluded that CD82 expression was interfering with the activation of the c-Met signaling pathway, the next step was to investigate the mode of inhibition. The first hypothesis was that CD82 and c-Met were physically interacting with one another due to their respective locations on the cell surface. When immunostaining was carried out on CD82-expressing cells, it was discovered that this was not the case. While CD82 and c-Met are located near one another, there is no co-localization between the two proteins

that would result in the de-activation of c-Met (5). This further supported the authors' hypothesis that CD82 was interfering with the integrin crosstalk responsible for c-Met activation within the cytoplasm.

After immunostaining ruled out the possibility of co-localization between the two proteins, the authors then tested the hypothesis of an indirect deactivation mechanism. c-Met is responsible for the regulation of many cellular activities and it does so by the activation of downstream signaling molecules. One of these molecules is Src, a non-receptor kinase that is known to transform healthy cells into malignant tumors when overexpressed (24). When HGF binds to c-Met, or c-Met is activated by integrin crosstalk, the phosphorylation of pY1349 leads to the activation of Src (23). Higher expression of Src and its downstream signaling partners have been demonstrated to be overexpressed in a variety of cancers, including breast cancer, pancreatic cancer, and prostate cancer (24). In the c-Met signaling pathway, Src substrates include focal adhesion kinase (FAK) and p130 Crk associated substrate (p130Cas). Fibroblasts from FAK-null mice demonstrate decreased migration behavior, but an increase in local adhesions to the surrounding cytoskeleton (25). Migration and invasion of tumor cells require an elaborate rearrangement of the cytoskeleton to form the lamellipodi (a cytoskeletal projection made of actin filaments), necessary for cell movement. The construction and maintenance of this actin fiber scaffolding is carried out through the formation of focal adhesions, a task in which FAK plays a critical role. The FAK-Src complex is then responsible for the phosphorylation of paxillin, and p130Cas (25). Both molecules mediate the organization of focal adhesions and the cytoskeleton, which ultimately allow the cell to migrate (25). Similar to the FAK-null mice, mice lacking

p130Cas demonstrated poor actin filament arrangement and decreased migration behavior (25).

Given the similarities between FAK-null mice and PC3 cells re-expressing CD82, the possibility that CD82 was interfering with Src activation was explored. Using western-blotting analysis, CD82 was found to regulate the phosphorylation of Src and subsequently the activation of FAK and p130Cas (24). The presence of the CD82 protein was linked to the inhibition of the tyrosine phosphorylation associated with p130Cas (24). When CD82 is present, there is a reduction in the integrin mediated activation of Src and related proteins, most likely contributing to the lack of migration seen in CD82 expressing tumors. Keeping in mind that the inhibition of c-Met also blocks invasion, the conclusion can be reached that CD82 expression reduces the invasive phenotype through the reduction of both c-Met and Src signaling (24).

Oncogenic c-Met Signaling and Tumor Development

The identification of c-Met as an oncogenic target originated from the discovery of a missense c-Met mutation in patients who had been diagnosed with hereditary papillary renal cell carcinomas (PRCCs) (26). These mutations include trisomy of chromosome 7, which has been identified as a common mutation in PRCC patients (27) (28). Somatic mutations of c-Met have been identified in a variety of cancers, including gastric, head and neck, liver, ovarian, and thyroid (29). Elevated levels of the c-Met ligand HGF lead to the over-activation of the c-Met signaling pathway. In addition to somatic mutations in c-Met being linked to cancer development, somatic mutations in HGF have also been identified in a variety of different cancers (29). One of the most

common mutations in HGF is in the promoter region of the HGF gene, resulting in the increased expression of HGF and the over-activation of the c-Met pathway (30). A study conducted by Ma et al. in 2008 successfully provided a direct link between the dysregulation of c-Met signaling and the development of tumors in human patients (31).

The degradation of the extracellular matrix (ECM) surrounding the tumor cell is a major milestone on the road to metastasis (32). Tumor cells are capable of releasing enzymes known as proteases that are capable of hydrolyzing peptide bonds in order to break down the ECM (32). Also crucial to the metastasis of cancer is the formation of invadopodia within the cytosol (32). Similar to lamellipodia, invadopodia are actin-rich cytoskeletal structures that are mediated by intracellular components such as focal adhesion kinase (FAK), paxillin, cdc42 and Rac1 (32). cdc42 and Rac1 are both members of the Rho GTPase family, which has been shown to promote the formation of lamellipodia and invadopodia (32). Rac1 is required for the formation of cell protrusions in both collectively and individually migrating cells (33). To degrade the ECM and invade new tissue, tumor cells must coordinate their actions with one another. Cell-to-cell communication is crucial to the development of invadopodia and lamellipodia by restricting the formation of protrusions to the leading cell (33). A lack of this communication between cells results in a chaotic disorganization that hinders the tumor cells' ability to organize their cytoskeletons to complement one another (33). cdc42 plays a major role in the cell-to-cell communication associated with the collective movement of migrating cancer cells (33). The c-Met signaling pathway stimulates the activation of the Ras pathway. The subsequent activation of Rac1/Rho/cdc42 leads to the reorganization of the cytoskeleton needed to form cellular structures needed for invasion and migration.

c-Met Activation of Rac1/Rho/cdc42 Necessary for Cell Migration

More recent studies have supported the hypothesis that CD82 is interfering with the integrin crosstalk responsible for the C-Met signaling pathway, but they have yet to identify the exact point in the pathway that CD82 is targeting. The re-expression of CD82 does not alter the activation of ERK (extracellular signal-regulated kinase), or AKT (RAC-alpha serine/threonine-protein kinase), which narrows down the list of possible targets for CD82 (24). The specific target this publication focuses on is the Rac1/Rho/cdc42 pathway. When the c-Met signaling cascade begins, growth factor receptor-bound protein 2 (Grb2) forms a complex with son of sevenless (SOS). After the complex has formed, the next step is the activation of Ras, which in turn activates the Rac1/Rho/cdc42 step. The end result of the activation of these proteins is actin cytoskeleton arrangement and changes in cell motility. Rac1 is a small GTPase that is involved in cell migration, cell cycle progression, and cytoskeleton arrangement (34). Specifically, Rac1 aids in the formation of lamellipodia and membrane ruffling (34). Lamellipodia formation can be is the first step in the cytoskeletal rearrangement required for migration; the actin fibers within the cell begin to extend through the cell lamella region, which is near the front of mobile cells (34).

Metastatic Signaling by c-Met via Rac1 Activation

As mentioned previously, the Rho-family of GTPases has been shown to promote metastatic growth via cytoskeleton dynamics and cell motility (35). These cellular activities are tied together with the growth receptor c-Met, the deregulation of which has been linked to the development of melanoma in mice models (36). The advancement of

tumors into a metastatic stage is made possible not only through c-Met, but the complex network of effector molecules that connect c-Met to the cellular controls for migration, invasion, and cytoskeleton dynamics (37) (26). This signaling network only functions through the association of c-Met with repulsive co-receptors (plexins), adhesion molecules such as the $\alpha 6\beta 4$ integrin, and the coordinated actions of the Rho family GTPases Rac1, Rho, and cdc42 (38) (39) (40).

Recently, a new concept regarding signal transduction maintained by growth factor receptors has begun to appear in literature. Specifically, there is growing evidence to suggest that receptors, as well as the adhesion molecules they communicate with, rely on reactive oxygen species (ROS) and hydrogen peroxide H_2O_2 as critical intermediate molecules in the downstream cascade (41) (42). In addition to cytochromes, Rac1 has been shown to be an important molecular piece of the oxidase complex found in mammalian cells (43). The Rac1/ROS interaction has been demonstrated to play a role in mitogenic signaling by PDGF and by oncogenic H-Ras (44) (45) (46). The Rac1/ROS signaling complex is activated by integrins upon cellular adhesion to the ECM, and is necessary for the development of proper focal adhesion assembly in NIH-3T3 cells (47) (48). It is reasonable to assume that the oxidant intermediates generated by Rac1 are responsible for the metastatic characteristics seen in prostate cancer (49).

The connection between Rho GTPases and the cytoskeleton has been extensively studied in the neuroblastoma line N1E-115. The activation of Rac1 and cdc42 in these cells results in the formation of lamellipodia and filopodia along the very edges of the neurite (50). Despite differences in cell types, the biochemical processes behind the lamellipodia and filopodia formation rely on the activation of Rho GTPases in to form the

actin filaments necessary for migration and invasion (50) (51). This relationship carries over to fibroblasts, neurons, and macrophages (52). The effect of Rho GTPase activation on the actin cytoskeleton can be observed in epithelial and endothelial tissue, as well as in circulating cells such as lymphocytes, mast cells, and platelets (53) (54) (55). The effects of Rho GTPase activation is not limited to rearranging already-present actin filaments; studies have shown that Rho GTPases, Rac1 plays important regulatory role in coordinating the cellular activities that rely on the disassembling and re-assembling of actin fibers (56). Rho activation and signaling is required to form and maintain focal adhesions, and integrin complexes propagate the movement of the cytoskeleton (57) (58). With keratinocytes, it has been discovered that both Rac1 and Rho activation are required for the assembly of the actin cytoskeleton (59).

