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MOLECULAR MECHANISM FOR CD82 SUPPRESSION OF HGF-INDUCED MET ACTIVATION AND INVASION

By

Susan M. Spotts

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
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ABSTRACT

MOLECULAR MECHANISM FOR CD82 SUPPRESSION OF HGF-INDUCED MET ACTIVATION AND INVASION

By

Susan M. Spotts

Met is up-regulated in metastatic prostate cancer which can lead to enhanced migration and invasion of tumor cells [1]. CD82 is a member of the tetraspanin family and has been identified as a metastasis suppressor in prostate cancer [2, 3]. CD82 can suppress ligand-induced Met activation and invasion in prostate tumor cells [4]. The objective of this project was to determine the mechanism by which CD82 suppresses ligand-dependent activation of Met and invasion.

We've determined that CD9, but not CD151, integrin α3 or α6, is required for CD82 suppression of HGF-induced Met activation. CD9 was not required for CD82 suppression of invasion. We have determined that CD151 and α3 integrin contribute to Met activation and invasion in PC3 cells. Integrin α6β1 and CD51 were found to be associated with Met in the absence of CD82. CD151and integrin α3 were also shown to associate in the absence of CD82. In the presence of CD82, CD151 and integrin α3 associations are reduced and CD82 is now able to associate with integrin α6 and CD151. Our current model is that without CD82; integrins α3β1 and α6β1 are able to interact with Met via interactions with CD151 to promote Met activation and invasion. In the presence of CD82, CD9 and CD151interact with CD82 drawing both CD151 and integrin α6β1 into the TERM resulting in suppression of Met activation and invasion.

ACKNOWLEDGEMENTS

There have been many people who have contributed to and supported me through the process of earning my master's degree. First, I would like to thank my mentor, Dr. Cindy Miranti, whose continued support, and guidance has made this possible. Thank you for encouraging me and giving me an opportunity to grow as a scientist.

I want to thank my committee members, Dr. John Wang and Dr. Kathy Gallo, for their advice and guidance throughout this process. I am grateful for your support and assistance.

To all past and current Miranti lab members, I am indebted to you all for the support and friendship you have shown me over the past three years. I will never forget your kind words and camaraderie throughout my time here. I will never be able to put into words all that you have meant to me and my appreciation.

I would also like to thank my family for their continuous love and support throughout my life. To my mother and father, thank you for believing in me even when I couldn't. I would have never made it without you. To my aunt and uncle, thank you for opening your home to me and providing love and support when times were difficult.

It's with your support that I am encouraged to continue my education to become a better scientist.

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INTRODUCTION

Prostate cancer is the second leading cause of cancer deaths in men in the United States [5]. While primary prostate tumors are often curable, metastatic prostate tumors are not and ultimately fatal. Understanding the mechanisms of metastasis development is crucial for developing novel therapeutic targets for this incurable disease.

Met is a receptor tyrosine kinase that has been shown to be up regulated in metastatic prostate cancer which can lead to proliferation and invasion of tumor cells [1]. According to a study in 2002, 51% of locally invasive prostate carcinomas were Met positive and 100% of prostate metastases were Met positive. Met can be activated through its ligand, hepatocyte growth factor (HGF), or through a ligand-independent mechanism mediated by cell adhesion via integrins.

CD82/KAI1 was first identified in a rat prostate cancer model as a metastasis suppressor gene [2]. CD82 expression is often lost in metastatic prostate tumors and correlates with poor prognosis. Re-expression of CD82 in human tumor cell xenografts suppresses the development of metastatic tumors with little to no effect on primary tumor growth [3]. *In vitro* studies have shown that functional CD82 can regulate cell aggregation, adhesion, motility, invasion, apoptosis, and morphogenic processes [3]. Normal prostate cells express CD82 which inhibits cross-talk between Met and integrins, but the loss of CD82 in prostate tumor cells allows for enhanced integrin- and ligand-mediated activation

of Met, which may promote metastasis [4]. These studies provide evidence that CD82 functions as an inhibitor of metastatic potential.

CD82, a member of the tetraspanin super family, has classical properties shared by other tetraspanin family members [3]. Tetraspanins possess short carboxy- and amino-terminal tails, four transmembrane domains, two extracellular loops, and a short intracellular loop. The larger extracellular domain (EC2) of the tetraspanin CD81 has been crystallized and molecular modeling studies have provided significant insight into the predicted structure of tetraspanins. Within the family, tetraspanins share very little sequence homology; however, there are a few key residues that all tetraspanins share which are important for maintaining their 3D structure. The EC2 domain is divided into a constant region which allows for dimerization and a variable region [6]. The variable region is thought to confer specificity via interactions with non-tetraspanin protein binding partners. The EC2 domain contains either four or six conserved cysteine residues which have been proposed to form 2 or 3 disulfide bridges giving tetraspanins their characteristic tertiary structure. A short region within the EC2 of the tetraspanin CD151 has been shown to be important for interacting with the extracellular domain of the α3 integrin subunit [7].

Tetraspanins lack catalytic activity and function as 'molecular facilitators' to organize surrounding membrane proteins into a complex known as the tetraspanin enriched microdomain (TERM) [3]. Tetraspanins associate laterally within the membrane to cluster various protein binding partners in tetraspanin-enriched microdomains to regulate downstream signaling pathways [8]. The

tetraspanin-enriched microdomain, TERM, is distinct from lipid rafts [9]. Various reports have found tetraspanin-enriched microdomains to be located on the plasma membrane, endosomes, and exosomes.

Various tetraspanins, integrins, receptor tyrosine kinases, and other membrane proteins have been shown to exist within tetraspanin-enriched microdomains. Our lab has previously shown that CD82 suppresses HGF-induced Met activation through an unknown mechanism. Supporting papers have since been published providing additional evidence that CD82 inhibits Met activation and its cross-talk with integrins [10, 11]; however, the mechanism is still elusive. We hypothesize that the suppressive function of CD82 is regulated by specific constituents of the tetraspanin-enriched microdomains.

Some of the tetraspanin-enriched microdomain associated proteins include CD151, CD9, and integrins. CD151 has been shown to up regulated in metastatic prostate cancer. CD151 is highly expressed in epithelial and endothelial cells where it localizes to cell-cell junctions to modulate cell migration and invasion [9]. Studies have shown that CD151 forms a stable, specific, and stoichiometric association with integrin α3β1 via the extracellular EC2 domain [7]. The same group has also determined that CD151 associates indirectly with integrin α6 [12].

CD9 has been implicated in cell adhesion, motility, proliferation, and differentiation with a functional role as a tumor suppressor [9]. CD9 has been shown to be up or down regulated in metastatic tumors depending on the study. In 2000, Shi *et al.* reported that CD9 associates with TGF-α and regulates TGF-

induced EGFR activation [25]. It has been reported that CD9 and CD151 associate with CD82 through conserved polar residues within the transmembrane domains, which are conserved throughout the tetraspanin super family [13]. This group also reports that CD9 and CD151 are not required for CD82 suppression of invasion.

Tetraspanins are palmitoylated and palmitoylation is important for the assembly of the tetraspanin-enriched microdomains [9]. Multiple palmitoylation sites have been indentified on CD9, CD151, and CD82. Palmitoylation partly stabilizes tetraspanin-tetraspanin interactions within the tetraspanin-enriched microdomain [14]. Mutations of CD9 palmitoylation sites impaired its association with CD81 and CD53 [14]. A previous paper showed that palmitoylation-deficient CD151 weakens its association with the α3β1 integrin within the tetraspanin web [15]. A palmitoylation-deficient CD82 mutant largely reverses the suppression function of CD82 on cell migration and invasion of prostate tumor cells [16]. In 2008, Sharma *et al.* identified DHHC2 as the enzyme responsible for palmitoylating CD9, CD151, and CD82. DHHC2-dependent palmitoylation aided CD9 and CD151 associations as well as associations with integrin α3 [17]. Induction of DHHC2 resulted in increased cell-cell contact [17]. There is also evidence to show that integrins may be palmitoylated which may aid in their ability to associate with tetraspanins.

Metastasis is a disease of abnormal cell adhesion in which a small population of tumor cells detaches from the primary tumor, migrate, and invade other tissues in the body [18]. These processes are linked to cell adhesion.

Normal epithelial cells balance cell-cell interactions, generally known to negatively regulate cell growth and migration, with cell-extracellular matrix (ECM) interactions that positively regulate cell growth, migration, invasion, and survival [18]. During metastasis development there is a shift in the balance towards increased cell-ECM interactions and decreased cell-cell interactions. Integrins are heterodimeric transmembrane proteins that are able to activate signaling transduction pathways by interacting with extracellular matrix proteins [19]. Integrins consist of an alpha subunit and a beta subunit that specifies which particular extracellular matrix protein the integrin will bind. Changes in integrin and matrix protein expression as well as changes in integrin affinity for their ECM substrate are observed in most cancers and this can lead to an increase in invasion and metastasis [19]. These particular integrins are specific for binding to the laminin extracellular matrix protein. Multiple groups have reported that integrin α3 and integrin α6 associate with CD151 via the EC2 domain [16]. In 2005, Zhang *et al.* reported that integrin α6 physically interacts with CD82 [16]. How CD82 association with integrins affects integrin function and whether this complex influences other signaling transduction pathways remains controversial.

Hypothesis

CD82 has been shown to suppress HGF-induced c-Met activation [4]; however, the molecular mechanism of this process is still elusive. CD82 lacks catalytic activity and has been suggested to function as a 'molecular facilitator' to organize tetraspanins, integrins, and other membrane proteins into a tetraspanin-

enriched microdomain [3]. If CD82 functions through its involvement in the TERM, we would predict that the specific constituents and specific protein interactions within the microdomain are important for the ability of CD82 to suppress Met activation.

Our lab has previously shown that in the absence of HGF, there are basal levels of Met activation when the cells are plated on matrix. This basal level of activation is through an integrin-mediated mechanism. In the absence of HGF and when the cells are in suspension, there is no Met activation. Therefore, our hypothesis is that CD82 suppresses Met activation by associating with integrins to displace them from a complex with Met. CD82 may regulate the spatial and temporal positions of integrin and Met associations.

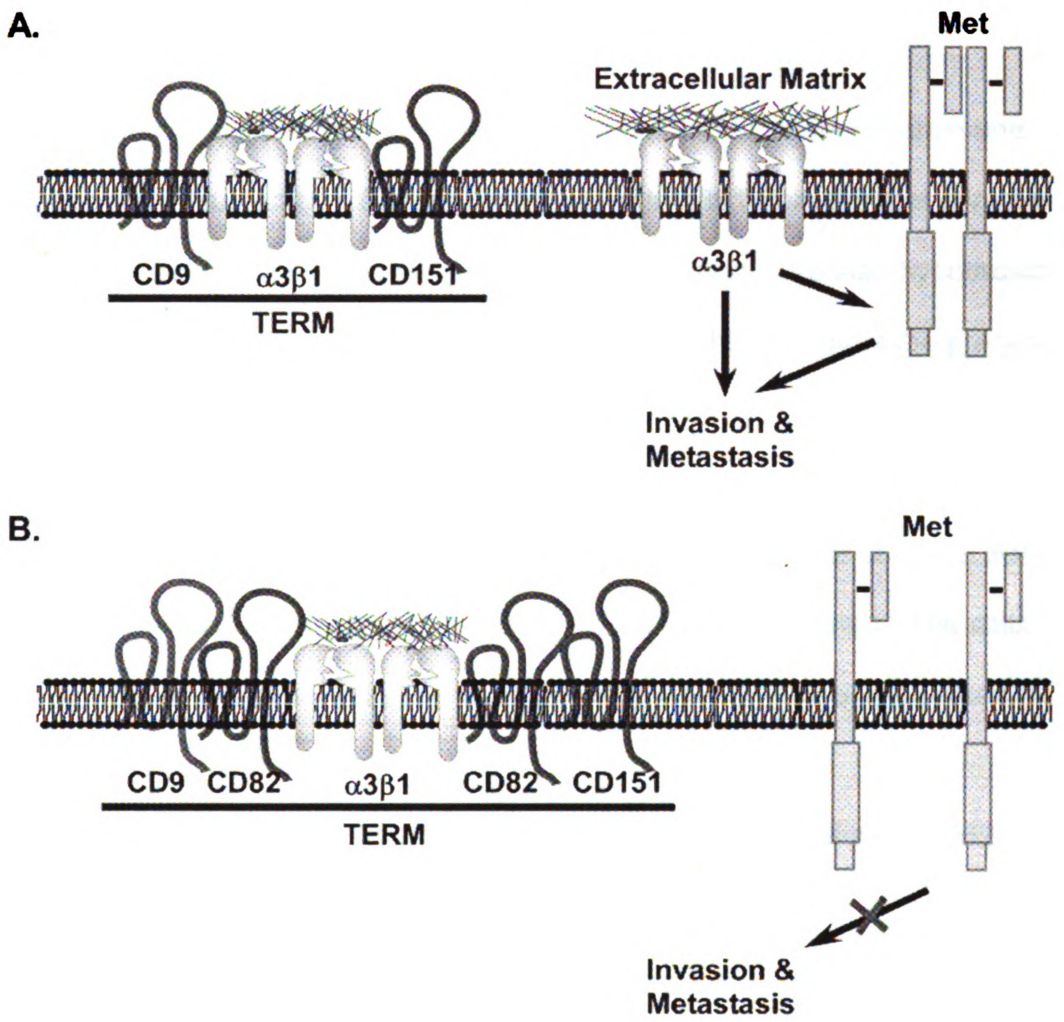
Preliminary data generated in the lab has shown through co-immunoprecipitation studies that CD82, CD9, and CD151 complex together when extracted with a mild detergent. Within these tetraspanin complexes, the α3β1 integrin, but not the α6β1 integrin, was found to be associated with these tetraspanins complexes as well.

My hypothesis is that in the absence of CD82, integrin α3β1 interacts with Met resulting in activation of downstream signaling pathways for invasion and metastasis (Figure 1A). In the presence of CD82, CD82 interacts with integrin α3β1 and draws them into the tetraspanin-enriched microdomain (Figure 1B). Withdrawal of the integrins from the Met complex disrupts the ability of Met to aggregate and to signal downstream. The specific composition of this

microdomain is essential for the ability of CD82 to suppress HGF-induced Met activation.

Figure 1. Proposed molecular mechanism for CD82 suppression of Met activation. (**A**) In CD82 non-expressing cells, α3β1 integrin associates with Met leading to Met aggregation and downstream signaling of cell invasion and metastasis. (**B**) In CD82-expressing cells, CD82 associates with the integrin α3β1 which becomes displaced into the tetraspanin-enriched microdomain (TERM). Therefore, Met is unable to aggregate and signal downstream.

Figure 1. Proposed molecular mechanism for CD82 suppression of Met activation.



CHAPTER 1: IDENTIFICATION OF REQUIRED TETRASPANIN-ENRICHED MICRODOMAIN CONSTITUTUENTS

Rationale

My hypothesis states that in the presence of CD82, Met activation and signaling are disrupted by CD82 displacing integrin α3β1 from the Met complex and drawing it into the tetraspanin-enriched microdomain. We also hypothesize that CD82 functions through a definite microdomain which requires the presence of specific protein-binding partners such as integrins and other tetraspanins.

To determine these particular protein-binding partners that are responsible for CD82 suppression, PC3 cells expressing either empty vector or CD82 were transiently transfected with siRNA specific to the protein of interest. The cells were stimulated with increasing concentrations of HGF, immunoprecipitated for Met, and then immunoblotted
for tyrosine phosphorylation and total Met protein. We expect that in CD82-expressing cells, a larger concentration of HGF will be required to reach an equivalent amount of Met activation in a non-expressing CD82 cell. We then predicted that PC3 cells expressing CD82 and have a reduction in a protein that is required for CD82 suppression function will have adequate Met activation at lower concentrations of HGF similar to non-CD82 expressing cells.

Results

Integrin α3 is not required for CD82 suppression of HGF-induced Met activation but contributes to Met activation.

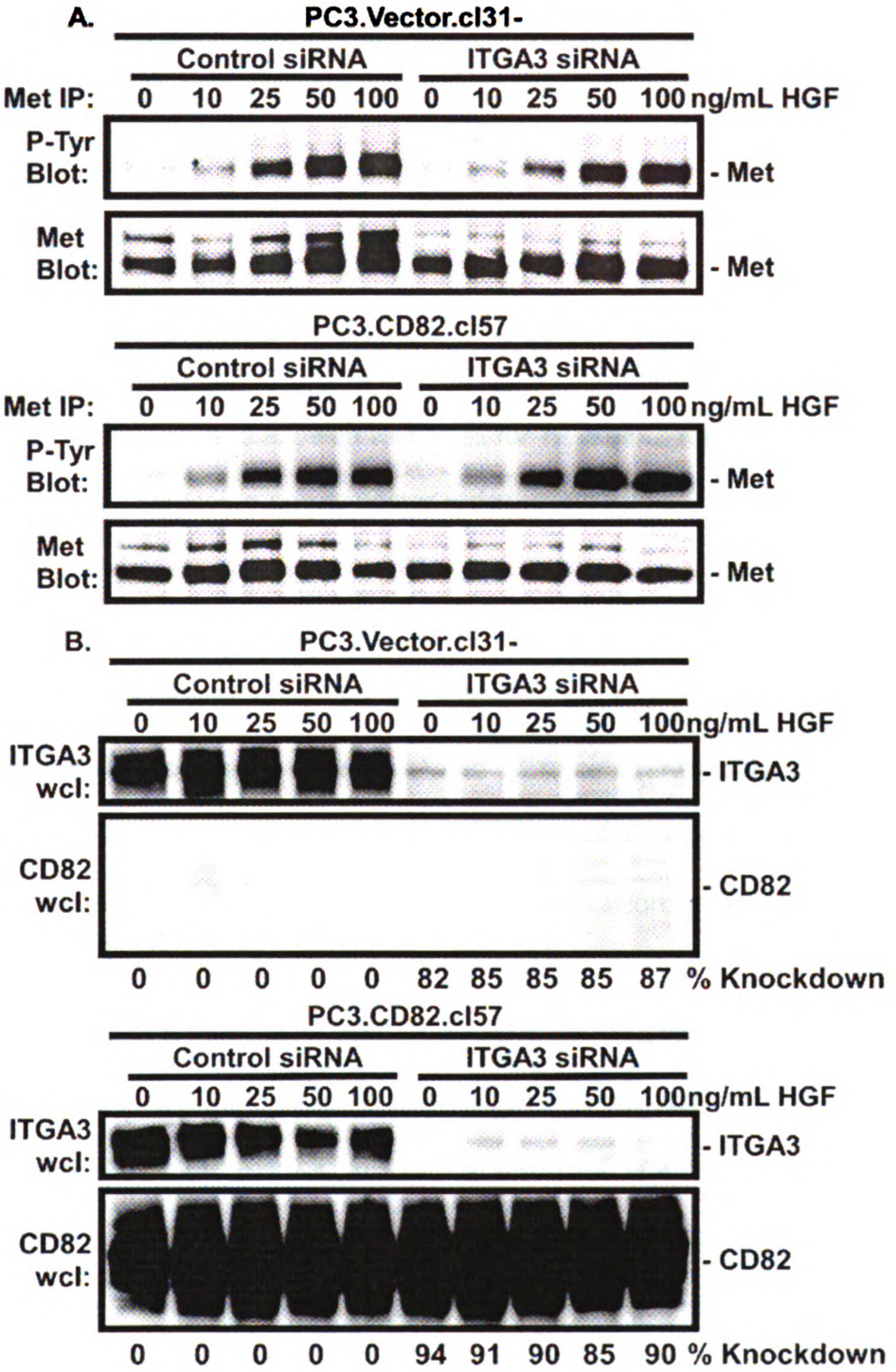
Based upon the model presented in Figure 1, it would be predicted that α3 integrin is necessary for dimerization of Met to stimulate its activity. To determine whether integrin α3 is required for Met activation, PC3 cells stably expressing CD82 or empty vector were transiently transfected with either a pool of integrin α3-specific siRNAs or a control siRNA. The control siRNA is a scrambled sequence that has no known target based on a BLAST search. The same control siRNA sequence will be used throughout all assays requiring siRNA. After transfection, cells were stimulated with increasing concentrations of hepatocyte growth factor (HGF). Met protein was immunoprecipitated from cell lysates and analyzed by immunoblotting for tyrosine phosphorylation and total Met protein. My data shows that approximately 85% of integrin α3 protein expression was knocked down in PC3 cells expressing empty vector resulting in reduced Met activation compared to cells transfected with control siRNA (Figure 2A,B). Knockdown of integrin α3 by approximately 90% had no effect on Met activation in CD82-expressing PC3 cells.

This data suggests that integrin α3β1 may be required for Met to reach maximal activation. However, this particular assay lacks an output to determine what level of Met activation results in increased migration, proliferation, or invasion. An experiment that includes a physiological output such as proliferation or migration

is required to firmly state that CD82 suppression of Met activation results in a specific physiological response.

Figure 2. Integrin α3 is not required for CD82 suppression of HGF-induced Met activation but contributes to Met activation. (**A**) PC3 cells stably expressing CD82 (cl57) or empty vector (cl31-) were transiently transfected with either a pool of 4 integrin α3 (ITGA3) specific siRNAs or scramble siRNA (control) and starved for 48 hours. The cells were then stimulated with 0, 10, 25, 50, and 100 ng/mL of HGF for 10 minutes. Met phosphorylation was measured by immunoblotting immunoprecipitates with a phospho-specific antibody. Total levels of Met protein in immunoprecipitates were measured by immunoblotting stripped blots with Met antibody. (**B**) ITGA3 and CD82 expression were monitored by immunoblotting of whole cell lysates. The numbers below the figures indicate the percent knockdown values of ITGA3, as determined by densitometry. *Susan Spotts, unpublished data*

Figure 2. Integrin α3 is not required for CD82 suppression of HGF-induced Met activation but contributes to Met activation.



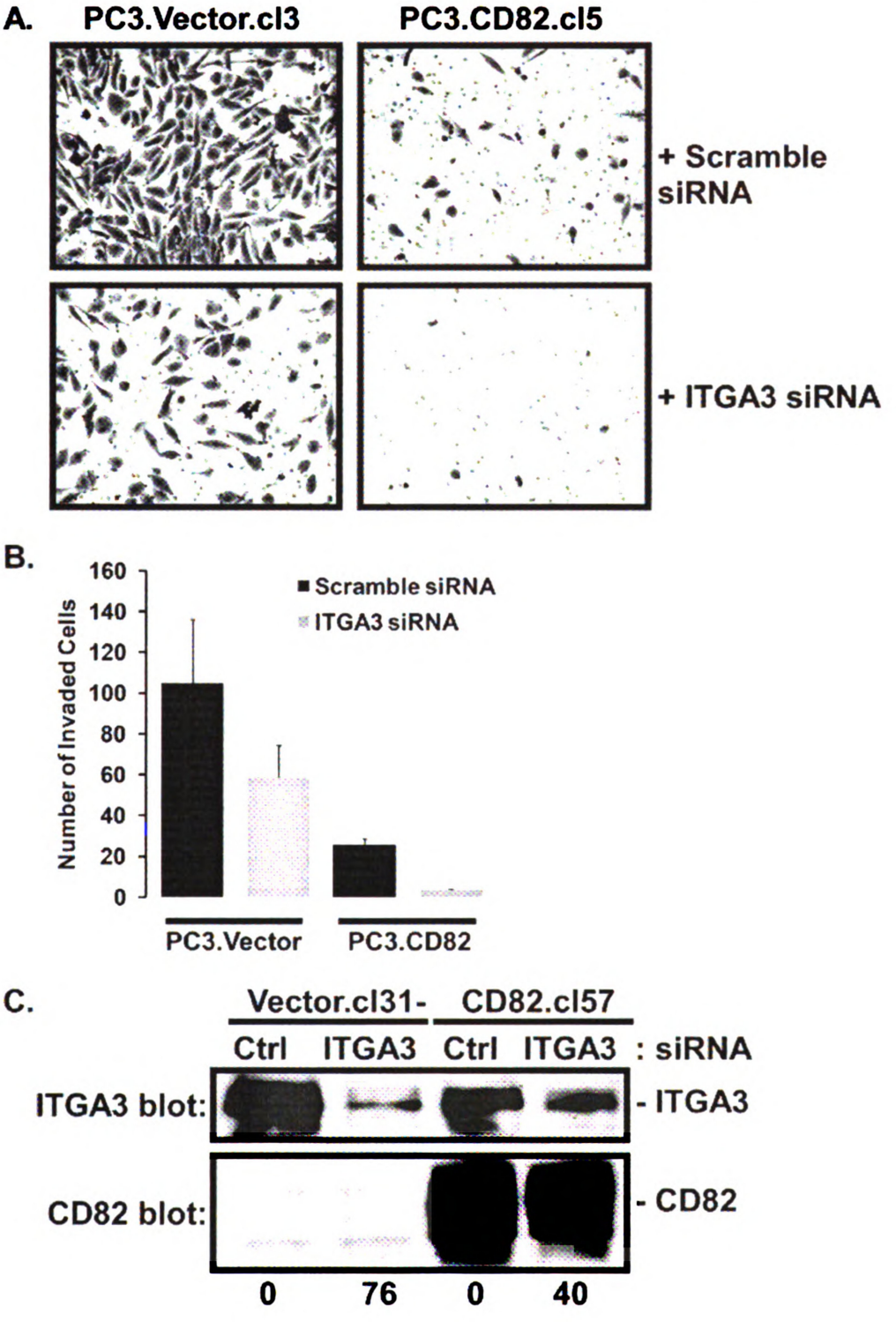
Integrin α3 is not required for CD82 suppression of invasion but contributes to PC3 cell invasion.

Our lab has previously demonstrated that Met is required for PC3 cell invasion of Matrigel [4]. Thus, if α3 integrin is required for Met activation, then blocking α3 integrin should also suppress invasion. To determine if integrin α3 is required for PC3 cell invasion, PC3 cells stably expressing either empty vector or CD82 were transiently transfected with a pool of integrin α3-specific siRNAs. The cells were then placed in a Boyden chamber containing Matrigel and laminin as a chemo-attractant and allowed to invade for 72 hours. Knockdown of integrin α3 in PC3 cells expressing empty vector reduced the number of cells that were able to invade the Matrigel, and appear in the lower chamber, compared to their control counterparts (Figure 3A). Knockdown of α3 integrin was quantified using Quantity One software to determine the density of the bands in Figure 3C. The bands present in the integrin α3 siRNA lanes were compared to the bands in the control siRNA lanes for each cell type. Knockdown of α3 integrin in empty-vector PC3 cells by 76% resulted in 1.8-fold decrease in invasion as shown in Figure 3B,C. PC3 cells expressing CD82 had only a 40% reduction in integrin α3 protein expression (Figure 3C). While the presence of CD82 suppressed invasion 4-fold, loss of α3 integrin did not restore the invasive capacity of PC3 parental cells, but rather resulted in a further reduction of invasive capabilities by an additional 6.5-fold (Figure 3A,B). These data suggest that integrin α3 partially contributes to PC3 invasion, and may be reflective of its action on Met based on the effect of α3 integrin on Met activation (see Figure 2). However, the presence

of α3 integrin with CD82 is not required for CD82 to suppress invasion. The further reduction in invasion in CD82 cells may reflect other α3-regulated events that are not controlled by CD82.

Figure 3. Integrin α3 is not required for CD82 suppression of invasion but contributes to PC3 cell invasion. (**A**) PC3 cells stably expressing CD82 (cl57) or empty vector (cl31-) were transiently transfected with either integrin α3 (ITGA3) specific siRNA or scramble siRNA (ctrl) and starved for 48 hours. Cells were placed in a Boyden chamber and allowed to invade for 72 hours. Cells which passed through the matrigel and appeared on the lower membrane were stained with crystal violet. (**B**) Cells were counted in 5 different fields and averaged. Three separate experiments were then averaged together. The error bar represents one standard deviation above the mean. (**C**) 72 hours after cells were placed in the Boyden chamber, parallel cultures of cells were lysed and immunoblotted for ITGA3 and CD82 expression. The numbers below the figure indicate the percent knockdown values of ITGA3, as determined by densitometry. *Susan Spotts, unpublished data*

Figure 3. Integrin α3 is not required for CD82 suppression of invasion but contributes to PC3 cell invasion.