Purpose

The focus of this research project was to identify the specific phosphorylation site(s) and the subsequent signaling pathways in c-Met regulated by CD82. To identify the specific pathways and to determine the overall role of CD82 in prostate cancer metastasis, the following objectives were addressed:

1. Identify the c-Met phosphorylation site being blocked by CD82 expression
 - a. Integrins within the cells were engaged by plating the cells onto a substrate of collagen or laminin. The cells were then lysed, and the levels of phosphorylated Met, along with the specific Met phosphorylation sites (PTyr 1234/1235 and Total P Tyr), were analyzed by gel electrophoresis and western blot.
2. Determine whether the Rac1 pathway is being regulated by CD82 expression
 - a. Integrins within the cells were engaged via an adhesion assay. The cells were then lysed, and the levels of both Rac1 and cdc42 were analyzed by gel electrophoresis and western blot.
3. Identify the Rac1 induced cellular changes that are being affected by CD82 expression: invasion, migration, adhesion, lamellipodia formation, and F-actin fiber arrangement.
 - a. The cells were fixed to a coverslip coated with either collagen or laminin, and then stained to visualize F-actin fibers and focal adhesions

Chapter Two: Materials and Methods

Cell Lines and Cell Culture

Human prostate cancer cells (PC3) were obtained from Dr. Cindy Miranti of the University of Arizona. From this parental cell line, three clones were generated: PC3-5V, (containing an empty vector), as well as PC3-29 and PC3-#1, both of which express CD82. The cells were cultured in F12K Media, (Gibco), which was supplemented with fetal bovine serum (FBS), streptomycin, penicillin, glutamate, and amphotericin B. The cells were plated in 10mL sterile flasks and kept in an incubator at 37°C with 5% CO₂. Cells not currently being used were flash frozen in liquid nitrogen and stored at -80°C until they were needed. Frozen cells were quickly thawed in a 37°C water bath, and then added to a sterile flask with F12K Media (Gibco) at the same temperature. The cells were then cultured until the flasks were 70-80% confluent, after which further experiments were performed.

Adhesion Assay

Each cell line was grown until flasks showed 70-80% confluency. Thereafter, the media was switched to serum-free media, (Gibco, RPMI Medium 1640, Product No.11875-093), and the cells were grown in this starvation media for an additional 24 hours before beginning the procedure. Substrate plates were also prepared 24 hours in advance of the procedure. A working stock of 10µg was made of the desired substrate stock, (1.0 mg/mL laminin or 2.3 mg/mL collagen), and then added to sterile 10mL flasks in an even layer. Plates were then kept at 4°C overnight. The following day, the remaining substrate was removed via aspiration, and the plates were washed two times

with PBS. Once the washes were complete, 1% BSA-PBS solution was added evenly to each plate. The coated plates were allowed to incubate at 37°C for at least 1 hour.

Approximately 15 minutes into the incubation period of the cells, the BSA solution was removed and the substrate plates were washed with PBS. The substrate plates were then incubated upside down to dry until the cells were ready.

For the preparation of the cultured cells, the plates were removed from the incubator and washed with PBS buffer two times. Once all the PBS had been aspirated, a 1:4 solution of 2X trypsin-EDTA was added to the plates. The plates were allowed to incubate at 37°C for 10-15 minutes in the 2X trypsin-EDTA solution, or until the majority of the cells had dislodged. A solution of PBS-EDTA/STI was added to each of the plates to stop trypsinization. Each cell line was separately pooled into a centrifuge tube and the plates were washed twice more with the same solution (PBS-EDTA-STI) and pooled together. The supernatant was discarded after centrifugation at 800 rpm for 8 minutes and the pellet was re-suspended in PBS-EDTA/STI and re-centrifuged. The supernatant was again removed and the cells resuspended in (without phenol red) DMEM media (Gibco, DMEM high glucose, Product No.31053-028). The tubes, with caps loosened, were placed in the 37°C incubator for 30-40 minutes. When the incubation period was complete, the cells were added to the substrate plates. The plates were incubated at 37°C, checking every 15 minutes for adhesion under a microscope. Meanwhile, the cell suspension was centrifuged and the supernatant removed. Lysis buffer (RIPA or MAPK) was added and the plates were kept on ice for 30 minutes. Once all of the plates developed signs of cell adhesion (actin projections, flattened appearance, etc.), the plates were removed from the incubator and carefully aspirated to remove any free-floating

cells. The same lysis buffer added to the suspension was also added to the substrate plates. After adding the lysis buffer, the plates were immediately placed on ice until all plates had been coated in the buffer. For the lysis to take place, the plates were placed at 4°C for at least 30 minutes. After the lysis had taken place, the cells were removed using a cell scraper and then transferred to a microfuge tube. The samples were centrifuged, including the cell suspensions, at maximum speed for 5 minutes at 4°C. Once the samples had been collected, a BCA protein assay (ThermoFisher Scientific, Catalog No. 23225) was performed to determine the concentration of protein in each of the samples. All unused samples were stored at -80°C.

BCA Protein Assay

On the day of the immunoprecipitation assay, all samples were removed from the -80°C environment. The samples were centrifuged at maximum speed for two minutes at 4°C. Keeping all reagents and samples on ice, a protein assay was performed using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Catalog No. 23225). Protein standards were prepared using the BCA protein solution provided in the kit. Exactly 10µL of each sample was added to 40µL of distilled water; 1mL of the mixed Reagent A and Reagent B was added after the samples had been thoroughly mixed. The solution was also added to the protein standards. The samples and standards were incubated in a 37°C water bath for 30 minutes. After the incubation period was complete, the absorbance of each sample was measured at 562 nm. This calculation allowed for the concentration of protein to be determined in each of the samples.

Immunoprecipitation Assay

Protein A-agarose beads (Pierce, Product No.20333), were prepared by washing the beads three times with 1% BSA in lysis buffer. In between the washes, the beads were placed in the centrifuge and spun at max speed for two minutes at 4°C. The cell samples were thawed and prepared for the assay by making a concentration of 1 ug/uL in the same lysis buffer as used above, then stored on ice until the primary antibody was added. After the primary antibody was added, the samples were left on the rotator at 4°C overnight. The beads were then washed three times with the lysis buffer, spinning at 4°C for two minutes in between each wash. The supernatant was then removed and the beads resuspended in 2X SDS buffer (800 uL of 2X sample buffer and 200 uL of beta-mercaptoethanol). The samples were boiled for five minutes, and then spun for two minutes at maximum speed at room temperature. The supernatant was then removed and added onto an SDS-gel (sodium dodecyl sulfate with distilled water) and the proteins separated by electrophoresis.

Table 1: Targets for immunostaining are given with their corresponding antibody, as well as the dilution that was used to achieve optimal results.

Target	Antibody Used	Dilution
Total Met	Met (D1C2) XP® Rabbit mAB, Cell Signaling Technologies, Product No. 8198.	1:500
Tyr 1234/1235	Anti-phosphor-Met (Tyr 1234/1235), Upstate Signaling Solutions, Product No. 07-211	1:1000
Rac1	Anti-Rac1 Monoclonal Antibody, Cytoskeleton Inc. Product No. ARC03-FS	1:500
Total P _{tyr}	4G10® Platinum, Anti-Phosphotyrosine, One World Lab, Product No. 18943	1:2500

Western blots

A polyacrylamide gel was loaded with equal concentrations of protein for each of the desired samples. In addition to each of the samples, a prestained molecular weight marker (Novex Benchmark™ Prestained Protein Ladder, Product No.10748-010) was loaded as well. SDS running buffer was added to the gel apparatus prior to the loading and running of the gel. The gel was run at 125V for 90 minutes, or until the samples had migrated $\frac{3}{4}$ of the length of the gel.

Once the gel had been run to completion, it was then blotted to a PVDF (polyvinylidene fluoride) membrane (Immobilon Transfer Membrane, Product No. IPFL07810). This was accomplished by placing the electrophoresis gel into a transfer tank for 3 hours at 25V using blot transfer buffer. The transfer buffer used for western blotting was Novex Tris-Glycine Transfer (ThermoFisher Scientific, Catalog No. LC3675) buffer plus methanol, and distilled water. When the gel had been blotted, any potential non-specific binding sites were blocked with 5% BSA/TBST and incubated for 2 hours at room temperature. The appropriate primary antibody was added to the blotting membrane (see Table 3), then incubated at room temperature for 2 hours. The blot was then washed four times with wash buffer (.1% PBS-2.0 Tween), spending 10 minutes on the shaker between each wash. After the final wash, the secondary antibody, (Li-Cor IRDye 800CW; donkey-anti-mouse Product No. 926-32212, or donkey-anti-rabbit Product No.926-32213), was then added to the membrane, which was allowed to rotate for one hour. The blot was again washed four times with the wash buffer, shaking 10 minutes in between each wash. The blot was illuminated at 800nm and imaged was captured using the Li-Cor Odyssey Imaging Suite (LI-COR Biosciences, Lincoln NE). In

the case of fixed cells, an adhesion assay was carried out on an endogenous matrix of either collagen or laminin. Once the cells were fixed and permeabilized they were stained for the appropriate targets.

Immunostaining

In order to carry out the immunostaining necessary to view cytoskeletal components, a “mini” adhesion assay must first be carried out. Rather than transferring the starved cells to plates that have been coated in collagen, cover slips must be prepared instead to visualize the cytoskeleton. Single cover slips were placed in a six well plate, and washed three times with 70% ethanol, followed by washing three times with PBS. After being coated with a 10 μ g solution of collagen, the cover slips were allowed to incubate at 4°C overnight. On the day of the assay, the cover slips were washed twice with PBS and then blocked with a 1% BSA in PBS. The plates containing the starvation media and the cells were prepared according to the adhesion assay protocol up until the point at which they were added to the collagen coated plates. The cover slips were washed two times with PBS, and then kept at 37°C for five minutes to dry. Once the cells had been allowed to incubate for roughly 30-40 minutes in clear DMEM medium, approximately 1mL of cell suspension was added to each of the cover slips. The coverslips were then placed in the incubator at 37°C until adhesion was observed, approximately for one hour. Cells were fixed with 2% paraformaldehyde for 20 minutes at room temperature. The fixed cells were washed three times with PBS. Once the remaining PBS had been removed, the cells were permeabilized with 0.1% Triton in PBS and left at room temperature for three to five minutes. The permeabilized cells were then

washed three to four times in PBS. The slides were blocked with 0.1% BSA in PBS for two hours at room temperature, after which they were washed three times with PBS. The cover slips were then carefully transferred from the six-well plates to a plastic tray lined with parafilm. Table 4 shows the immunostaining targets with their appropriate primary and secondary antibodies with the correct dilution in PBS with 1% BSA and incubation period. Before the addition of each antibody, cells were washed three times with 0.1% BSA, 0.05% Tween 20 in PBS. The washes lasted approximately 10 minutes each. After the secondary antibody had been washed off, the cover slips were once again washed three times with 18 M Ω water and carefully dried. A drop of anti-fade reagent (ThermoFisher Scientific, ProLong Antifade Reagent, Product No. P36930) was added to each slide and the cover slip was inverted on top of it. The slides and cover slips were sealed together using clear nail polish and stored in a dark environment at 4°C overnight. Images of the slides were captured using the Olympus BX51 Upright Compound Microscope, with a QImaging Retiga 2000R FAST1394 camera.

Table 2: Immunostaining targets with corresponding primary and secondary antibodies. Dilutions and incubation times and temperatures are given for each individual target.

Target	Primary Antibody	Dilution	Secondary Antibody	Dilution
CD82	Anti-CD82 Antibody [TS82b], Abcam, Product No. ab59509 Incubate at 4°C overnight	1:500, 1% BSA in PBS	Alexa-Fluor 488 (goat antimouse) Incubate for 1 hour at room temperature in a dark environment	1:10,000 5% BSA in PBS
Phalloidin	Actin-Stain 555 Phalloidin, Cytoskeleton, Inc., Product No. PHDH1-S Incubate for 90 minutes at room temperature in a dark environment	1:500, 1% BSA in PBS	No secondary antibody necessary	N/A

Chapter Three: Results

c-Met Activation is Dependent on CD82 Expression

Before the levels of c-Met were measured and compared across cell lines, the expression of CD82 in the PC3-#1 and the PC3-29 cell lines needed to be confirmed. An adhesion assay was carried out, and the CD82 protein was immunoprecipitated from all three experimental cell lines. CD82 produces a broad band centered at approximately 37 kDa. The PC3-5V do not show evidence of this band, while there is a dark band visible in the PC3-#1 and PC3-29 lanes (Figure 3). This indicates that the cell lines containing the CD82 vector were expressing CD82 as expected.

To confirm that each of the three cell lines expressed c-Met under our growth conditions, an adhesion assay was carried out. Using collagen as the substrate, both the suspension and collected lysates of the samples were transferred to a PVDF membrane and stained for total c-Met expression. This includes both the phosphorylated, active form of c-Met, and the unprocessed c-Met. The cell suspensions do not show signs of activated proteins (Figure 4). Without any matrix to adhere to, there is no signaling through integrins. When the cell adheres to a matrix, such as laminin, the amount of signaling increases. The substrates used in this study were collagen and laminin. There is a higher concentration of both forms of the c-Met protein in the sample from the PC3-5V cell line.

The blot seen in Figure 4 was stripped of primary and secondary antibodies, and then was stained for total c-Met concentration, rather than total P_{tyr}. All of the cell lines show equal concentrations of total c-Met (Figure 5). As mentioned above, CD82 has not been found to interfere with the overall concentration of c-Met. The levels of functional c-Met are not dependent on the levels of CD82 found in the cell. The data does

demonstrate that CD82 is affecting c-Met activation; cells expressing CD82 have lower levels of activated c-Met. The levels of phosphorylated proteins appear to decrease when CD82 is returned to physiological levels, demonstrating that CD82 is interfering with integrin crosstalk.

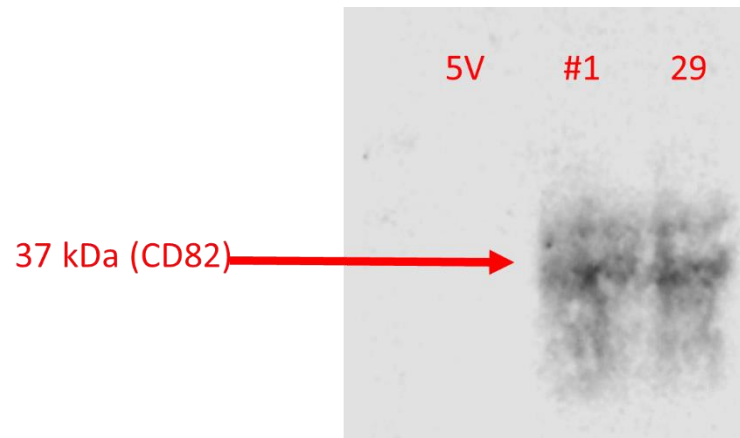


Figure 3: Western blot demonstrating the expression of CD82 in the experimental cell lines. Suspensions of all three cells lines were lysed and the CD82 protein immunoprecipitated. The protein was transferred to a PVDF membrane for western blotting. The blot was visualized at 800nm using a Licor Imaging System. The 5V cell line carries only an empty vector, and does not express CD82. Both PC3-#1 and PC3-29 cells have vectors that contain functional CD82.

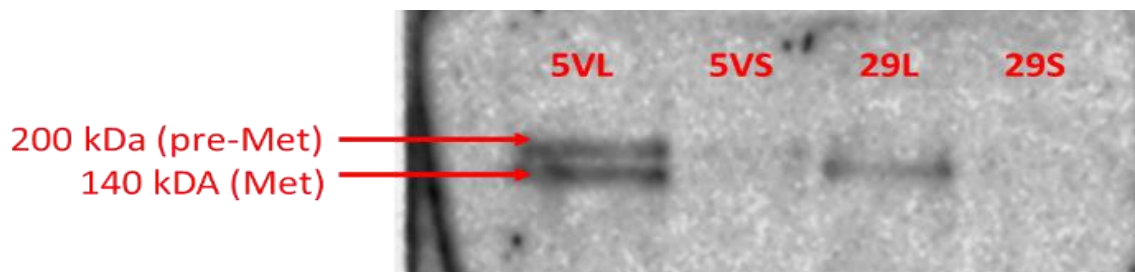


Figure 4: Western blot depicting the activation of processed c-Met in the experimental cell lines with laminin as a substrate and a PTyr probe. Cells were allowed to adhere to an endogenous matrix until adhesion was observed. Lysates were collected and kept frozen at -80°C until use. Lysates were also collected from suspensions of each cell line to serve as a control. Protein was analyzed via western blot, and stained with 4G10® Platinum, Anti-Phosphotyrosine, Millipore, Product No. 05-1050. 5VL-PC3 cell line engineered with an empty vector, adhered to the endogenous matrix laminin; 5VS-PC3 cell line engineered with an empty vector, sample collected from cell suspension for control; 29L- PC3 cell line engineered with a CD82-expressing vector, adhered to the endogenous matrix laminin; 29S- PC3 cell line engineered with a CD82-expressing vector, sample collected from cell suspension for control. The reduced intensity of the processed c-Met band is indicative of reduced c-Met activation in the PC3-29 cells.

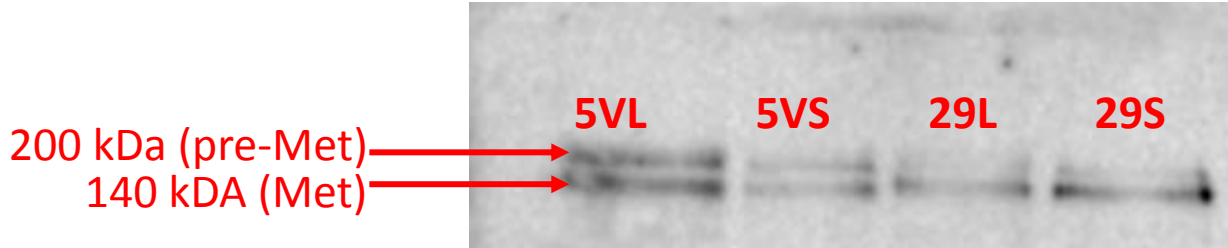


Figure 5: Western blot depicting the total c-Met found in each of the samples, with laminin as a substrate. Cells were allowed to adhere to an endogenous matrix until adhesion was observed. Lysates were collected and kept frozen at -80°C until use. Lysates were also collected from suspensions of each cell lines to serve as a control. Protein was analyzed via western blot, and stained with c-28 c-Met (Santa Cruz Biotechnology, Product No. sc-161). “S” indicates a cell suspension, which serves as a negative control. “L” indicates an adhered cell lysate with laminin as a substrate.