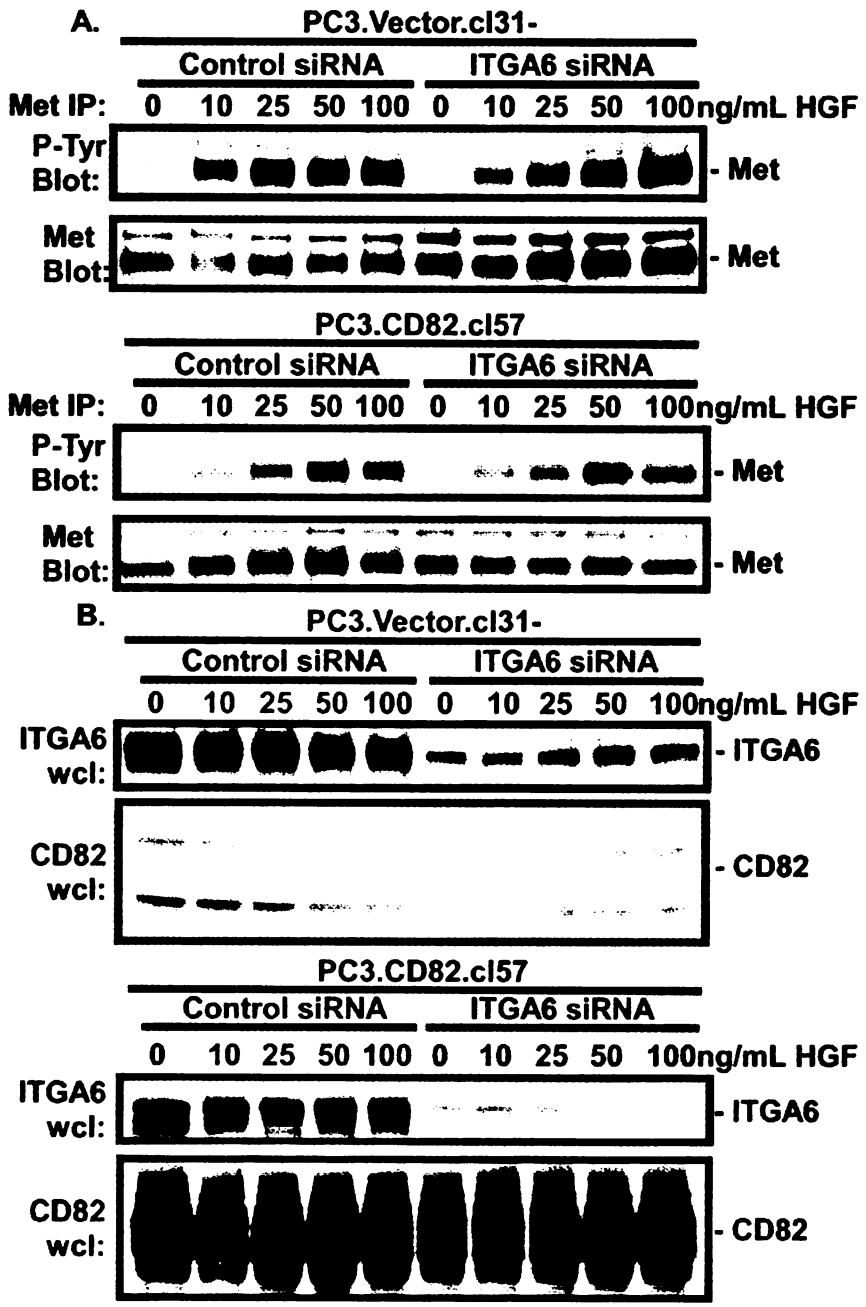


Integrin α6 is not required for Met activation nor for CD82 suppression of Met.

Loss of integrin α3 did not fully suppress invasion or completely block Met activation, and CD82 still maintained its capacity to suppress Met activation. However, loss of α3 integrin strengthened CD82's inhibition of invasion which suggests that CD82 may also suppress invasion by an additional mechanism. Integrin α6 is another laminin-specific integrin endogenously expressed on prostate cells [4]. Preliminary data generated in the lab had also shown that integrin α6 was present in CD82 complexes, albeit at much lower levels. Thus, CD82 might be controlling Met and invasion by regulating both α3 integrin and α6 integrin. To determine if α6 integrin can also control Met activation, PC3 cells stably expressing CD82 or empty vector were transiently transfected with either a pool of integrin α6-specific siRNAs or a control siRNA. The cells were stimulated with increasing concentrations of HGF prior to immunoprecipitation of Met and immunoblot analysis of Met tyrosine phosphorylation. Knockdown of integrin α6 protein expression by 75 to 92% in PC3 cells expressing empty-vector seems to have had no effect on Met activation. PC3 cells expressing CD82 with a 35-72% reduction in α6 integrin protein did not have altered Met activation (Figure 4A,B). These data suggest that integrin α6 does not contribute to the ability of CD82 to suppress Met activation nor does it play a role in stimulating Met activation. However, the knockdown efficiency in control PC3 cells was not optimal to make a final conclusion (Figure 4B). Knockdown efficiency was determined by densitometry using

Figure 4. Integrin α6 is not required for Met activation nor for CD82 suppression Met. **(A)** PC3 cells stably expressing CD82 (cl57) or empty vector (cl31-) were transiently transfected with either integrin α6 (ITGA6) specific siRNA or scramble siRNA (ctrl) and starved for 48 hours. The cells were then stimulated with 0, 10, 25, 50, and 100 ng/mL of HGF for 10 minutes. Met phosphorylation was measured by immunoblotting immunoprecipitates with a phospho-specific antibody. Total levels of Met protein in immunoprecipitates were measured by immunoblotting stripped blots with Met antibody. **(B)** ITGA6 and CD82 expression were monitored by immunoblotting of whole cell lysates. The numbers below the figures indicate the percent knockdown values of ITGA6, as determined by densitometry. *Susan Spotts, unpublished data*

Figure 4. Integrin α6 is not required for Met activation nor for CD82 suppression of Met.



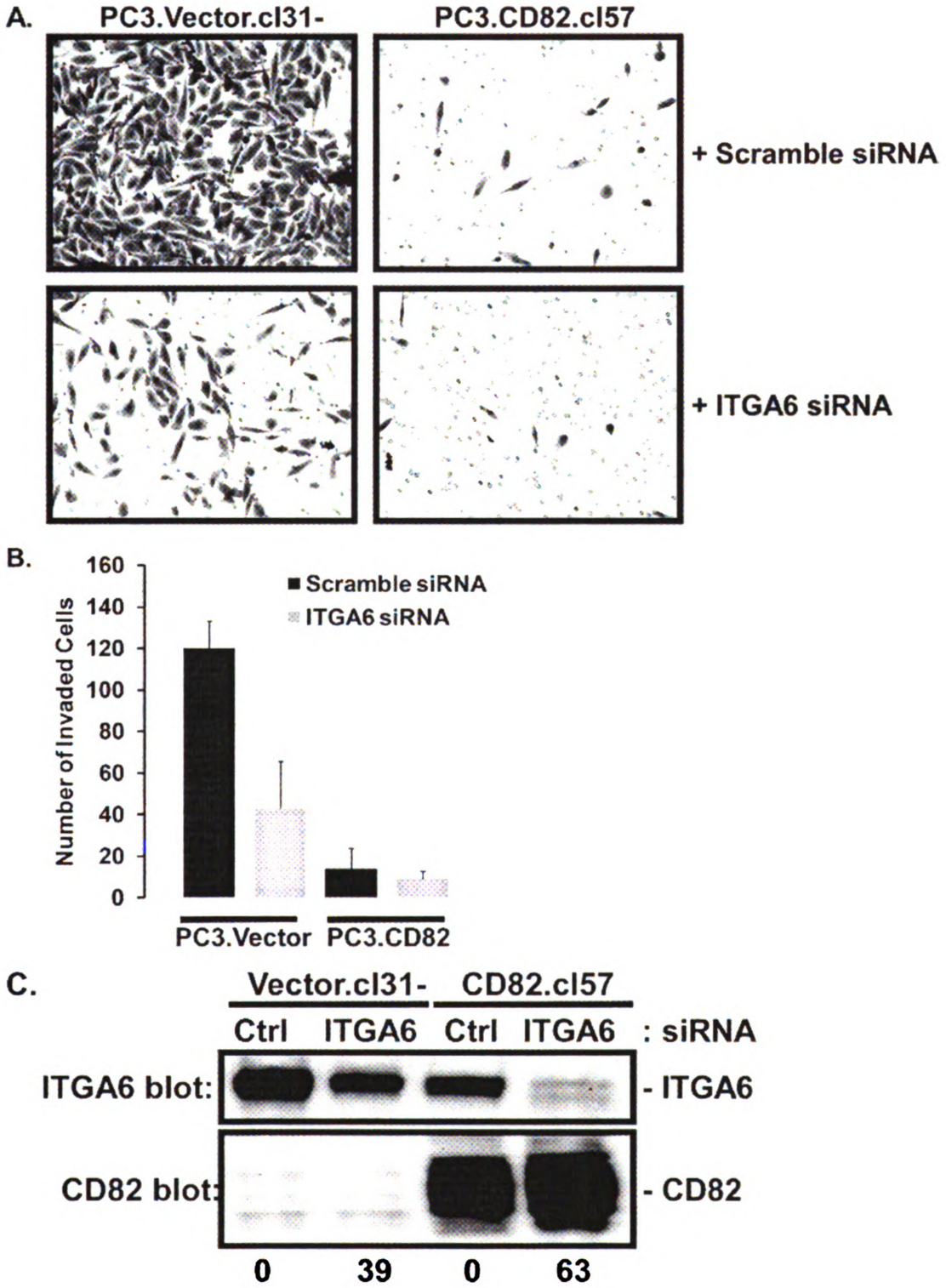
Quantity One software and comparing control cells with HGF treatments to integrin α6 siRNA treated cells stimulated with equal concentrations of HGF.

Integrin α6 is not required for CD82 suppression of invasion but contributes to PC3 cell invasion.

Integrin α6β4 has previously been shown to be essential for HGF-induced invasion in a highly tumorigenic human gastric cancer cell line [20]. To determine if integrin α6 is required for PC3 cell invasion, PC3 cells stably expressing either empty vector or CD82 were transiently transfected with a pool of four integrin α6-specific siRNAs. The cells were then placed in a Boyden chamber containing Matrigel and laminin as a chemo-attractant and allowed to invade for 72 hours. A 39% reduction of integrin α6 in PC3 cells expressing empty vector and integrin α6 siRNA displayed a 2.8-fold reduction in invasiveness compared to their control counterparts (Fig 5A,C). The difference in invasiveness may have been more significant had I been able to achieve a greater reduction in α6 integrin protein. Loss of α6 integrin in CD82 expressing cells had no effect on the ability of CD82 to inhibit invasion (Figure 5A,B). These data suggest that integrin α6, like α3, is required for invasion of PC3 cells, but its interaction with CD82 is not required for CD82 to suppress invasion.

Figure 5. Integrin α6 is not required for CD82 suppression of invasion but contributes to PC3 cell invasion. (**A**) PC3 cells stably expressing CD82 (cl57) or empty vector (cl31-) were transiently transfected with either integrin α6 (ITGA6) specific siRNA or scramble siRNA (ctrl) and starved for 48 hours. Cells were placed in a Boyden chamber and allowed to invade for 72 hours. Cells which passed through the matrigel and appeared on the lower membrane were stained with crystal violet. (**B**) Cells were counted in 5 different fields and averaged. Three separate experiments were then averaged together. (**C**) 72 hours after cells were placed in the Boyden chamber, parallel cultures of cells were lysed and immunoblotted for ITGA6 and CD82 expression. The numbers below the figure indicate the percent knockdown values of ITGA6, as determined by densitometry. *Susan Spotts, unpublished data*

Figure 5. Integrin α6 is not required for CD82 suppression of invasion but contributes to PC3 cell invasion.

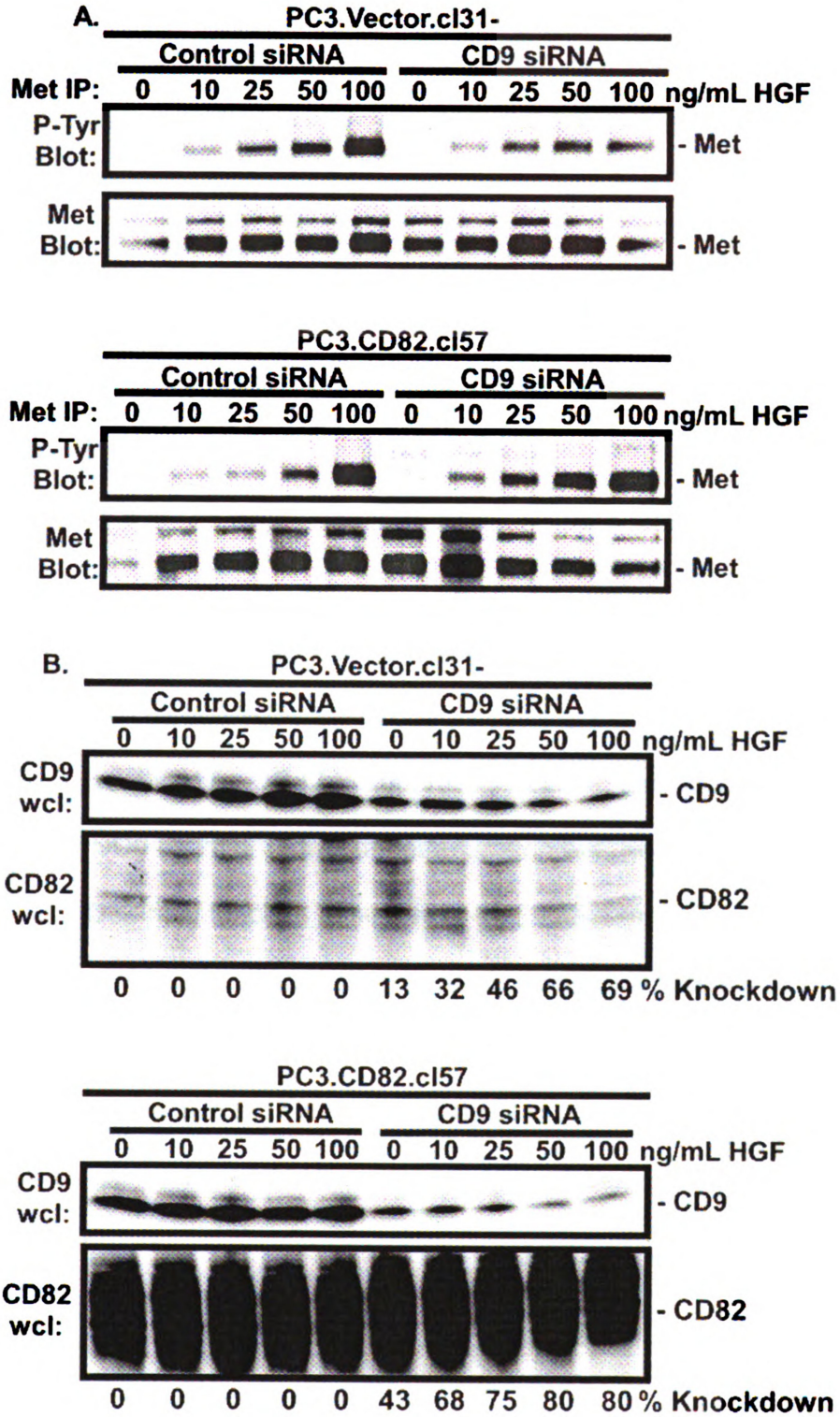


CD9 is required for CD82 suppression of HGF-induced Met activation.

If CD82 functions through the tetraspanin-enriched microdomain, we predict that the specific constituents within the microdomain are important for the ability of CD82 to suppress Met activation. Preliminary co-immunoprecipitation data generated in the lab had shown that CD9 protein in tetraspanin-enriched microdomains was increased in the presence of CD82. To determine if CD9 is required for CD82 suppression of Met activity, PC3 cells stably expressing CD82 or empty vector were transiently transfected with either CD9-specific siRNA or a control siRNA. These experiments were repeated multiple times using two unique siRNA sequences that are specific to CD9 based upon a BLAST search. The cells were stimulated with increasing concentrations of HGF, Met was immunoprecipitated from cell lysates, and analyzed by immunoblotting for tyrosine phosphorylation. An approximately 75% knockdown of CD9 resulted in increased Met activation in PC3 cells expressing CD82 compared to CD82 expressing PC3 cells transfected with control siRNA (Figure 6A,B). Knockdown of CD9 in PC3 cells expressing empty vector appears to have had little to no effect on Met activation compared to cells transfected with control siRNA. However, the knockdown of CD9 in empty vector–expressing PC3 cells was not very efficient with only a 13-70% reduction in CD9 protein levels. Greater knockdown of CD9 may be required to see a change in Met activation. These data suggest that CD82 requires the presence of CD9 to suppress HGF-induced Met activation.

Figure 6. CD9 is required for CD82 suppression of HGF-induced Met activation. **(A)** PC3 cells stably expressing CD82 (cl57) or empty vector (cl31-) were transiently transfected with either CD9 specific siRNA or control siRNA (ctrl) and starved for 48 hours. The cells were then stimulated with 0, 10, 25, 50, and 100 ng/mL of HGF for 10 minutes. Met phosphorylation was measured by immunoblotting immunoprecipitates with a phospho-specific antibody. Total levels of Met protein in immunoprecipitates were measured by immunoblotting stripped blots with Met antibody. **(B)** CD9 and CD82 expression were monitored by immunoblotting of whole cell lysates. The numbers below the figure indicate the percent knockdown values of CD9, as determined by densitometry. *Susan Spotts, unpublished data.*

Figure 6. CD9 is required for CD82 suppression of HGF-induced Met activation.

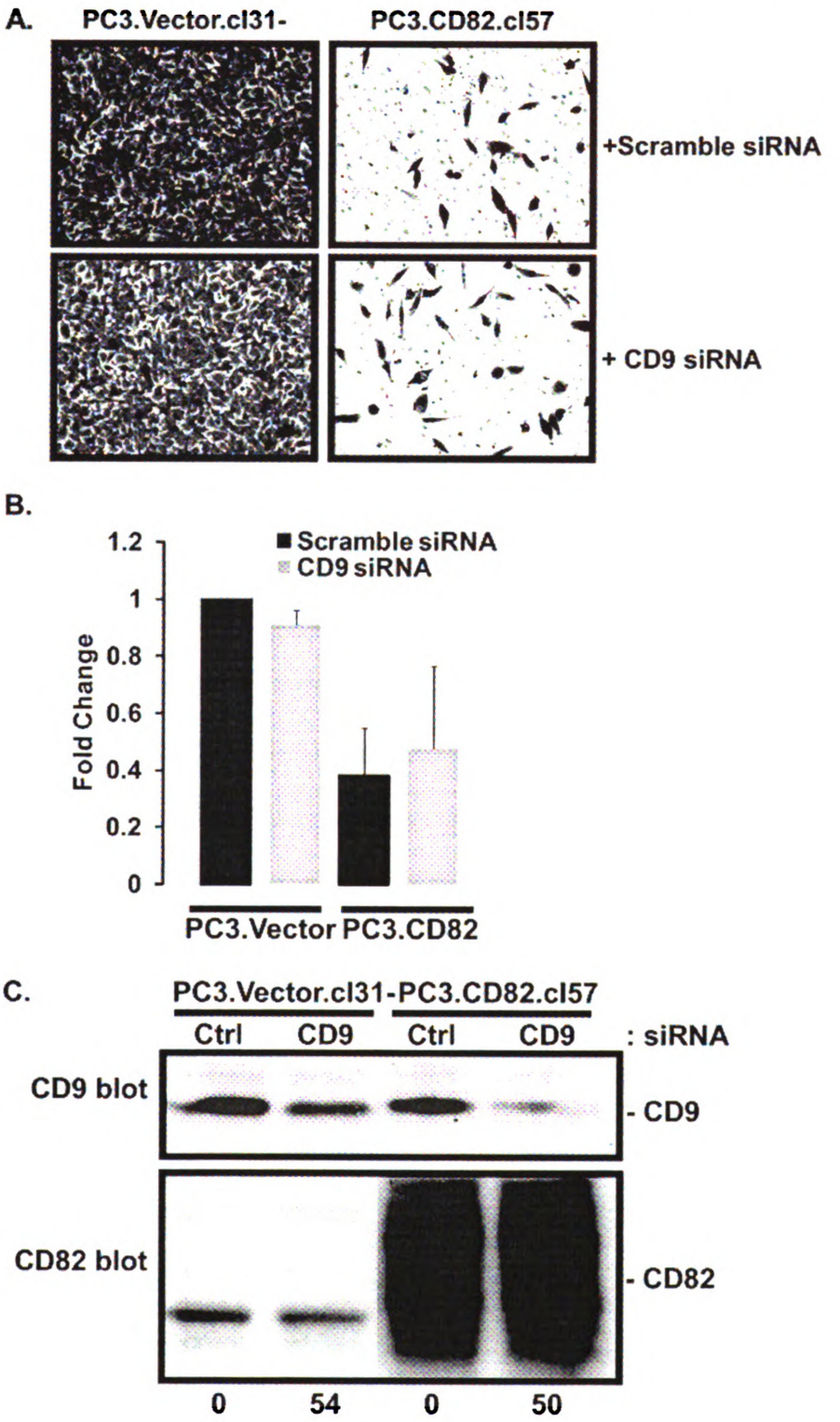


CD9 is not required for CD82 suppression of invasion.

Since CD9 is required for CD82 suppression of HGF-induced Met activation and Met is required for PC3 cell invasion, we predicted that knockdown of CD9 in PC3 cells expressing CD82 would result in increased invasion. To determine if CD9 is required for CD82 suppression of invasion, PC3 cells stably expressing either empty vector or CD82 were transiently transfected with either CD9-specific siRNA or control siRNA. The cells were then placed in a Boyden chamber containing Matrigel and laminin as a chemo-attractant and allowed to invade for 72 hours. A 50% reduction of CD9 protein in PC3 cells expressing CD82 resulted in a minimal increase in invasion that was not statistically significant compared to control siRNA cells (Figure 7A,B,C). PC3 cells expressing empty vector had a 54% reduction in CD9 protein and displayed no change in invasion compared to their control counterparts. These data suggest that CD9 is not required for CD82 to suppress invasion. Due to CD82's requirement of CD9 for HGF-induced Met activation but not for invasion, it appears that CD82 may function to suppress Met activation and invasion through two separate mechanisms. Met activation leads to multiple pathways being activated to regulate cell proliferation, migration, and invasion. An assay that measures both Met activation via tyrosine phosphorylation and a physiological output such as growth is needed to understand the disconnection in the requirement for CD9.

Figure 7. CD9 is not required for CD82 suppression of invasion. (**A**) PC3 cells stably expressing CD82 (cl57) or empty vector (cl31-) were transiently transfected with either CD9 specific siRNA or scramble siRNA (ctrl) and starved for 48 hours. Cells were placed in a Boyden chamber and allowed to invade for 72 hours. Cells which passed through the matrigel and appeared on the lower membrane were stained with crystal violet. (**B**) Cells were counted in 5 different fields and averaged. (n=6 for all samples except PC3.Vecter.cl31- + Scramble siRNA where n=3) (**C**) 72 hours after cells were placed in the Boyden chamber, parallel cultures of cells were lysed and immunoblotted for CD9 and CD82 expression. The numbers below the figure indicate the percent knockdown values of CD9, as determined by densitometry. *Susan Spotts, unpublished data.*

Figure 7. CD9 is not required for CD82 suppression of invasion.



CD151 is not required for CD82 suppression of HGF-induced Met activation, but contributes to Met activation in PC3 cells.

Once we had determined that CD9 was required for CD82 suppression of Met activation, I assessed the requirement of other tetraspanins for CD82 inhibition of Met activation. CD151 is often up-regulated in a variety of cancers and CD151 ablation results in decreased cell migration, invasion, spreading, and signaling [21]. CD151 has been shown to directly interact with integrin α3 and indirectly with integrin α6. Klosek *et al.* reported that CD151 associates with the α3 integrin and Met, providing a functional link for integrins to associate with Met in human salivary gland tumors [12]. Knowing that integrin α3 can regulate Met, we hypothesized that CD82 may suppress Met activation by drawing integrin α3 and/or integrin α6 and CD151 into tetraspanin enriched microdomains. To determine whether CD151 is required for CD82 to suppress Met activation, PC3 cells stably expressing CD82 or empty vector were transiently transfected with either a pool of CD151-specific siRNA or a control siRNA. These cells were then stimulated with increasing concentrations of HGF. Met protein was immunoprecipitated from cell lysates and analyzed by immunoblotting for tyrosine phosphorylation and total Met protein. The results show that a 70-90% knockdown of CD151 had no effect on CD82-expressing PC3 cells' ability to suppress HGF-induced Met activation (Figure 8A,B). However, a reduction of CD151 protein by approximately 90% in PC3 cells expressing empty vector reduced Met activation compared to cells transfected with control siRNA (Figure

8A,B). These data suggest that CD151 is required for Met to reach maximal activation, but that CD151 is not required for CD82 suppression of Met.

CD151 is not required for CD82 suppression of invasion, but contributes to PC3 cell invasion.

Since CD151 ablation has been shown to reduce invasion, we expected to see a reduction in the number of cells that were able to invade in the PC3 cells expressing empty-vector. However, we also sought to determine if CD151 is required for CD82 to suppress invasion. PC3 cells stably expressing either empty vector or CD82 were transiently transfected with either a pool of four CD151-specific siRNAs or control siRNA. The cells were then placed in a Boyden chamber containing Matrigel and laminin as a chemo-attractant and allowed to invade for 72 hours. A 88% knockdown of CD151 in vector-expressing PC3 cells reduced invasiveness by 1.7-fold compared to their control counterparts (Figure 9A,B,C). Loss of CD151 in CD82 expressing cells did not restore the invasive capacity of control PC3 cells, but resulted in an additional 3.5-fold reduction in invasive capabilities (Figure 9A,B). The CD151 knockdown invasion assay results mimic the α3 integrin knockdown invasion assay data. These data suggest that CD151 and integrin α3 may be present in the same pathway regulating invasion. Overall, these results suggest that CD151 is not required for CD82 to suppress invasion, but contributes to invasion of PC3 cells.

Figure 8. CD151 is not required for CD82 suppression of HGF-induced Met activation, but contributes to Met activation in PC3 cells. **(A)** PC3 cells stably expressing CD82 (cl57) or empty vector (cl31-) were transiently transfected with either CD151 specific siRNA or control siRNA (ctrl) and starved for 48 hours. The cells were then stimulated with 0, 10, 25, 50, and 100 ng/mL of HGF for 10 minutes. Met phosphorylation was measured by immunoblotting immunoprecipitates with a phospho-specific antibody. Total levels of Met protein in immunoprecipitates were measured by immunoblotting stripped blots with Met antibody. **(B)** CD151 and CD82 expression were monitored by immunoblotting of whole cell lysates. The numbers below the figure indicate the percent knockdown values of CD151, as determined by densitometry. *Susan Spotts, unpublished data*

Figure 8: CD151 is not required for CD82 suppression of HGF-induced Met activation but contributes to Met activation in PC3 cells.