Phosphorylation Levels of Tyr1234 and Tyr1235 Are Not Dependent on CD82

Expression in Experimental Cell Lines

One of the most common hypotheses regarding the interaction of CD82 and c-Met is that CD82 interferes with the phosphorylation of the four sites located at the base of the intercellular portion of the growth receptor. To test this hypothesis, an immunoprecipitation technique was carried out to analyze the expression levels of the activated phosphorylation sites Tyr1234 and Tyr1235 (Figure 6). Analysis of the resulting western blot revealed similar levels of phosphorylated protein between the two experimental cell lines.

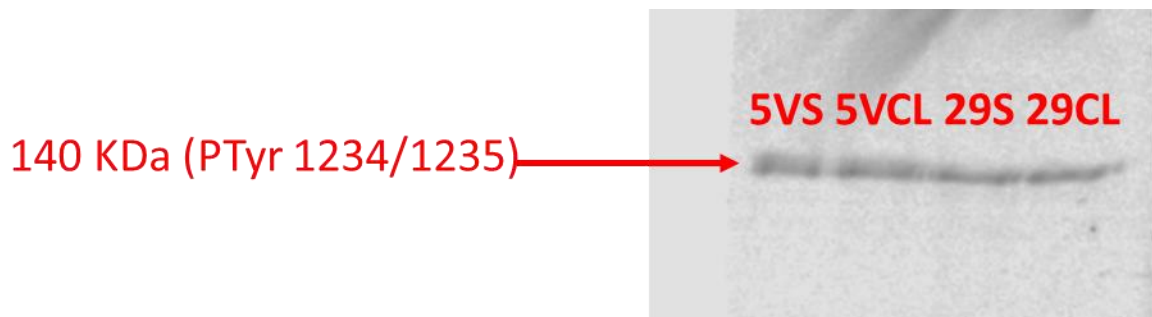


Figure 6: Expression levels of phosphorylated Tyr1234 and Tyr1235. These two phosphorylation sites are located on the intercellular portion of the c-Met receptor, and begin the signaling cascade that eventually leads to Rac1 activation. The levels of PTyr1234 and PTyr 1235 remain constant despite CD82 expression. “S” indicates a cell suspension, which serves as a negative control. “CL” indicates an adhered cell lysate with collagen as a substrate.

cdc42 Expression Remains Unchanged in the Presence of CD82 Expression in Experimental Cell Line

cdc42 is a small GTPase with functions that are similar to those of Rac1, and whose location in the c-Met signaling pathway are predicted to be the same.

Immunoprecipitation was carried out to isolate only the activated cdc42 from the sample lysates, and the protein was visualized via western blotting. A positive cdc42 cell lysate with a His tag attached and a cdc42 cell lysate without the His tag served as positive controls. The levels of activated cdc42 appear to be unchanged, both between and within the cell lines (Figure 7). However, the presence of a faint band in the negative control indicates that a follow-up assay may be necessary. To confirm the results seen in the cdc42 assay, a simplified western blotting procedure, (immunoprecipitating the target protein from only the cell suspensions), was carried out to visualize the levels of activated cdc42 protein in only the cell suspension samples (Figure 8). The levels of

activated cdc42 protein appear to be independent of CD82 expression, as the cell suspensions and lysates all display comparable levels of the protein.

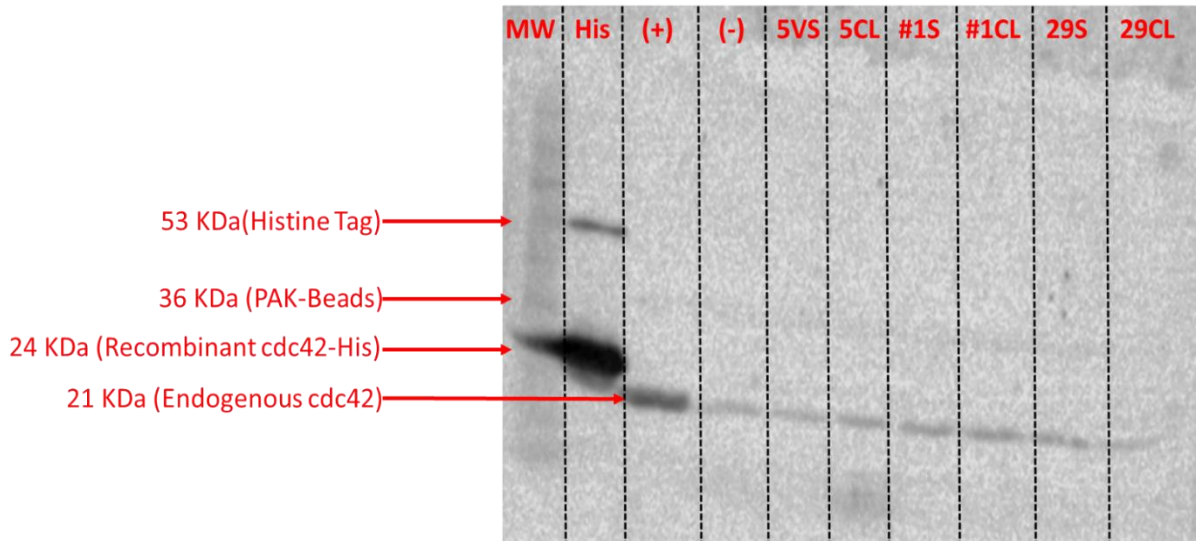


Figure 7: cdc42 activation assay results showing the expression levels of recombinant cdc42-His, (i.e. the positive control with His tag added for identification) and endogenous cdc42 found naturally in the cell lines. Lysates from both cell suspensions and adhesion assays were used to analyze the levels of cdc42 present in the cells, with the cell suspensions serving as a control. The levels of cdc42 remain constant in the presence and absence of CD82 expression. Lane borders have been added by the author for clarity.

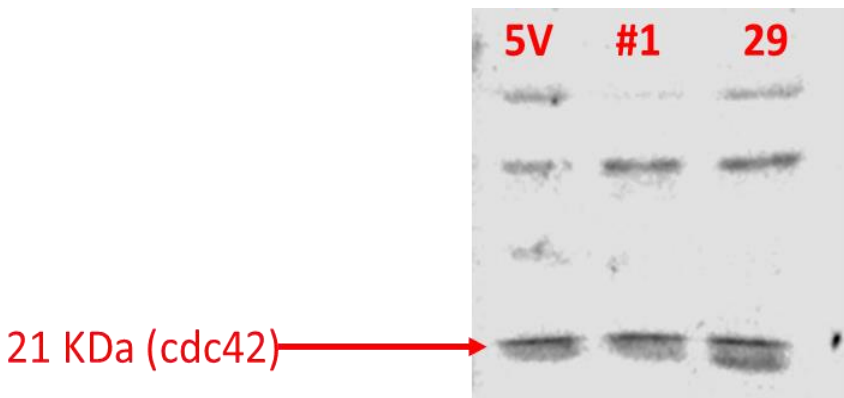


Figure 8: cdc42 assay results showing the expression levels of endogenous cdc42 in its activated form, using only cell suspensions. Results from the previous assay have been simplified to show the levels of activated cdc42 found in the suspensions in the experimental cell lines. The levels of activated cdc42 are comparable across the three different cell lines.

Correlation of Rac1 Activation With CD82 Expression

A Rac1 assay was used to isolate the activated form of the Rac1 protein from cellular suspensions. Cell suspensions were collected from each of the three cell lines, and the activated Rac1 protein was isolated from each of the samples. The isolated protein was visualized using standard western blotting techniques. Analysis of the protein levels showed higher expression levels of activated Rac1 protein in the PC3-5V suspension sample, which does not express CD82 (Figure 9). The PC3-29 and PC3-#1 cell lines both express CD82, and these cell lines showed lower levels of activated Rac1 protein than the PC3-5V suspension sample.

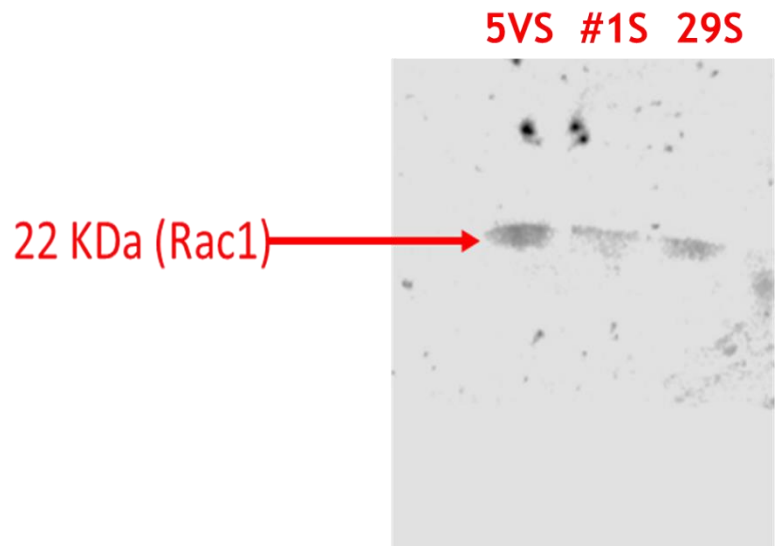


Figure 9: Rac1 assay results showing the expression levels of Rac1 in its activated form in a pull-down Rac1 assay using cell suspensions. The western blot show the levels of activated Rac1 found in the suspensions in all of the experimental cell lines. The levels of activated Rac1 appear to be elevated in the 5VS sample, which does not express CD82.