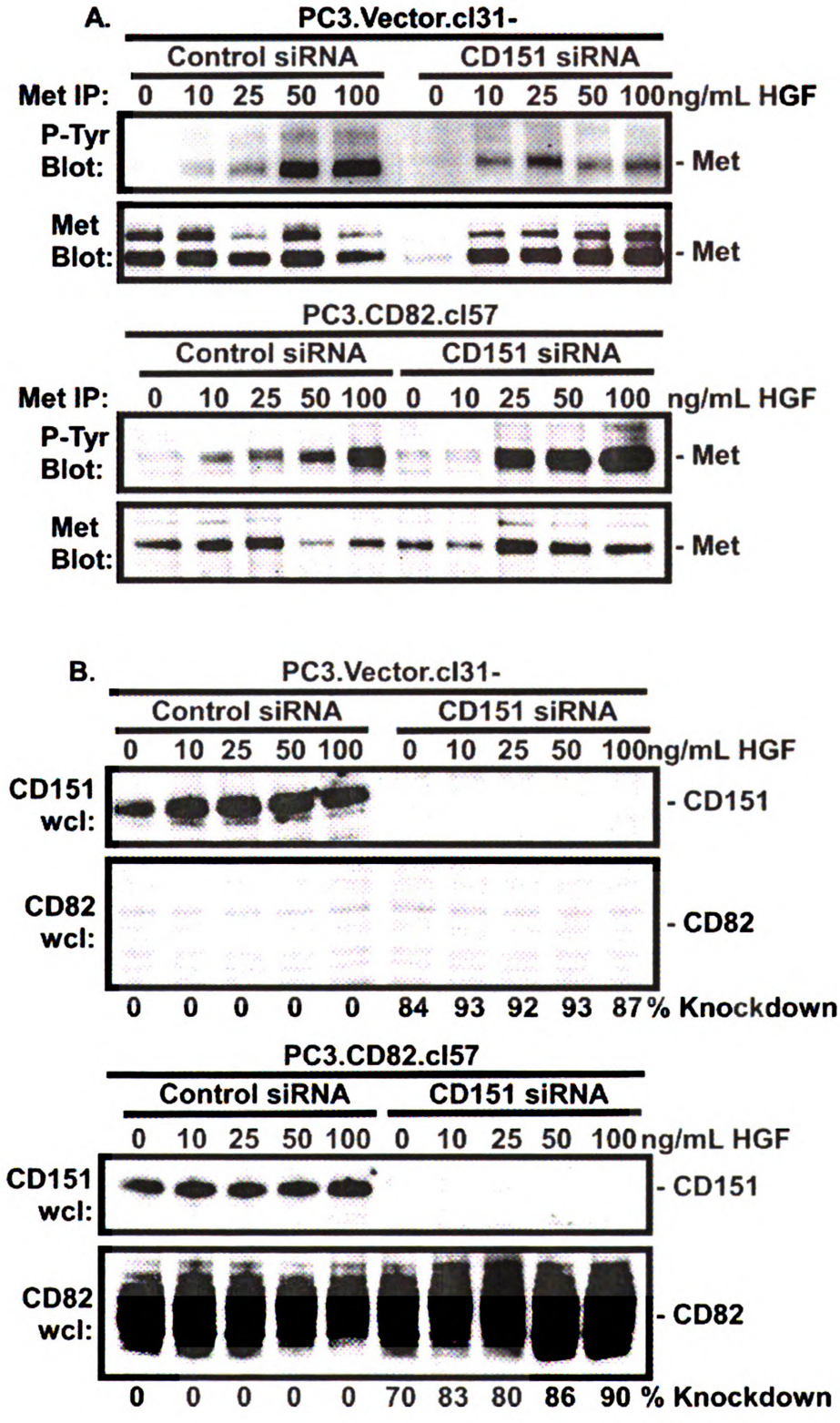
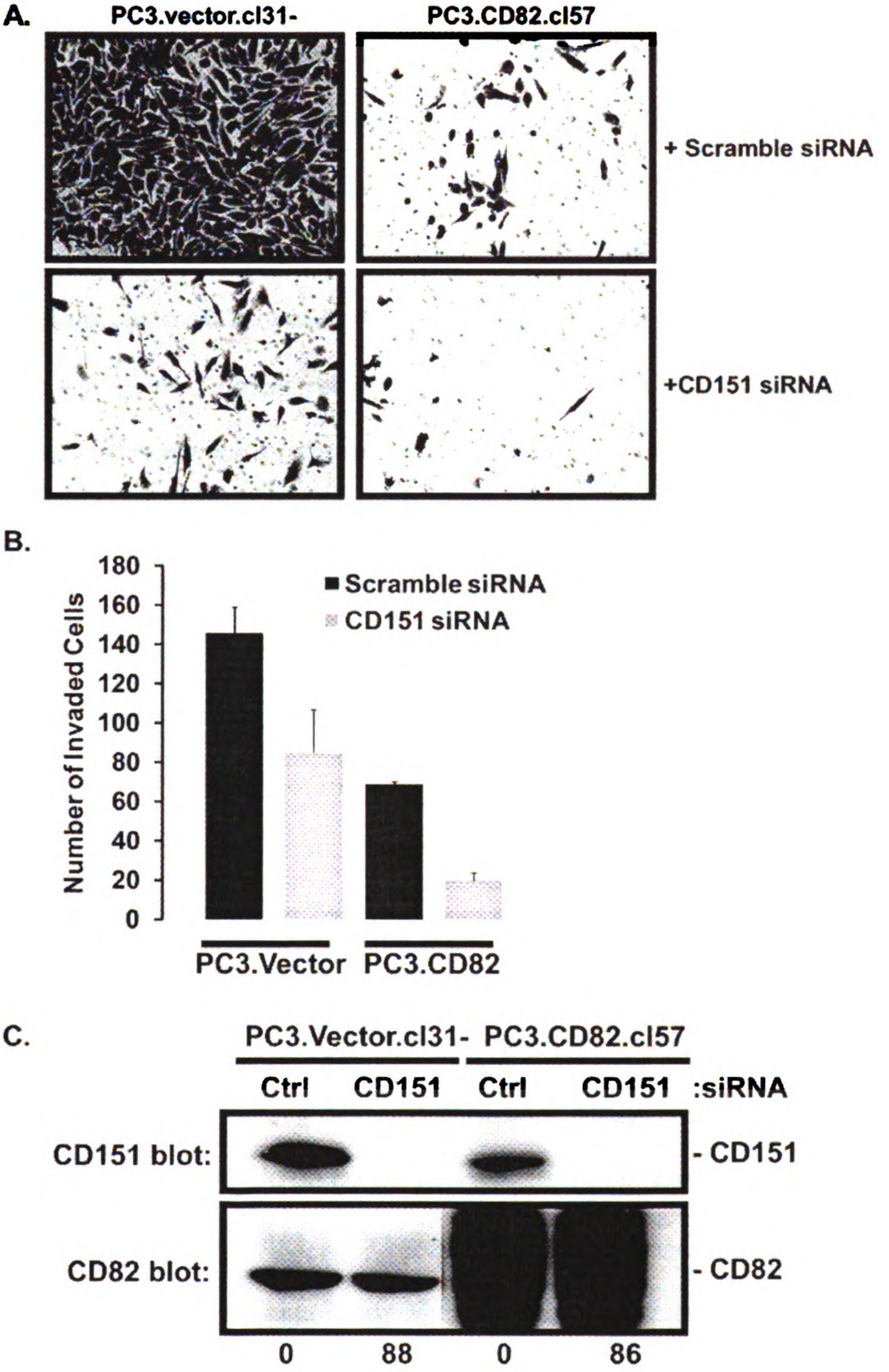


Figure 9. CD151 is not required for CD82 suppression of invasion, but contributes to PC3 cell invasion. (**A**) PC3 cells stably expressing CD82 (cl57) or empty vector (cl31-) were transiently transfected with either CD151 specific siRNA or scramble siRNA (ctrl) and starved for 48 hours. Cells were placed in a Boyden chamber and allowed to invade for 72 hours. Cells which passed through the matrigel and appeared on the lower membrane were stained with crystal violet. (**B**) Cells were counted in 5 different fields and averaged. Four separate experiments were then averaged together. (**C**) 72 hours after cells were placed in the Boyden chamber, parallel cultures of cells were lysed and immunoblotted for CD151 and CD82 expression. The numbers below the figure indicate the percent knockdown values of CD151, as determined by densitometry. *Susan Spotts, unpublished data*

Figure 9: CD151 is not required for CD82 suppression of invasion but contributes to PC3 cell invasion.



Discussion

Our lab had previously established that CD82 is capable of suppressing both integrin-mediated and ligand-induced Met activation and that Met requires the cross-talk with integrin to achieve maximal activation [4]. Multiple labs within the tetraspanin field have provided information regarding the requirement for the correct composition of tetraspanins and their protein-binding partners within the tetraspanin-enriched microdomain to achieve functional tetraspanins [22].

I was interested in determining which constituents within the tetraspanin-enriched microdomain were required for CD82 to suppress HGF-induced Met activation. Based upon these results, we have determined that CD82 requires the presence of CD9 for suppression of HGF-induced Met activation and its function is independent of the presence of CD151, α3 integrin, and α6 integrin. On the other hand, CD151, and integrins α3 and α6 do not appear to be required for CD82 suppression of Met activation.

However, the experiment chosen to determine the requirement for other tetraspanins and integrins on CD82 suppression of HGF-induced Met activation contains a major flaw. This experiment does not establish the threshold of Met activation required to result in a physiological change within the cell. To provide a more concrete understanding of which proteins are required for CD82 function, Met activation studies should be conducted simultaneously with an assay that has a physiological output such as a wound healing assay or scratch test that measures HGF-dependent migration. Multiple tests may need to be completed

to determine whether CD82 only suppresses migration, proliferation, or invasion, or whether it's a combination of pathways it affects.

Met contains multiple tyrosine residues within the cytoplasmic domain. The phosphorylation states of these residues in specific patterns results in regulation of different signaling transduction pathways. Identifying the specific tyrosine residues which are regulated by CD82 and result in decreased cell invasion or proliferation will provide valuable insight into CD82's molecular mechanism. Birchmeier *et al.* have shown that mutations of the tyrosine residues in the C-terminal tail result in reduced ligand-induced cell motility and branching morphogenesis [1]. Preliminary studies to identify the tyrosine residues being modulated by CD82 has been completed in our lab and the data suggest that CD82 regulates tyrosine residue 1365. Studies involving knockdown of various tetraspanins and integrins should be completed in which the phosphorylation state of specific tyrosine residues on Met would be monitored.

CD82 has also been shown to suppress invasion to the same extent as loss of Met has on reduced invasiveness [4]. Therefore, we sought to understand whether the same proteins required for CD82 suppression of Met activation was also required for CD82 inhibition of invasion. The results suggest that CD9, CD151, integrin α3, and integrin α6 are not required for CD82 suppression. However, α3 and α6 integrins and CD151 contribute to invasion of PC3 cells. Interesting, the loss of CD151 and integrin α3 in addition to the presence of CD82 resulted in further reduced invasion. These data seem to

further strengthen the evidence that CD151 and integrin α3 associate to regulate Met as well as other receptor tyrosine kinases.

CHAPTER 2: IDENTIFICATION OF PROTEIN-PROTIEN INTERACTIONS

Rationale

The literature has suggested that CD82 functions as a 'molecular facilitator' to organize tetraspanins, integrins, and other membrane proteins into a tetraspanin-enriched microdomain [3]. If CD82 functions through its involvement in the TERM, we would predict that specific protein interactions within the microdomain are important for the ability of CD82 to suppress c-Met activation.

Met can be activated through an integrin-mediated mechanism and CD82 was shown to co-immunoprecipitate with integrins. In addition, I have demonstrated that α3 integrin is required for efficient Met activation (see Figure 2). Thus our hypothesis is that CD82 suppresses Met activation by drawing the integrins out of the Met complex and into a tetraspanin-enriched microdomain.

Protein-protein interactions within the tetraspanin-enriched microdomain have been divided into three types based on the strength of the detergent required to disrupt their interactions [6]. Type 1 interactions are direct physical interactions which include tetraspanin homodimers and some heterodimer interactions such as CD151 and integrin α3 interactions [6]. Type 2 interactions are stable under mild lysis conditions and consist of tetraspanin-tetraspanin heterodimer interactions and tetraspanin-integrin interactions which are stabilized

mainly by palmitoylation [6]. Type 3 interactions are very weak associations between tetraspanins and receptor protein-binding partners which are stabilized also by palmitoylation [6].

To identify the protein-protein interactions which exist in the presence and absence of CD82, we chose to conduct co-immunoprecipitation experiments. While co-immunoprecipitation assays allow for the identification of protein-protein associations, it does not provide any information regarding direct physical interactions. PC3 cells expressing either CD82 or empty vector were stimulated with 0 or 25 ng/mL of HGF for 10 minutes prior to lysing. Since we are interested in determining hetero-tetraspanin interactions and tetraspanin-integrin associations which are Type 2 interactions, we chose to use the mild detergent 1% CHAPS in our lysis buffer. I immunoprecipitated the protein of interest, then immunoblotted for the corresponding proteins of interest before immunoblotting for total protein. We expected to see a decrease in Met-integrin associations in the presence of CD82 and an increase in associations with HGF stimulation. We also expected to see an increase in tetraspanin-integrin interactions in the presence of CD82 with little change occurring with HGF treatment.

Results:

Met associates with integrin α6β1 but not with integrin α3β1.

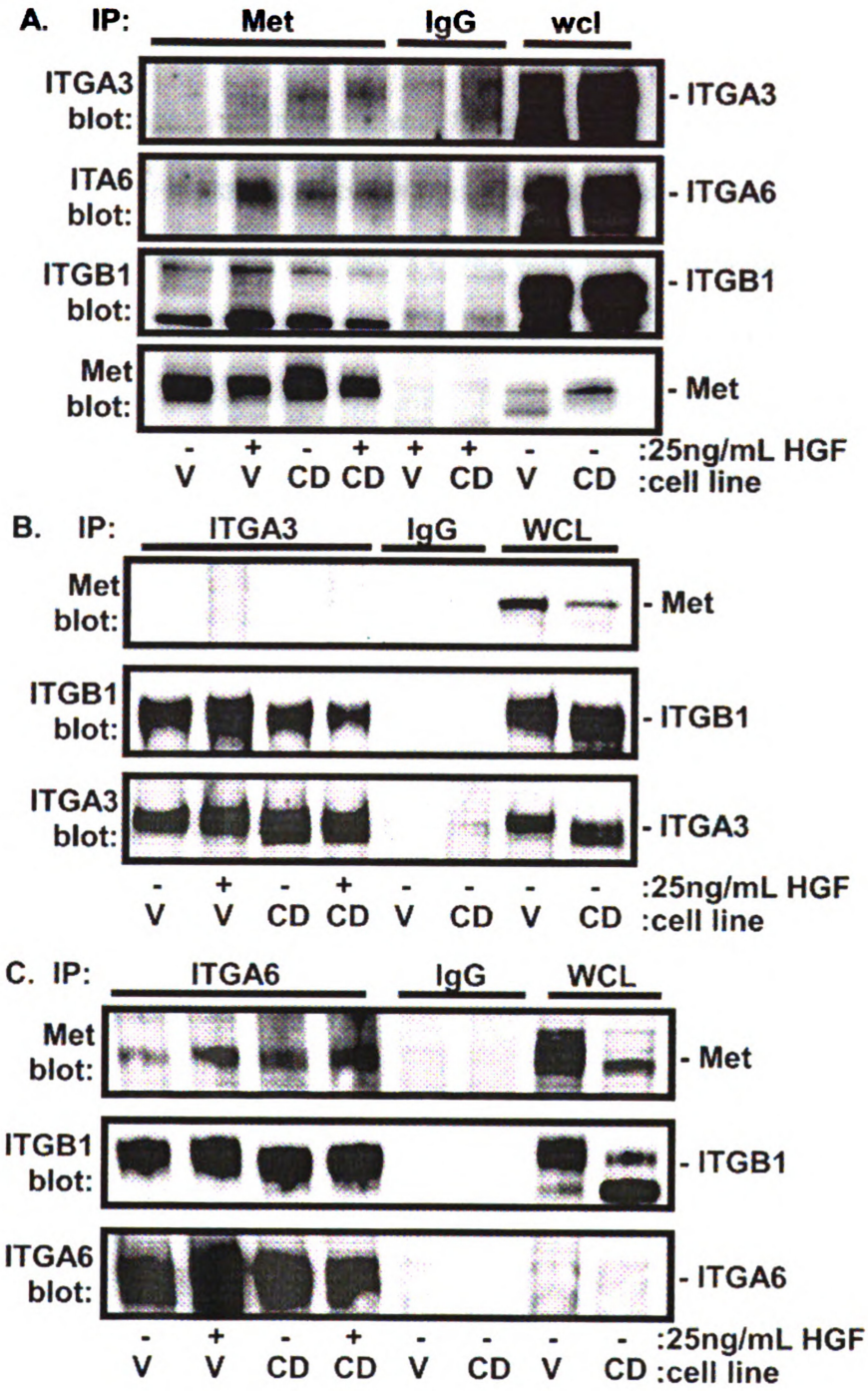
Met can be activated through an integrin-mediated mechanism and CD82 was previously shown to co-immunoprecipitate with integrins. I predict that integrin α3 will be the predominant integrin α subunit to associate with Met since

we have data suggesting that integrin α3 contributes to both HGF-induced Met activation and invasion of PC3 cells (see Figure 2). To determine which integrins are present in the Met complex and whether the presence of CD82 has an effect on Met/integrin complexes, PC3 cells expressing either empty vector or CD82 were left unstimulated or stimulated with 25 ng/mL of HGF for 10 minutes and lysed under mild conditions using 1% CHAPS lysis buffer. Met, integrin α3, integrin α6, and integrin β1 protein was then immunoprecipitated and immunoblotted for various integrin subunits and tetraspanins. I chose the HGF concentration of 25 ng/mL based on the fact that this concentration is where I saw the largest differential effect on HGF-induced Met activation between vector and CD82 expressing cells. I was unable to detect integrin α3 or α6 subunits and the integrin β1 subunit within the Met immunoprecipitates (Figure 10A). While it appears that small amounts of integrin α3 and α6 might be associating with Met, the integrin β1 subunit, which is the other half of the integrin dimmer, was not detected. Integrin β1 serves as a positive control throughout these co-immunoprecipitations since integrins function as heterodimers consisting of an alpha and a beta subunit. However, I was also able to detect Met and β1 within integrin α6 immunoprecipitates (Figure 10C). Upon HGF treatment, there appears to be a slight increase in the amount of Met associating with α6 integrin in both CD82-expressing cells and non-CD82 expressing cells. I was able to detect β1 integrin, but not Met within the integrin α3 immunoprecipitates (Figure 10B). Overall these data suggest that in PC3 cells, Met predominantly associates with integrin α6β1. This result conflicts with our original hypothesis of

Met associating predominantly with integrin α3 subunit since integrin α3 contributes to HGF-induced Met activation and invasion.

Figure 10. Met associates with integrin α6β1 but not with integrin α3β1. PC3 cells stably transfected with either empty vector (V) or CD82 (CD) were serum starved for 48 hours and then stimulated with 25 ng/mL of HGF for 10 minutes. The cells were lysed in 1% CHAPS lysis buffer for 30 minutes. (**A**) Lysates were immunoprecipitated with anti-Met antibody, then immunoblotted with anti-integrin α3 subunit (ITGA3), anti-integrin α6 subunit (ITGA6), anti-integrin β1 subunit (ITGB1), and anti-Met antibodies. (**B**) Lysates were immunoprecipitated with anti-integrin α3 (ITGA3) antibodies, then immunoblotted with anti-Met, anti-integrin β1 (ITGB1), and anti-integrin α3 (ITGA3) antibodies. (**C**) Lysates were immunoprecipitated with anti-integrin α6 (ITGA6) antibodies, then immunoblotted with anti-Met, anti-integrin β1 (ITGB1), and anti-integrin α6 (ITGA6) antibodies. Whole cell lysates (WCL) served as a positive control. Respective whole IgG molecules served as negative controls. *Susan Spotts, unpublished data*

Figure 10: Met associates with integrin α6β1 but not with integrin α3β1.



In the presence of CD82, integrin α6 is drawn into the tetraspanin-enriched microdomain and associates with CD82 and CD151.

Once we had determined which integrin associates with Met, we sought to identify which tetraspanins associate with Met, and the α3 and α6 integrin subunits. I was able to detect CD82 within α6 integrin immunoprecipitates (Figure 11A), but I was unable to detect CD82 within α3 integrin or Met immunoprecipitates (Figure 11B,C). I was also able to detect an association between CD9 and both integrin α subunits. However, there was a reduction in the amount of CD9 that was able to associate with the α integrin subunits in the presence of CD82 (Figure 11A,B). However, in the reverse co-immunoprecipitations, in which CD9 was pulled down in immunoprecipitates and then immunoblotted for integrin α3 and integrin α6, I was unable to detect Met, α3 integrin, or α6 integrin within the CD9 immunoprecipitates (Figure 11D). I also evaluated the associations between CD151 and α integrin subunits. I predicted that there would be an association between CD151 and both α3 integrin and α6 integrin. Both integrin α3 and α6 were found to interact with CD151; however in the presence of CD82, there was a reduction in the amount of CD151 interacting with integrin α3 (Figure 11B). In the reverse co-immunoprecipitation, CD151 associated with both integrin α subunits with some decrease in the amount of association due to the presence of CD82 (Figure 11E). I was also able to detect a weak association with Met within the CD151 immunoprecipitates; however, because of the low signal, I am unable to determine if there is a change in Met-CD151 interactions due to CD82 or HGF stimulation.

Figure 11. In the presence of CD82, integrin α6 is drawn into the tetraspanin-enriched microdomain and associates with CD82 and CD151. PC3 cells stably transfected with either empty vector (V) or CD82 (CD) were serum starved for 48 hours and then stimulated with 25 ng/mL of HGF for 10 minutes. The cells were lysed in 1% CHAPS lysis buffer for 30 minutes. (**A**) Lysates were immunoprecipitated with anti-integrin α6 (ITGA6) antibody, then immunoblotted with anti-CD82, anti-CD9, and anti-CD151 antibodies. (**B**) Lysates were immunoprecipitated with anti-integrin α3 (ITGA3) antibodies, then immunoblotted with anti-CD82, anti-CD9, and anti-CD151 antibodies. (**C**) Lysates were immunoprecipitated with anti-Met antibodies, then immunoblotted with anti-CD82, anti-CD9, and anti-CD151 antibodies. (**D**) Lysates were immunoprecipitated with anti-CD9 antibody, then immunoblotted with anti-Met, anti-integrin α3 subunit (ITGA3), and anti-integrin β1 subunit (ITGB1) antibodies. (**E**) Lysates were immunoprecipitated with anti-CD151 antibody, then immunoblotted with anti-Met, anti-integrin α3 subunit (ITGA3), and anti-integrin β1 subunit (ITGB1) antibodies. Whole cell lysates (WCL) served as a positive control. Respective whole IgG molecules served as negative controls. *Susan Spotts, unpublished data*

Figure 11: In the presence of CD82, integrin α6 is drawn into the tetraspanin-enriched microdomain and associates with CD82, and CD151

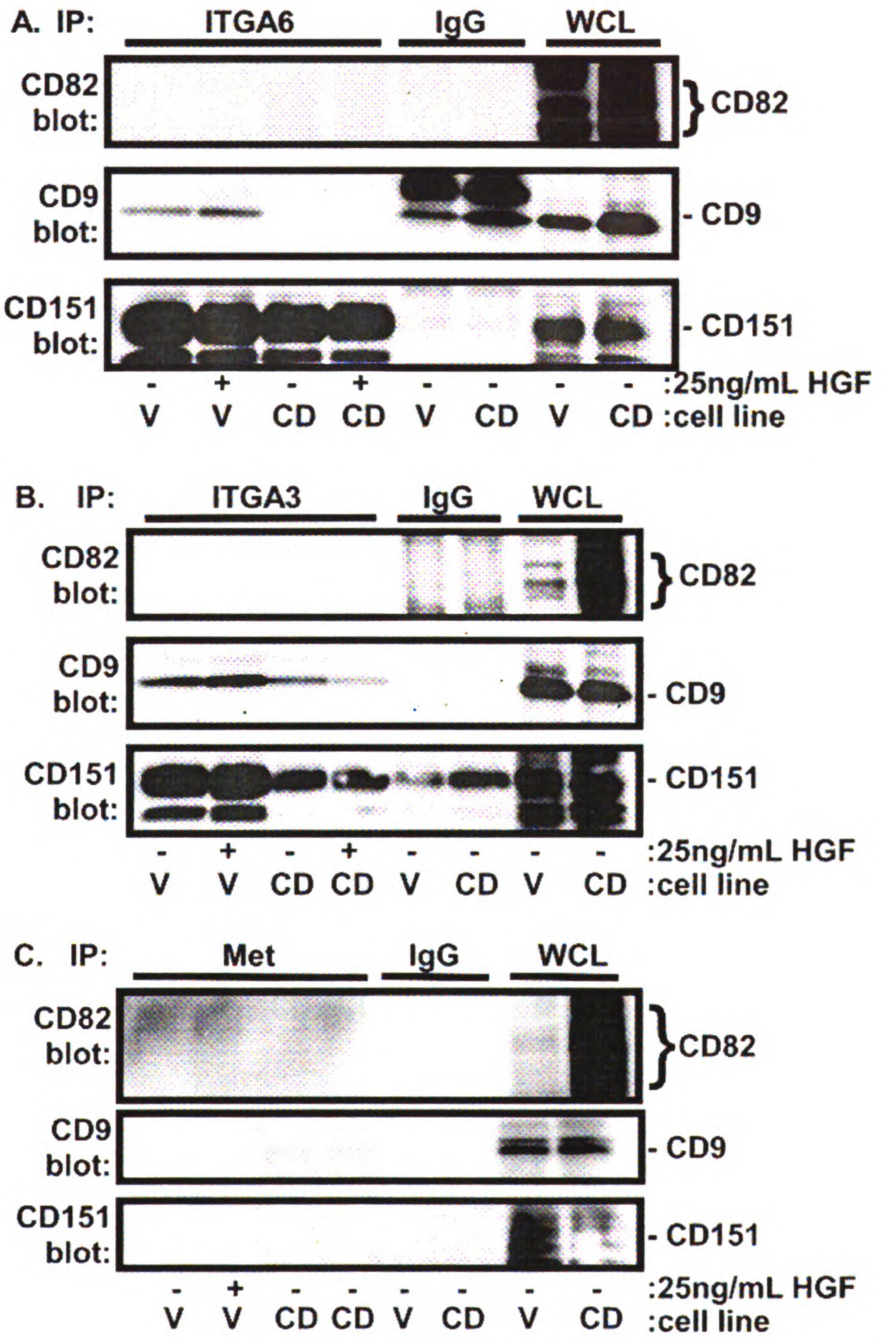
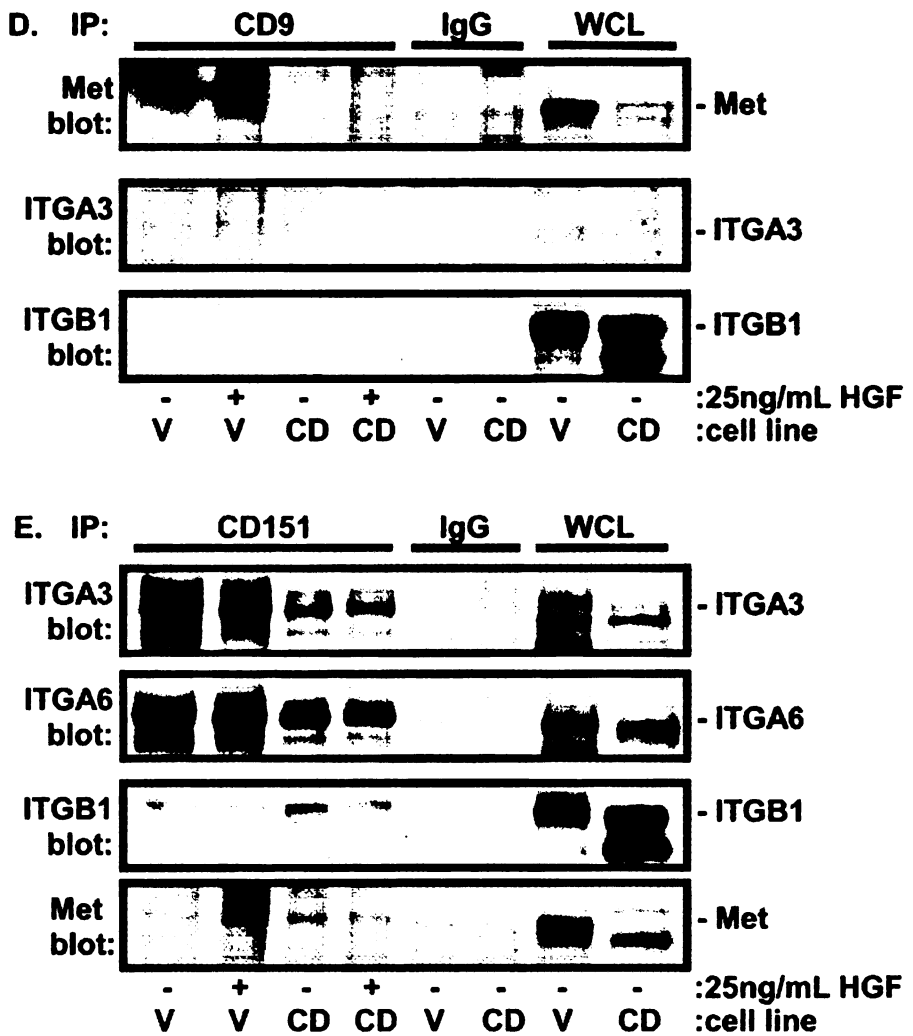


Figure 11 con't: In the presence of CD82, integrin α6 is drawn into the tetraspanin-enriched microdomain and associates with CD82, and CD151



In the presence of CD82, there is a reduction in the number of CD9 and CD151 associations.