Visualization of CD82 Expression in Experimental Cell Lines

The PC3-5V cell line contains an empty vector, and does not express CD82. To validate the findings of the c-Met and PTyr activation assays, cell staining was carried out to identify which cells were actively expressing CD82. If the activation of c-Met was

truly being influenced by the expression of CD82, there would be observable changes in the cytoskeleton. Part of the c-Met signaling pathway includes signaling migration and cytoskeletal rearrangement, mediated by Rac1 activation. If the express of CD82 is interfering with the activation of either of these proteins, there will be observable characteristics. Figure 10B demonstrates the results of the immunofluorescent staining of PC3-29 cells for CD82 expression. The staining pattern indicates that CD82 is being expressed across the cell membrane, with little fluorescence visible within the cell itself. Figure 10A shows the result of CD82 staining for the PC3-5V cells. Little to no fluorescence is visible, within the cytosol or on the cell membrane, indicating a lack of CD82 expression in the PC3-5V cell line. When it had had been experimentally validated that the PC3-29 cells were expressing CD82, the next step was to evaluate cytoskeleton changes.

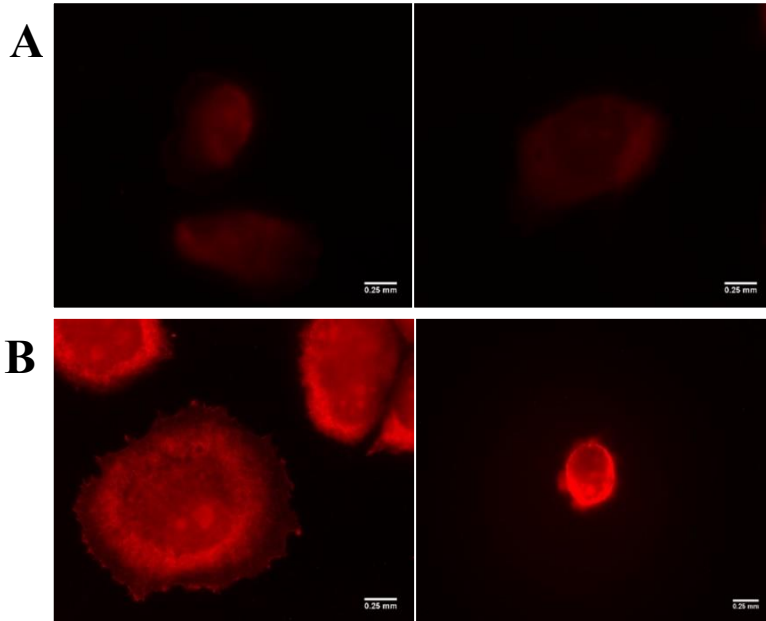


Figure 10: Visualization of CD82 in experimental cell lines. A) PC3-5V cells were treated with anti-CD82 antibody [TS82b] Abcam Product No. ab59509. PC3-5V have been transfected with an empty vector, and thus do not express CD82 on the surface. B) PC3-29 cells were treated with anti-CD82 antibody [TS82b] Abcam Product No. ab59509. PC3-29 have been engineered with a vector that contains the CD82 expression system. Areas of CD82 expression have been highlighted on the surface of the cell.

Visualization of F-actin Stress Fibers in Experimental Cell Lines

A comparison was made between the actin cytoskeleton arrangement in the presence and absence of CD82 expression. This was done by staining the cytoskeleton using rhodamine phalloidin to locate F-actin within the cell. Figure 13 depicts the results of staining PC3-29 cells with rhodamine phalloidin to visualize the location of F-actin fibers, polymerized actin, and stress fibers within the cell. The irregular surface of the cell indicates that it has begun to adhere to the endogenous matrix on the cover slip; however, there is no evidence of F-actin on the outer edges of the cell. There is a limited amount of fluorescence that can be seen towards the center of the cell, but there does not appear to be any significant structural arrangement. In contrast, Figure 11 shows F-actin fibers scattered throughout the cytosol, and is of a higher intensity in the than the PC3-29 cells.

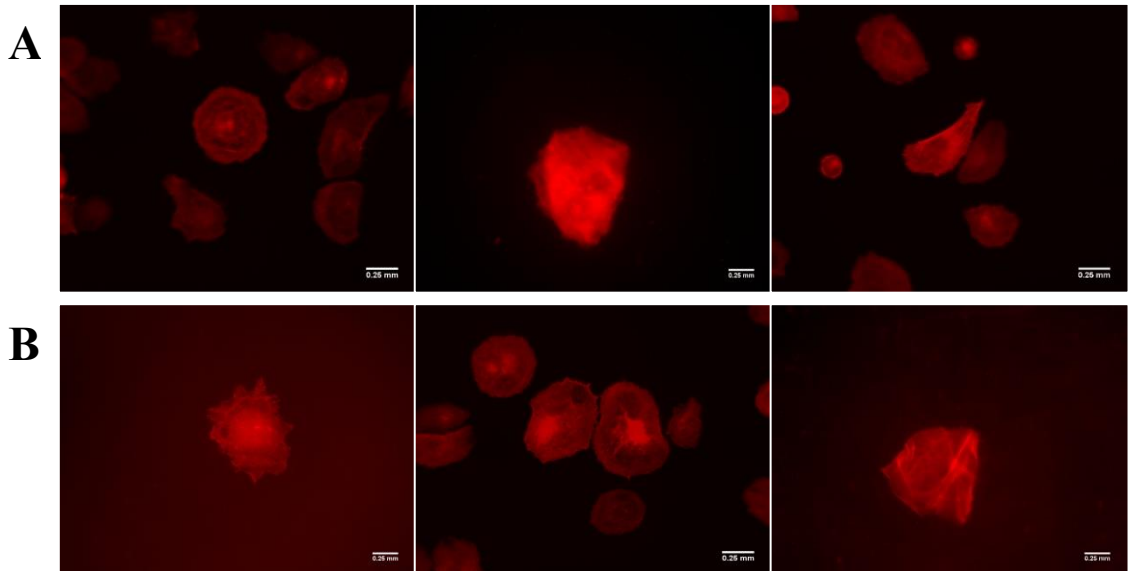


Figure 11: Visualization of F-actin fiber arrangement in experimental cell lines. Both cell lines were stained with rhodamine phalloidin to highlight the location of F-actin fibers and focal adhesions with the cell and on the cellular membrane. A) Rhodamine phalloidin staining of PC3-5V cells. F-actin fibers are visible throughout the entire cytosol, with evidence of focal adhesions forming within the cytosol. In the far right micrograph, the cytoskeleton has arranged the F-actin fibers such that the cell has formed lamellipodia in preparation for migration. B) Rhodamine phalloidin staining of PC3-29 cells. The F-actin fibers are concentrated in the center of the cytosol, as well as on the edges. Focal adhesions have formed on the edges of the cell membrane indicating the adhesion of the cell to the

Chapter Four: Discussion

The metastasis of malignant tumors is the leading cause of mortality amongst cancer patients (32). Prostate cancer shows a high affinity for metastasis to both brain and bone tissue as it progresses to more advanced stages. Metastasis is made possible by two factors: the degradation of the extracellular matrix, and the remodeling of the actin cytoskeleton. In order for the cells in the primary tumor site to migrate to other tissues, they must make their way through the basement membrane and into underlying blood vessels. Tumor cells are capable of producing matrix metalloproteins, such as MMP-2, MMP-9, and plasmin, to break down the extracellular matrix (60). When the extracellular matrix has sufficiently degraded, tumor cells can pass through the basement membrane and into blood vessels through a process known as intravasation (60).

The movement of the tumor cells across the matrix and between the cells lining the blood vessels is aided by the arrangement of the actin cytoskeleton. F-actin fibers within the cytosol can be arranged to form invasive structures such as lamellipodia and filipodia. These invasive projections can be extended from the main cell body, and when the cytoskeleton contracts, the cell will migrate across the matrix. The cytoskeleton can also be arranged to allow the cell to pass between the tightly connected cells forming the blood vessels. Actin fibers will gather along the edges of the cell to prepare for lamellipodia formation.

The growth factor receptor c-Met is responsible for a signaling cascade that results in the promotion of metastatic characteristics. These characteristics include invasion, migration, proliferation, cell survival, and cytoskeleton rearrangement (Figure 12). In 2006, a study conducted by Sridhar and Miranti demonstrated that the tumor

suppressor protein CD82 is able to regulate c-Met activation in the metastatic prostate cancer cell line PC3 (5). Both CD82 and c-Met expression is heavily regulated in normal cells. When uncontrolled cell growth progresses to cancer, the downregulation of CD82 is observed in a variety of cancer types, including prostate cancer (5). Metastatic prostate cancer has also demonstrated an overexpression of c-Met (5). When CD82 is restored to physiological levels in metastatic prostate cancer cells, the result is a decrease in observable metastatic characteristics and the deactivation of important signaling proteins (5).

Structure and Function of c-Met

The ability to identify proteins and/or signaling molecules involved in prostate cancer metastasis would open up an entirely new area of research into drug therapeutics. One potential drug target is the c-Met signaling pathway, which has been shown to be involved in cancer cell migration and invasion (3). c-Met is a member of the tyrosine kinase receptor family, and is found bound in the cellular membrane (29). The receptor is composed of two subunits, α and β , that are linked to one another via a disulfide bond (29). The β chain is made up of three regions: an extracellular domain, a transmembrane region, and cytoplasmic region that extends through the membrane and into the cell itself (29). The pathway begins by the binding of the ligand, hepatocyte growth factor (HGF), to the membrane-bound receptor on the outside of the cell membrane. c-Met can also be activated by integrin signaling within the cell as well. Elevated levels of these integrins are associated with tumor formation and metastasis (5). The binding of HGF to the c-Met receptor activates the kinase function of the protein, which dimerizes and phosphorylates two tyrosine residues at the two key phosphorylation sites this project explored (Tyr 1234

and Tyr 1235) (29). The activation of the c-Met receptor results in the phosphorylation of four key sites within the cytosol. The phosphorylation of these four sites then sets off a signaling cascade, with each subsequent molecule activating the next. When the cascade has been triggered, a docking site forms via the phosphorylation of two more tyrosine molecules, Tyr 1349 and Tyr 1356. The site is located on the C-terminal tail, and when the site is phosphorylated the tyrosine molecules recruit downstream effector molecules (61). These major signal relay molecules include Src, growth factor receptor bound protein 2 (GRB2), GRB2-associated binding protein 1 (GAB1), phosphoinositide-3-kinase (PI3K), SHC, SHP2, and signal transducer and activator of transcription 3 (STAT3) (61) (29). The end result of this cascade is the stimulation of cellular activities associated with metastasis, including dissociation, migration, invasion, and cytoskeletal rearrangement.

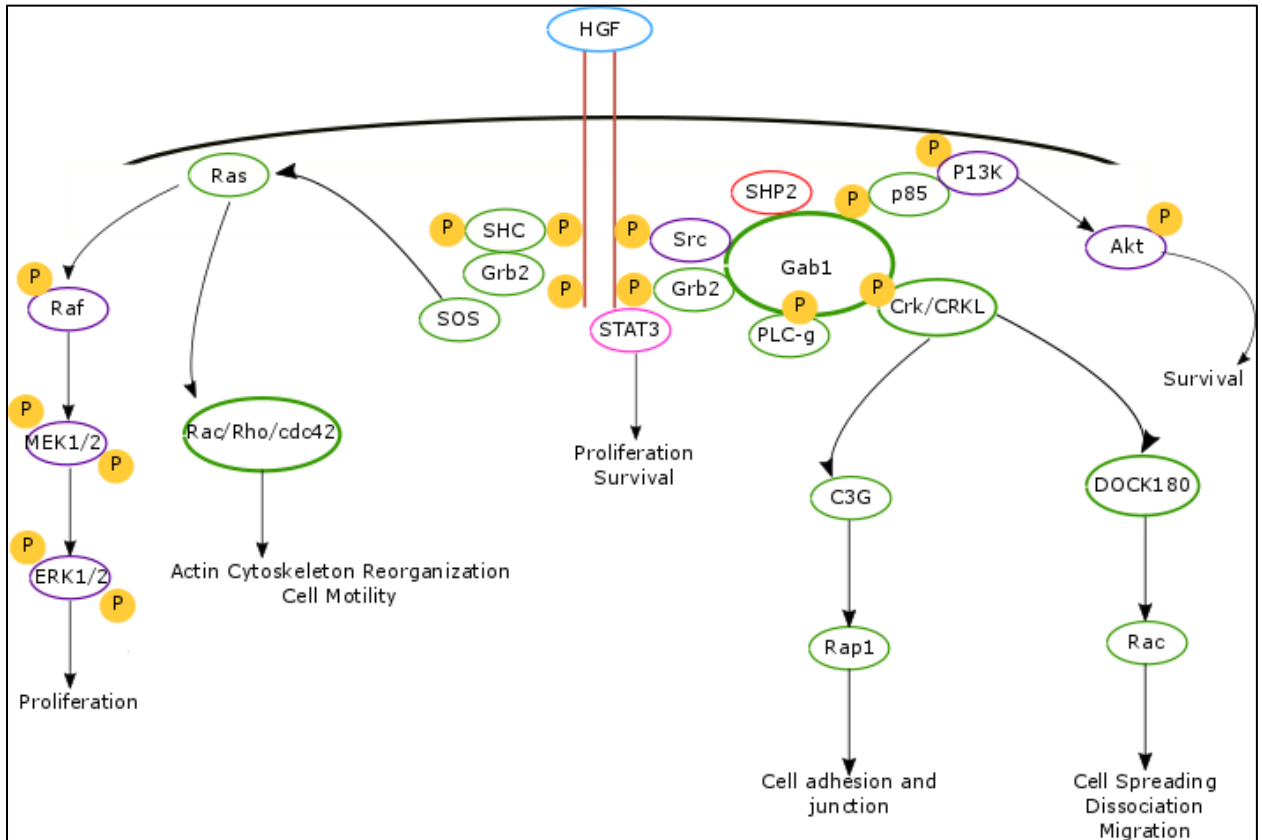


Figure 12: Extended diagram showing the full c-Met signaling pathway. The binding of hepatocyte growth factor (HGF) leads to the phosphorylation of four key sites within the cytosol, which initiates a signaling cascade that stimulates cellular activities associated with tumor metastasis, such as dissociation, migration, invasion, and cytoskeletal rearrangement.

Regulation of c-Met Downstream Signaling by CD82

The first objective of this study was to identify the phosphorylation site being regulated by CD82 expression. The levels of activated c-Met have been shown to be dependent on the expression of CD82 in the experimental cell lines, both in previous research and in this study (Figure 4) (5). The PC3-5V, which do not express CD82, have significantly higher levels of activated c-Met in the sample. The c-Met protein was isolated from cell suspensions and adhered cell lysates, and then treated with a PTyr

probe. This probe is used to highlight the presence of phosphorylated proteins. The use of the PTyr probe assumes the phosphorylation of c-Met is equivalent with activation. There is a higher concentration of phosphorylated proteins in the cell line that is lacking CD82 expression. This is demonstrated by the increased band intensity at 140 kDa in the 5VL sample (Figure 4). Integrin signaling is silenced when the cells are not adhered to a matrix. The cell suspensions do not show any evidence of activated c-Met. The levels of activated c-Met appear to decrease in the presence of CD82 expression in PC3 cell lines (Figure 4).

While CD82 may play a role in the regulation of c-Met activation, it does not appear to affect the overall expression of c-Met (Figure 5). The levels of total c-Met present in the cell lines was observed using a total Met probe. There is an important distinction that must be made between the probe used to observe total c-Met and the PTyr probe. As mentioned previously, the PTyr probe is used to look at phosphorylated proteins that have been isolated from samples using immunoprecipitation. The total Met probe also requires immunoprecipitation, but it cannot differentiate between active and inactive c-Met. Therefore, the results of the total c-Met western blot cannot be used to draw conclusions regarding the activated state of c-Met. The levels of total c-Met do not appear to change in the presence of CD82 expression (Figure 5). The 140 kDa band produced by the total Met probe does not lose intensity between the PC3-5V and PC3-29 cell lines (Figure 5). This indicates that the production of c-Met is not dependent on CD82 expression, but CD82 does appear to be regulating c-Met activation.

This research project was successful at demonstrating that the expression of the tumor suppressor protein CD82 is regulating the activation of c-Met without influencing

the overall production of c-Met. The relationship between CD82 expression and c-Met observed in this study is supported by previous literature (5). An interesting question posed by these results is if this relationship would prove to be true across different types of extracellular matrix proteins. Laminin was used to look at the activation of c-Met, while collagen was used to investigate the PTyr 1234/1235 phosphorylation site. There was no noticeable change in the level of phosphorylation between the different cell lines. Further research would include using both laminin and collagen for the PTyr 1234/1235 phosphorylation sites. For example, in addition to exploring PC3 cellular adhesion to collagen, laminin, and fibronectin, further research could be done using attachment factor protein matrix. This matrix contains gelatin as an attachment factor, and enhances the growth of endothelial cells. If the starved cells are placed on a surface that has been coated with an attachment factor, it is possible that even cells displaying migratory characteristics would demonstrate higher levels of attachment. An increase in the binding affinity of the cell to the substrate could have an observable effect on metastatic characteristics, as well as the levels of activated c-Met found in the cells.