To further understand the protein-protein interactions that are specific for CD82 suppression of HGF-induced Met activation, I sought to identify the tetraspanins that were interacting in the presence and absence of CD82. I was able to detect CD82 and CD151 in CD9 immunoprecipitates (Figure 12A); however, less CD151 was pulled down in the presence of CD82. I was also able to detect CD9 and CD82 in CD151 immunoprecipitates (Figure 12B). While CD151 appears to only associate with CD82 when the cells are stimulated with HGF, CD151 may also be able to associate with CD82 in the absence of HGF. I may not have immunoprecipitated enough CD151 in the control, non-HGF treated sample to determine if the interaction is dependent on cell stimulation with HGF.

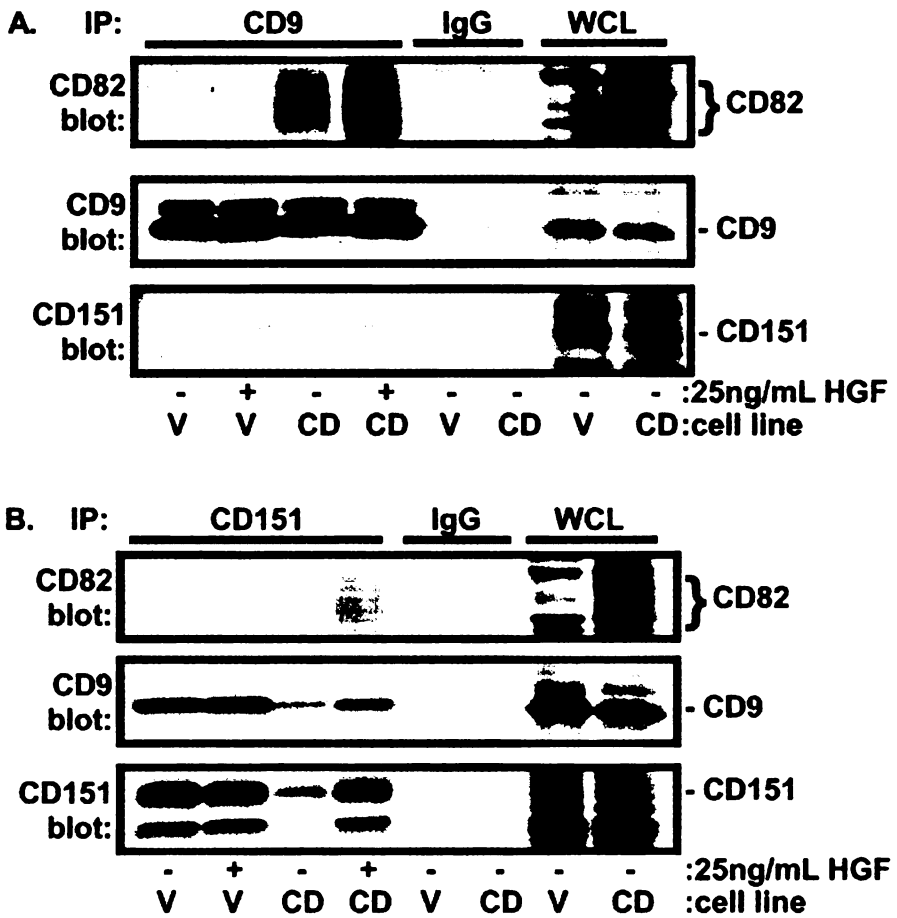
Discussion

These data suggest that in the absence of CD82, CD151 interacts with integrin α3, integrin α6, and Met within the tetraspanin enriched microdomain. However, while the co-immunoprecipitation data did not show an association between Met and CD9, I did detect a decrease in CD9-CD151 interactions in the presence of CD82. Therefore, I am assuming that CD9 may also exist within the Met complex in association with CD151. It is also possible that CD9 and CD151 form a complex that is separate from the Met and integrin complex. These data further suggest that when CD82 is present within the cell; CD82, CD151, and

integrin α6 associate within a complex and that CD9, CD151, and integrin α3 may form a separate complex.

Figure 12. In the presence of CD82, there is a reduction in the number of CD9 and CD151 associations. PC3 cells stably transfected with either empty vector (cl31) or CD82 (cl57) were serum starved for 48 hours and then stimulated with 25 ng/mL of HGF for 10 minutes. The cells were lysed in 1% CHAPS lysis buffer for 30 minutes. (**A**) Lysates were immunoprecipitated with anti-CD9 antibody, then immunoblotted with anti-CD82, anti-CD9, and anti-CD151 antibodies. (**B**) Lysates were immunoprecipitated with anti-CD151 antibodies, then immunoblotted with anti-CD82, anti-CD9, and anti-CD151 antibodies. Whole cell lysates (WCL) served as a positive control. Respective whole IgG molecules served as negative controls. *Susan Spotts, unpublished data*

Figure 12: In the presence of CD82, there is a reduction in the number of CD9 and CD151 associations.



However, the data still fails to show certain interactions that are needed to completely validate my working hypothesis. While integrin α6 did show an association with Met, I would have expected that in the presence of CD82 there would be a reduction in the amount of integrin α6 able to associate with Met. I would also assume that integrin α3 should be detected within the Met complex independent of the absence or presence of CD82. Some of these interactions may not have been captured because these microdomains are transient and dilute within the membrane making them hard to capture in a co-immunoprecipitation. The antibodies used for these experiments may also recognize the site in which Met and the integrins interact and therefore would not be able to bind or disrupt the binding.

Yamada *et al.* conducted a monoclonal antibody study to further understand the interaction between CD151 and integrin α3β1 [23]. They reported that the antibodies had distinct reactivities towards this particular tetraspanin-integrin complex and the antibodies were divided into three categories [23]. Group 1 antibodies were devoid of sufficient protein when integrin α3 was co-immunoprecipitated, but had increased affinity when unbound integrin α3 was immunoprecipitated [23]. This suggests that the epitope of bound integrin α3β1 may be blocked by the association with CD151 [23]. Based on this observation, I may not have been able to detect an association between integrin α3β1 and Met, because the epitope may have been blocked by association with Met.

Alternatively, my initial hypothesis may have been incorrect and based upon the data I have I would propose the following model that integrins α6β1 and α3β1, CD151, and Met form a complex in the absence of CD82. However in CD82-expressing cells, there is a partial shift of integrin α6β1 and CD151 from the Met complex into the tetraspanin-enriched microdomain with CD82.

Upon stimulation with HGF, I was able to detect a small increase in Met associating with integrin α6 in both the absence and presence of CD82. However, all other observable interactions seemed to be independent of HGF treatment. I expected to see an increase in integrin-Met associations with HGF stimulation because HGF aids in clustering Met for increased auto-phosphorylation of the receptor. While HGF increases Met clustering, it may also draw more integrins into the complex to further increase Met activation. However, for the most part I did not detect this.

I was careful to also include reverse co-immunoprecipitations to help validate the interactions; but in some cases, the associations were not detected in both immunoprecipitations. If an association can only be seen in one direction, I may have to conclude that the interaction is not real but an artifact of the co-immunoprecipitation conditions I used. However, the interaction may only be observed in one direction due to antibody epitopes being blocked by a particular association. Time courses may also be required to understand the kinetics of these interactions and how this regulates CD82's function.

CHAPTER 3: CONCLUSIONS AND PERSCPECTIVES

The overall objective of the project was to determine the molecular mechanism by which CD82 suppresses tumor metastasis. Based upon the literature, we had initially hypothesized that CD82 suppresses both HGF-induced Met activation and invasion by disrupting integrin α3β1-Met associations and drawing the integrins into the tetraspanin-enriched microdomain. The decrease in Met-integrin interactions would result in reduced Met activation which would lead to a decrease in downstream invasion. We also hypothesized that the specific constituents within the tetraspanin-enriched microdomain would be critical for CD82 function.

To determine which integrins and tetraspanins were required for CD82 suppression, we conducted Met activation studies on PC3 cells expressing CD82 or empty vector that were also transiently expressing either a specific siRNA or control siRNA. The results suggest that only CD9 is required for CD82 suppression of HGF-induced Met activation and that CD82 function is independent of integrins α3 and α6, and CD151. However, these data also suggest that integrin α3 and CD151 may contribute to Met activation in PC3 cells.

Once we had completed the Met activation studies, we wanted to assess the requirement of these proteins for CD82 suppression of invasion. Surprisingly, the invasion assay data was not entirely consistent with the Met activation studies. The data suggest that CD151 and integrin α3 and α6 are not required for CD82 suppression of invasion but may contribute to invasion of PC3

cells. We also observed that knockdown of either CD151 or integrin α3 in CD82-expresing cells had a further similar reduction in the number of cells able to invade the Matrigel. These data suggest that CD151 and integrin α3 may regulate invasion through the same pathway. Overall, the results seem to suggest that CD82 suppression of HGF-induced Met activation is an independent process of CD82 suppression of invasion.

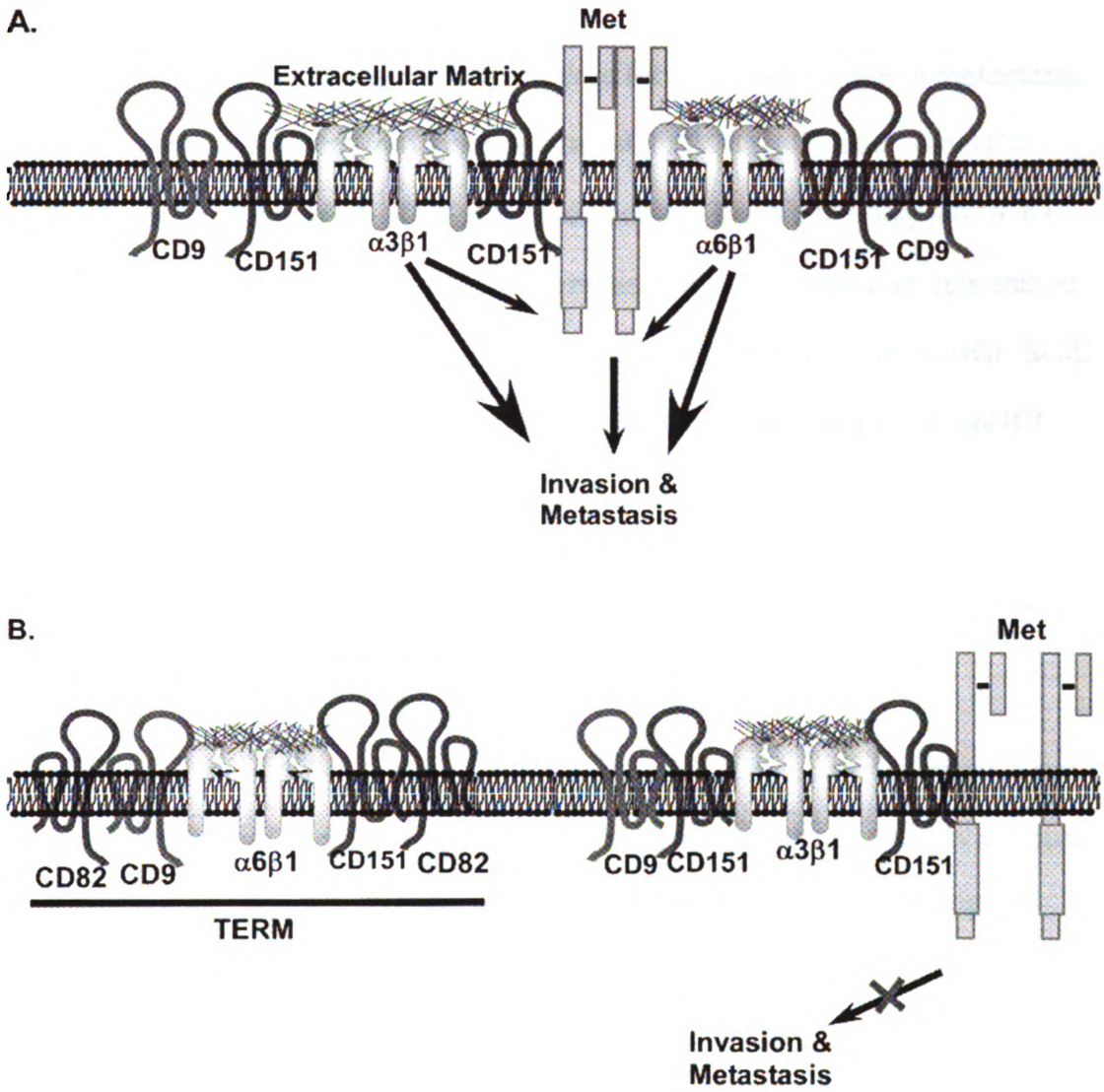
We sought to determine which integrins are present in the Met complex and how the presence of CD82 changes these interactions. These data suggest that in the absence of CD82, CD151 interacts with integrin α3, integrin α6, and Met within the tetraspanin enriched microdomain. However, while the co-immunoprecipitation data did not show an association between Met and CD9, I did detect a decrease in CD9-CD151 interactions in the presence of CD82. Therefore, I am assuming that CD9 may also exist within the Met complex in association with CD151; however, it may exist in a complex with CD151 that is separate from Met-integrin complexes. These data further suggest that when CD82 is present within the cell; CD82, CD151, and integrin α6 associate within a complex and that CD9, CD151, and integrin α3 may form a separate complex.