Regulation of c-Met Phosphorylation by CD82

The second objective of this study was to identify the specific c-Met phosphorylation site being regulated by CD82 in PC3 cells. A special focus was placed on the Tyr1234/1235 phosphorylation site. This is one of four sites that become phosphorylated once c-Met is activated by the binding of HGF or integrin activation. Tyr1234/1235 was chosen due to previous research showing that AKT and ERK are not affected by CD82 expression in PC3 cells (5). The activation of AKT and ERK can be

traced to their respective phosphorylation sites, neither of which includes the Tyr1234/1235 site. Preliminary data suggest that CD82 does not affect the phosphorylation of the other three sites, indicating that the activation of Tyr1234/1235 could be dependent on CD82 expression. This possible relationship was explored by immunoprecipitating c-Met, isolating Tyr1234/1235 from the cell lysates and probing for phosphorylated proteins. The phosphorylated version of Tyr1234/1235 creates a band at approximately 140kDa when run through an SDS-PAGE gel. There is a distinct band visible at 140 kDa in both the experimental and control cells, indicating the presence of phosphorylated Tyr1234/1235 protein (Figure 6). The intensity of this band remains constant across the four sample lanes. There does not appear to be any differential phosphorylation amongst the cell lines. The expression of CD82 does not seem to have an effect in the phosphorylation of the Tyr1234/1235 site.

From the data collected by this project, it can be determined that the phosphorylation of Tyr1234/1235 site was not affected by CD82 expression. However, this study was unsuccessful at determining which c-Met phosphorylation site is being regulated by CD82. If the phosphorylation of the Tyr1234/1235 site is not being regulated by the expression of CD82, the question remains as to which site is being regulated by CD82 expression. Considering the preliminary studies mentioned at the beginning of this section, it suggests the question if the phosphorylation of the four key sites is being affected by CD82 expression. In non-small cell lung carcinoma and oral squamous cell carcinoma, CD82 was found to attenuate the association between c-Met and several adaptor molecules, such as Grb2 and p85 (62). The arrangement of the actin cytoskeleton observed when CD82 is expressed suggests that CD82 is interfering with

migratory signaling. Cell migration is a complex phenomenon that is regulated by adaptor proteins, which recruit GTP-binding proteins cdc42, Rho, and Rac1 (62). Rather than interfere with the phosphorylation of the initial four sites, it is plausible that CD82 is instead regulating the recruitment of adaptor proteins. This hypothesis could be validated by observing the levels of adaptor proteins such as p85 and Grb2, which are responsible for activating cdc42 and Rac1, in each of the experimental cell lines. If CD82 expression is associated with lower levels of adaptor proteins, it would provide an explanation as to why the phosphorylation of the four key sites in c-Met remain unchanged in the presence of CD82.

CD82 Expression Regulates Downstream Signaling Events in the c-Met Signaling Pathway

The decision to explore the relationship between CD82 expression and Rac1 activation was influenced by previous research. As mentioned before, a study published in 2006 demonstrated that the ERK and AKT pathways were not influenced by CD82 expression in PC3 cells (5). Even if CD82 was interfering upstream in these two pathways, it would still not have the observed impact on migration and invasion characteristics. The activation of ERK is required for cell proliferation, and AKT activation leads to the development of survival characteristics in prostate cancer cells. Previous research conducted in our lab showed that the expression of CD82 in prostate cancer led to observable effects in the arrangement of the actin cytoskeleton. The ability of CD82 to influence the formation of F-actin fibers, polymerized actin, and stress fibers and their arrangement suggests that CD82 is interfering with a portion of the c-Met

signaling pathway involved in cell motility. Rac1 is a small Rho GTPase that is involved in cell motility and actin cytoskeleton reorganization (Figure 12). The role of Rac1 in the recruitment of F-actin fibers and chemotaxis has been well documented (63). This makes an ideal target for CD82 interaction, as the result of Rac1 inactivation is poor cell motility (63). The protein cdc42 also plays a role in cell migration, and due to its similarity to Rac1 in size and function, cdc42 activation was investigated.

cdc42 Expression Remains Unchanged in the Presence of CD82 Expression in Experimental Cell Lines

cdc42 is one of three Rho GTPases that are involved in the c-Met signaling pathway. Rho GTPases are involved in regulating the dynamics involved in cell adhesion and the actin cytoskeleton (64). The expression of these GTPases are often altered during the formation of malignant tumors, but researchers are still working to determine the exact way they perform their regulatory functions (65). Previous studies have shown that cdc42 promotes the attachment of cancer cells to endothelial tissue and the basement membrane by influencing the cells during the transendothelial migration of tumor cells (65). This invasion strategy is made possible by cdc42 upregulating the expression of the adhesion receptor known as β 1 integrin, a receptor that has already been shown to play a role in cancer metastasis (66). When cdc42 expression is reduced in metastatic tumor cells, the cancer cells fail to invade the basement membrane and form attachments to the surrounding tissue (65).

All three of the PC3 cell lines used in this study are prostate cancer cells derived from bone metastasis. A cdc42 activation assay was carried out on all three of the cell

lines, two of which carried a vector containing a function copy of the CD82 gene (PC3-29 and PC3-#1). The levels of activated cdc42 within the CD82-expressing cells, (PC3-#1 and PC3-29), were compared against the levels found in the PC3-5V cells in order to see if CD82 was interfering with the activation of cdc42. It was found that the level of activated cdc42 did not vary amongst the three different cell lines, nor did it appear to vary between adhered lysates and cell suspensions (Figure 8). If CD82 was influencing the activation of cdc42, there would be higher levels of activated cdc42 in the PC3-5V cells, both in the suspension and the adhered lysates. Despite its role in cell migration and invasion in metastatic prostate cancer, our results indicate that cdc42 activation is not dependent on the expression of a functional CD82 gene (67). It should be noted that a faint band does appear in the negative control lane. This is most likely due to experimental error. However, to confidently state the results of the cdc42 assay, the assay would need to be repeated. The cdc42 activation assay using just the cell lysates does not display any change in activation in the presence of CD82 expression. This lends evidence to the hypothesis that the expression of CD82 is interfering with the activation of the Rac1 protein, which also plays a major role in invasion and migration in the c-Met signaling pathway.

Rac1 Activation in CD82-Expressing Cell Lines

The activation of Rac1 and related GTPases is required for the assembly and placement of the actin fibers that make up the cytoskeleton (56). The up-regulation of c-Met in prostate cancer correlates with an increase in the activation of downstream effector molecules, such as Rho, cdc42, and Rac1. It was demonstrated that the levels of

cdc42 remain constant whether or not CD82 is being expressed by the cell (Figure 8). This evidence indicates that the decrease in migration seen in the PC3-29 and PC3-#1 cells is not due to CD82 interfering with cdc42. Because there is an observed change in the arrangement of F-actin fibers in the cytosol, CD82 must be affecting the activation of the signaling molecule necessary for migration. Rac1 has also been shown to play a role in the arrangement of the actin cytoskeleton prior to invasion. The isolation of Rac1 from each of the three experimental cell lines showed a slight increase in the concentration of activated Rac1 in the PC3-5V cell suspension (Figure 9). PC3-5V does not express CD82. PC3-#1 and PC3-29, have been engineered to carry a vector with a functional copy of CD82. The levels of activated Rac1 appear to decrease in the presence of CD82 in cell suspensions. It should be made clear that the decrease in Rac1 activation seen in this research applies only to the cell suspensions. The cdc42 activation assay was performed on both adhered cell lysates and cell suspensions. The apparent decrease in Rac1 activation seen in Figure 9 is only relevant when put into the context of the cell suspensions. More research is needed to determine if CD82 is involved in the downstream regulation of Rac1 activation by CD82.

CD82 is Expressed on the Peripheral Cell Surface in PC3-29 Cells

To test for a correlation between CD82 expression and cytoskeletal changes, it was critical that the expression of CD82 could be visualized in the experimental cell lines that were engineered to express it. Both cell lines were treated with anti-CD82 antibody [TS82b] Abcam Product No. ab59509, and visualized using a FIT-C filter under an oil-immersion lens. CD82 is a tumor suppressor protein that is categorized as a tetraspanin

protein, having four distinct regions that are embedded in the cell membranes of multicellular organisms (18). It is expressed in the cell membranes of all healthy tissues, but its expression is down-regulated or nonexistent in cancer cells, particularly in prostate cancer cells (7). PC3-5V showed little to no evidence of CD82 expression on the cell surface (Figure 10A). Even when the contrast of the micrographs was enhanced, there was no sign of CD82 expression at the cell surface in the PC3-5V (Figure 10A). In contrast, the PC3-29 cells showed high levels of CD82 expression (Figure 10B). Much of the CD82 expression could be seen along the edges of the cell membrane, particularly in those cells that showed the development of adhesive filaments (Figure 11B).

Lack of CD82 Expression Leads to Invasive Cytoskeletal Changes

Much as the human skeleton supports the structure of the body, the cytoskeleton supports the structure of the cell body. Changes in the actin cytoskeleton are required for a cancer cell to metastasize beyond its native tissue. The remodeling of the cytoskeleton involves the production and arrangement of polymerized actin, the creation of stress fibers, and the overall formation of lamellipodia. PC3-5V cells were stained with rhodamine phalloidin to visualize F-actin fibers and stress fibers. Rhodamine phalloidin is a high-affinity F-actin probe that can be used to stain for both F-actin fibers and focal adhesions within the cell and on the cell surface. Staining of PC3-5V shows that actin fibers are visible through the cytosol, with little evidence of focal adhesion formation (Figure 11A). Adhesive filaments are not visible on the edges of the cell, indicating that the cell is preparing to migrate. The micrograph on the right shows the formation of lamellipodia; the cell is beginning to take on a sickle-shaped appearance, and actin fibers

have begun to congregate along the edge of the cell and the tip of the lamellopodia (Figure 11A).

CD82 Expression Leads to Development of Adhesion Characteristics

All PC3-29 cells displayed in the micrographs show signs of adhesive filaments forming around the edge of the cell, indicating that the cell has begun to adhere to the endogenous matrix (Figure 11B). The F-actin fibers are concentrated in the center of the cytosol, with a structured scaffolding extended to the edges of the cell (Figure 11B). Overall, the cytoskeletons of the PC#-29 cells are far more organized than the PC3-5V cells. The PC3-5V cells do not show signs of adhering to the matrix. They demonstrate evidence of cellular structures that are associated with invasion and migration. The difference in the cytoskeletal organization between the two cell lines is assumed to be the expression of CD82. This lends evidence to the hypothesis that CD82 is interfering with the c-Met signaling pathway involved in cytoskeletal rearrangement, invasion, and migration.

In addition to interfering with the activation of Rac1, further research could be done to investigate other migratory characteristics affected by CD82. Not only would it be interesting to look at other physical structures, such as F-actin protrusions, levels of E-cadherin could be studied. There is also the possibility that CD82 has a regulatory function in another pathway involved in cancer cell metastasis, such as fibroblast growth factor pathways (68). The evidence gained from this study demonstrates no apparent decrease in cdc42 activation, leading to the hypothesis that CD82 could have other regulatory roles. In 2015, a study showed that the expression of CD82 reduces the

adhesion of cancer cells to blood vessels in Sialyl Lewis antigen-mediated adhesion (8). It is clear that CD82 has regulatory functions outside of preventing the rearrangement of the actin cytoskeleton needed for metastasis that are worth investigating.

Overall Conclusions

The results of this study indicated that CD82 has a regulatory effect on c-Met activation. The overall levels of total c-Met remain unchanged in the presence of CD82, but the expression of CD82 leads to a decrease in the expression of activated c-Met. While this project was unable to identify the specific c-Met phosphorylation site being regulated by CD82, the evidence suggests that the Tyr1234/1235 remains unaffected by CD82 expression. CD82 does appear to influence downstream events in the c-Met signaling pathway. We have shown that the levels of cdc42 expression do not change across the three experimental cell lines, and remain constant in suspension as well. We investigated the levels of activated Rac1 in cell suspensions, and the levels of activated Rac1 appear to decrease with CD82 expression. Further research is needed to conclusively state whether CD82 is influencing the activation of Rac1 in the c-Met signaling pathway. The results of this study provide evidence to suggest that CD82 is playing a role in regulating the formation of metastatic characteristics in PC3 cells.

Chapter Five: Research Summary

This project focused on the role of the metastasis suppressor protein CD82 in the deactivation of the c-Met signaling pathway in metastatic prostate cancer. Prostate cancer is currently the second leading cause of death among men in the United States (1). There are two key players in the metastasis of prostate cancer: tumor suppressor proteins, and growth factor receptors. Tumor suppressor genes are expressed in normal, healthy cells, and serve as key regulators of the cell cycle. A mutation in these genes often lead to a loss or reduction in function, which allows cells to transition into malignant tissues. The loss of the tumor suppressor protein CD82 has been implicated in approximately 70% of prostate cancer cases and has been linked to metastasis and poor patient prognosis (69).

Growth factor receptors also play a major role in the regulation of growth signals in normal cells. The importance of their function means that they are highly regulated in healthy cells. The overexpression of growth factors has been observed in many different types of cancer, including prostate cancer. Of the many growth factors, whose malfunction has been linked to the development of cancer, c-Met is a particularly attractive target for prostate cancer therapeutics. c-Met has one endogenous ligand: hepatocyte growth factor (HGF). When HGF binds to the c-Met receptor on the extracellular surface of the cell, it signals the beginning of a metastatic cascade. This cascade can also be initiated by engaging integrins. The activation of c-Met leads to the phosphorylation of four key sites connected to the β -subunit of the c-Met receptor: pY1003, pY1234/1235, pY1349, and pY1356. The phosphorylation of these four sites leads to the recruitment of downstream effector molecules that trigger metastatic

characteristics in prostate cancer such as invasion, migration, cell adhesion, and actin cytoskeleton re-arrangement (Figure 1).

The first objective in this study was to determine which downstream signaling pathway found in c-Met was being regulated by CD82. The levels of total c-Met appear to remain unchanged in the presence of CD82 expression. However, CD82 does appear to be regulating the activation of c-Met. There are decreased levels of activated c-Met in the cell lines that are expressing CD82. Overall, this study was successful at concluding that CD82 expression is regulating the activation of c-met, but not the overall production of c-Met.

Identification of the specific c-Met phosphorylation site being regulated by CD82 proved to be rather difficult. The Tyr1234/1235 phosphorylation site was chosen for investigation due to previous research suggesting the other three sites are unaffected by CD82 expression. The results of this study indicate that the phosphorylation of the Tyr1234/1235 site is not regulated by CD82 expression. There is no differential phosphorylation of Tyr1234/1235 amongst the cell lines used in this study. We were unsuccessful at determining which c-Met phosphorylation site is being regulated by CD82, if any. The question does arise as to whether the phosphorylation of these four sites is being regulated by CD82 expression. Further study is needed to determine which site, if any, is regulated by CD82.

The final objective of this study was to identify the overall downstream event that is being regulated by CD82. Previous research showed that the expression of CD82 led to observable changes in the cytoskeleton of the cell lines. The GTPase known as Rac1 was investigated as being regulated by CD82 expression. Rac1 is involved in the signaling of

actin cytoskeleton rearrangement and cell motility. cdc42 is a protein of similar size and function to Rac1, and the effect of CD82 expression on cdc42 was also investigated. The levels of activated cdc42 appear to be unchanged in the presence of CD82. Levels of activated Rac1 in the cell suspensions appeared to decrease with CD82 expression. While the suspensions appear to indicate an inverse relationship between Rac1 activation and CD82 expression, the results remain inconclusive. Further research is needed to look at the levels of activated Rac1 in both cell suspensions and adhered cell lysates. The expression of CD82 also appears to lead to the development of adhesion characteristics. PC3-29 cells demonstrated the formation of actin projections that suggest adherence to the substrate. They also displayed fewer stress fibers and polymerized actin. The lack of CD82 expression in the PC3-5V cells appears to lead to the development of invasive cytoskeletal changes. The PC3-5V showed evidence of more stress fibers and polymerized actin than seen in the PC3-29 cells. PC3-29 cells showed evidence of adhering to the substrate, whereas the PC3-5V cells demonstrated physical characteristics that indicate cell migration.

The ability of CD82 to associate with other proteins and regulate their functions is an important aspect of research into prostate cancer metastasis. The results of this study indicate that CD82 appears to be regulating the development of migratory characteristics associated with the activation of the growth factor receptor known as c-Met. Specifically, CD82 appears to be interacting with the GTPase Rac1, a signaling molecule that has been shown to have a role in cell motility. Further research is needed to validate the relationship between CD82 expression and Rac1 activation, but this study provides

important information regarding the regulatory function of CD82 in prostate cancer metastasis.

Chapter Six: Future Directions

While this project represents an investigation into the role of CD82 as an inhibitor of Rac1 in the c-Met signaling pathway, there still much that remains to be explored. A more exhaustive analysis of Rac1 activation in prostate cancer cell lines is required to support the conclusions drawn by this research. Part of this analysis would include performing a large scale Rac1 activation assay on the three experimental PC3 cell lines. Rac1 is a very difficult protein to isolate from cell lysates, as it is only expressed for a limited amount of time while the actin fibers of the cytoskeleton are being rearranged. A more thorough assay would involve plating multiple plates of the same cell line and harvesting the lysates from those cells at different time points. This would give more information as to how long it takes Rac1 to be activated and how long it is expressed in the presence and absence of CD82. The actual activation of Rac1 could be traced in real time, and conclusions drawn regarding the effect of CD82 expression on its activation. Based on the results of this investigation, one would expect to see a decrease in Rac1 activation in the cell lines that express physiological levels of CD82 if it is serving as an inhibitor (as seen in Figure 9).

Validation of the small-scale assay could then be followed by attempting to quantify the decrease in Rac1 activation between PC3-29 cell sand PC3-5V cells. Quantifying the effect that CD82 has on the activation of Rac1 could be instrumental in developing a more effective inhibitor of Rac1 and related Rho GTPases in the future. The effect of CD82 expression should also be compared across prostate cancer cell lines, and potentially across different kinds of human cancers, to see if the inverse relationship between CD82 expression and Rac1 activation holds true.

There still are questions to be answered regarding the effect of CD82 expression on the phosphorylation of the four key sites on the c-Met receptor. When placed in the context of previous research studies, the results of this study suggest that CD82 does not interfere with the phosphorylation of those four sites. This raises the question of whether CD82 is interfering with phosphorylation at all. It also demonstrates a need to investigate if phosphorylation is an adequate method for determining the activation levels of c-Met.

In addition to observing F-actin fibers, future work could potentially include observing other invasive characteristics seen in metastatic prostate cancer. This would include the development of F-actin protrusions and measuring the levels of E-cadherin in the cells. There is also the possibility that CD82 possess another regulatory function that has yet to be described. A better understanding of how small Rho GTPases become inhibited by tumor suppressor proteins such as CD82 could potentially lead to the development of more effective chemotherapies capable of targeting metastatic characteristics.

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