Our current hypotheses are that in the absence of CD82, integrins α3β1 and α6β1 are able to interact with Met via interactions with CD151 (Figure 13A). In the presence of CD82, CD82 interacts with CD9 and CD151 which draws both CD151 and integrin α6β1 into the tetraspanin-enriched microdomain (Figure 13B). With fewer integrins within the Met complex, Met is unable to reach full activation and signal downstream for invasion and metastasis.

CD82 has been also been shown to inhibit EGF receptor signaling by directly associating with CD82 and co-internalizing via the CD82's internalization motif and its "sorting motif" [24]. The increased rate of EGFR internalization results in decreased EGFR signaling and migration [24]. Yang *et al.* has shown that CD151 ablation in immortalized and metastatic breast cancer cell lines results in decreased migration, invasion, and signaling and disruption of integrin α6β4-EGFR associations [21]. Although this paper is taken in the context of breast cancer, this system could be applied to prostate cancer in which Met up-regulation results in increased metastatic potential.

Figure 13: Working model of molecular mechanism for CD82 suppression of Met activation. (**A**) In the absence of CD82, integrin α6β1 associates with Met and integrin α3β1 is able to interact with Met via interactions with CD151. (**B**) In the presence of CD82, CD82 interacts with CD9 and CD151 which draws both CD151 and integrin α6β1 into the tetraspanin-enriched microdomain. With fewer integrins within the Met complex, Met is unable to reach full activation and signal downstream for invasion and metastasis.

Figure 13. Working model of molecular mechanism for CD82 suppression of Met activation.



Future Work

The Miranti lab has *in vivo* data, demonstrating that CD82 suppresses metastases but not primary tumors in SCID mice expressing human HGF. To determine if human HGF (huHGF) is required for the development of metastasis, DU145 cells were injected into the prostates of 20 SCID mice and 20 huHGF SCID mice. Of these mice, 100% produced primary tumors but only the SCID mice expressing human HGF developed metastases. Once we had determined that human HGF is required for the development of metastases, 20 huHGF SCID mice were injected with DU145 cells expressing CD82 and another 20 huHGF SCID mice were injected with control DU145 cells. Of these mice, 100% developed primary tumors and only the mice injected with control Du145 cells developed metastases. These experiments provide *in vivo* evidence that CD82 suppresses HGF-dependent processes.

My thesis work has all been completed in cell culture and therefore is not a very good "*in vivo*" model. These experiments need to be repeated in a mouse model to provide further evidence for our model of the mechanism for CD82 suppression. PC3 cells stably expressing either CD82 or empty vector should be transfected with short hairpin RNAs that are specific for CD9, CD151, integrin α3, integrin α6, or a control sequence. PC3 clones which stably express both CD82 or empty vector and a specific shRNA should be placed into SCID mice expressing human HGF and monitored for the development of primary tumors and metastases. Based upon my results, I would expect that injection of control PC3 cells expressing shRNAs of CD151, integrin α3, or integrin α6 would result

in reduced size or number of primary tumors and metastases compared to mice injected with control PC3 cells expressing control shRNA. If this experiment were repeated with PC3 cells expressing CD82, I would expect there would be a reduction in primary tumors and metastases compared to PC3 cells expressing CD82 and control shRNA.

APPENDIX A: MATERIALS AND METHODS

Antibodies

The antibody against Met for immunoprecipitation (Met-D1) was obtained from Dr. Brian Cao at the monoclonal antibody core facility at the Van Andel Institute. The CD82 and CD9 antibodies used for immunoprecipitation and immunoblotting were purchased from Diaclone. Antibodies against CD151 and Met used for immunoblotting were purchased from Santa Cruz. The anti-phosphotyrosine monoclonal antibody 4G10 was purchased from Upstate. The CD151 antibody utilized in co-immunoprecipitations was purchased from Abcam. The integrin α3 used for immunoprecipitations and from immunoblotting was a generous gift from Dr. Christopher Stipp (University of Iowa, Iowa City, IA, USA). Integrin α6 antibody used for immunoblotting was a generous gift from Dr. Anne Cress (University of Arizona, Tucson, AZ, USA).

Cell Culture and Transfection

PC3 cells were obtained from the American Type Culture Collection (ATCC). PC3 cells were maintained in F12K medium (Invitrogen) supplemented with 10% fetal bovine serum (Gibco), 2mM glutamine, 50U of penicillin, and 50μg of streptomycin/ml. PC3 cells were transfected with a plasmid construct pcDNA3.1.CD82. PC3 cells at $2x10^6$ per 10-cm diameter plate were transfected with 5 μg of DNA using Lipofectamine 2000 lipid as described by the manufacture (Invitrogen). At 6 hours following transfection, the culture media

was changed to regular F12K medium with supplements and replaced 48 hours later with the same culture medium containing puromycin (2μg/ml). Puromycin-resistant clones were picked 10 days later and screened for CD82 expression by immunoblotting with TS82b antibody. CD82-expressing clones were maintained in growth medium containing puromycin and used in assays described below.

Immunoprecipitation and Immunoblotting

PC3 cells expressing either vector or CD82 were serum starved for 48 hours and then treated for 10 minutes with increasing concentrations of hepatocyte growth factor (HGF) at 37°C (Calbiochem and a generous gift from George Vande Woude). Cells were lysed in either 1X MAPK lysis buffer (50mM Tris pH 7.5, 100mM EDTA, 1% TritonX-100, 50mM NaF, 50mM β-glycerophosphate, 5mM sodium pyrophosphate, 1mM Na3VO4, 1mM PMSF, 100U/mL aprotinin, 10 ug/mL pepstatin, and 10 ug/mL leupeptin) or 1% CHAPS lysis buffer. Protein concentration of the samples was determined using either the bicinchoninic acid assay (Pierce) or Bradford assay (BioRad). Immunoprecipitation mixtures containing 500-2000μg of protein were incubated with the appropriate antibodies for 3h at 4°C with protein A-conjugated agarose beads (Pierce) to capture complexes. All immunoprecipitated complexes were washed three times with their respective lysis buffer. Immunoprecipitated samples were resuspended in 2X SDS sample buffer, boiled for 5-10 minutes. For immunoblotting of CD82 in whole cell lysates, 50μg of protein was resuspended in 2X SDS sample buffer under non-reducing conditions. All resuspended samples were then subjected to

SDS poly-acrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (PVDF). The PVDF membranes were blocked with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween20 (TBST) for 2 hours, followed by a 2 hour incubation with the appropriate primary antibodies. After several washes, blots were incubated with a horseradish peroxidase-conjugated secondary antibody (BioRad) for 1 hour and visualized with a chemiluminescence reagent. Blots were stripped in low-pH 2% SDS at 65°C for 60 minutes, rinsed, and reprobed for total levels of protein in the immunoprecipitates or cell lysates.

Invasion Assay

For invasion assays, the underside of the membrane of the upper chamber of matrigel invasion chambers (BD Biosciences) was coated with 10µg/ml of laminin (Invitrogen). Approximately 62,500 cells were serum starved for 48 hours and then added to the upper chamber. The cells were incubated at 37°C for 72 hours prior to the cells being washed, fixed, and stained with 0.09% crystal violet stain (Chemicon) for 20 minutes. Cells remaining on the upper side of the membrane were removed with a cotton swab. Stained cells that had invaded through to the underside were visualized on a Nikon TE300 microscope and pictures taken using a CCD camera (Hammamatsu) attached to an Apple Macintosh G5. Images were compiled using OpenLab software (Improvision). Pictures were collected from five different fields of each chamber and counted. Excess dye was removed from the exterior sides of each chamber with 70% ethanol. Crystal

violet stain was eluted from chambers using extraction buffer [25% Acetate Buffer pH4.5 (.122M Acetic Acid, .078M Sodium Acetate), 50% Reagent Agent, 25% DI water] and then read the OD at 570ηm.

Small Interfering RNA Transfections

Two unique small interfering RNA (siRNA) sequences targeting CD9 and a non-targeting control sequence were designed and purchased from IDT. (CD9 sequence 1: GAGCAUCUUCGAGCAAGAA; CD9 sequence 2: UGCUGUUCGGAUUUAACUU; Scramble sequence: ACUACCGUUGUUAUAGGUGTT) A pool of four siRNA against CD151, integrin α3, and integrin α6 were purchased from Dharmacon. Approximately $7.5x10^5$ cells were transfected with either 10nM (Integrin α3, Integrin α6, and CD151), 25nM (CD9 Sequence 1), or 50nM (CD9 Sequence 2) siRNA in F12K serum free media using siLentFect lipid reagent (Bio-Rad) and Opti-MEM (Invitrogen) media following manufacturer's directions. The media was changed 24 hours after transfection.

REFERENCES

1. Birchmeier, C., et al., *Met, metastasis, motility and more.* Nat Rev Mol Cell Biol, 2003. **4**(12): p. 915-925.

2. Dong, J.T., et al., *KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2.* Science, 1995. **268**(5212): p. 884-886.

3. Tonoli, H. and J.C. Barrett, *CD82 metastasis suppressor gene: a potential target for new therapeutics?* Trends in Molecular Medicine, 2005. **11**(12): p. 563-570.

4. Sridhar, S.C. and C.K. Miranti, *Tetraspanin KAI1//CD82 suppresses invasion by inhibiting integrin-dependent crosstalk with c-Met receptor and Src kinases.* Oncogene, 2005. **25**(16): p. 2367-2378.

5. *Cancer Facts & Figures 2006.* Atlanta (American Cancer Society), 2006.

6. Zoller, M., *Tetraspanins: push and pull in suppressing and promoting metastasis.* Nat Rev Cancer, 2009. **9**(1): p. 16.

7. Yauch, R.L., et al., *Direct extracellular contact between integrin alpha 3 beta 1 and TM4SF protein CD151.* J. Biol. Chem., 2000. **275**(13): p. 9230-9238.

8. Levy, S. and T. Shoham, *Protein-protein interactions in the tetraspanin web.* Physiology, 2005. **20**(4): p. 218-224.

9. Lazo, P.A., *Functional implications of tetraspanin proteins in cancer biology.* Cancer Science, 2007. **98**(11): p. 1666-1677.

10. Todeschini, A.R., et al., *Ganglioside GM2-tetraspanin CD82 complex inhibits Met and its cross-talk with integrins, providing a basis for control of cell motility through glycosynapse.* J. Biol. Chem., 2007. **282**(11): p. 8123-8133.

11. Miho Takahashi, T.S.M.A.K.I.K.S., *Regulation of c-Met signaling by the tetraspanin KAI-1/CD82 affects cancer cell migration.* International Journal of Cancer, 2007. **121**(9): p. 1919-1929.

12. Klosek, S.K., et al., *CD151 regulates HGF-stimulated morphogenesis of human breast cancer cells.* Biochemical and Biophysical Research Communications, 2009. **379**(4): p. 1097-1100.

13. Bari, R., et al., *Transmembrane interactions are needed for KAI1/CD82-mediated suppression of cancer invasion and metastasis.* Am J Pathol, 2009. **174**(2): p. 647-660.

14. Yang, X., et al., *Palmitoylation supports assembly and function of integrin-tetraspanin complexes.* J. Cell Biol., 2004. **167**(6): p. 1231-1240.

15. Berditchevski, F., et al., *Expression of the palmitoylation-deficient CD151 weakens the association of alpha 3 beta 1 integrin with the tetraspanin-enriched microdomains and affects integrin-dependent signaling.* J. Biol. Chem., 2002. **277**(40): p. 36991-37000.

16. He, B., et al., *Tetraspanin CD82 attenuates cellular morphogenesis through Ddown-regulating integrin alpha6-mediated cell adhesion.* J. Biol. Chem., 2005. **280**(5): p. 3346-3354.

17. Sharma, C., X.H. Yang, and M.E. Hemler, *DHHC2 affects palmitoylation, stability, and functions of tetraspanins CD9 and CD151.* Mol. Biol. Cell, 2008. **19**(8): p. 3415-3425.

18. Kinch, M.S.a.K.C.-K., *Overexpression and functional alterations of the EphA2 tyrosine kinase in cancer.* Clinical and Experimental Metastasis, 2003. **20**(1): p. 59-68.

19. Cheresh, H.J.D.a.D.A., *Role of integrins in cell invasion and migration.* Nature Reviews Cancer, 2002. **2**(2): p. 91-100.

20. Klosek, S.K., et al., *CD151 forms a functional complex with c-Met in human salivary gland cancer cells.* Biochemical and Biophysical Research Communications, 2005. **336**(2): p. 408-416.

21. Yang, X.H., et al., *CD151 accelerates breast cancer by regulating alpha6 integrin function, signaling, and molecular organization.* Cancer Res, 2008. **68**(9): p. 3204-3213.

22. Hemler, M.E., *Targeting of tetraspanin proteins -] potential benefits and strategies.* Nat Rev Drug Discov, 2008. **7**(9): p. 747-758.

23. Yamada M, T.Y., Sanzen N, Sato-Nishiuchi R, Hasegawa H, Ashman LK, Rubinstein E, Yanez-Mo M, Sanchez-Madrid F, Sekiguchi K, *Probing the interaction of tetraspanin CD151 with the integrin alpha 3 beta 1 using a panel of monoclonal antibodies with distinct reactivities toward CD151-integrin alpha 3 beta 1 complex.* Biochem J., 2008. **415**(3): p. 417-27.

24. Odintsova, E., T. Sugiura, and F. Berditchevski, *Attenuation of EGF receptor signaling by a metastasis suppressor, the tetraspanin CD82/KAI-1.* Current Biology, 2000. **10**(16): p. 1009-1012.

25. Shi, W., H. Fan, L. Shum, and R. Derynck, *The tetraspanin CD9 associates with transmembrane TGF-alpha and regulates TGF-alpha-induced EGF receptor activation and cell proliferation.* J Cell Biol, 2000. **148**(3): p. 591-602.

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