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Mechanisms of KAI1/CD82 - Induced Prostate Cancer Metastasis

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14. ABSTRACT Our basic understanding of how prostate cancer metastasis develops is limited. The recent identification of genes, whose expression suppresses metastasis but not growth in xenograft models, has provided a potential avenue for better understanding the metastatic process. The overall objective of this proposal is to determine how loss of the metastasis suppressor, KAI1/CD82, promotes the development of metastatic prostate cancer. Elevated expression of integrins a6b1 and a3b1 is highly correlative with the invasive and metastatic phenotype of prostate cancer. It has been proposed that migration of tumor cells on laminin-enriched nerve fibers via integrins facilitates prostate cancer spread. The metastasis suppressor KAI1/CD82 is known to associate with a6b1 and a3b1 integrin laminin receptors. We previously demonstrated that adhesion of metastatic prostate cancer cells to laminin induces activation of the metastasis associated receptor tyrosine kinase c-Met, and that re-expression of KAI1/CD82 suppresses both laminin- and HGF-induced c-Met activation. c-Met is up-regulated in all metastatic prostate cancers and is a physio

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INTRODUCTION

Prostate cancer is the most frequently diagnosed cancer and the third leading cause of cancer deaths in men in the United States (7). Death is due to invasion and metastasis beyond the prostate gland, primarily into the bone and occasionally to other peripheral organs. Our basic understanding of how prostate cancer metastasis develops is limited. The recent identification of genes, whose expression suppresses metastasis but not growth in xenograft models, has provided a potential avenue for better understanding the metastatic process (8). **The overall objective of this proposal is to determine how loss of the metastasis suppressor, KAI1/CD82, promotes the development of metastatic prostate cancer.**

Elevated expression of two integrins $\alpha6\beta1$ and $\alpha3\beta1$ is highly correlative with the invasive and metastatic phenotype of prostate cancer. It has been proposed that migration of tumor cells on laminin-enriched nerve fibers via $\alpha6\beta1$ and $\alpha3\beta1$ integrins facilitates prostate cancer spread (4). The metastasis suppressor KAI1/CD82 is known to associate with $\alpha6\beta1$ and $\alpha3\beta1$ integrin laminin receptors. We previously demonstrated that adhesion of metastatic prostate cancer cells to laminin induces activation of the metastasis associated receptor tyrosine kinase c-Met, and that re-expression of KAI1/CD82 suppresses both laminin- and HGF-induced c-Met activation (9). c-Met is up-regulated in all metastatic prostate cancers and is a physiological mediator of cell migration and invasion (6). **Thus we hypothesize that loss of CD82/KAI1 expression in primary prostate cancer results in enhanced activation of c-Met via both its ligand HGF and laminin integrins, which influences downstream signaling required to promote metastasis.**

BODY

Summary of Aim 1. The goal of aim 1 is to identify the mechanism by which CD82 regulates c-Met activity. Our *working hypothesis* is that CD82 negatively regulates c-Met activation through CD82-specific association with integrins and other tetraspanin molecules. Our **first task** is to determine the region on CD82 that interacts with integrins. To accomplish this we first needed to generate mutants of CD82 that we predict would no longer interact with integrins. We initially focused on the second extracellular domain of CD82, a region found in other tetraspanins to be important for a direct integrin interaction (13). In addition to a direct interaction, some tetraspanins interact with integrins via other tetraspanins when they form heterodimers. The formation of tetraspanin heterodimers depends on their palmitoylation (12). Thus we will also investigate this interaction.

As reported last year we were initially successful at generating a few stable PC3 cell lines expressing a C-terminally HA-tagged extracellular deletion and its wild-type tagged counterpart (Fig 1). Initially we were able to generate a couple of cell lines expressing the palmitoylation mutant, but soon discovered that this protein was not stable in these cells. We sequenced the construct that was provided to us from another lab (15) and discovered several mutations unrelated to the palmitoylation sites. We have regenerated this mutant in our own lab and validated its sequence. We are currently generating stable cell lines.

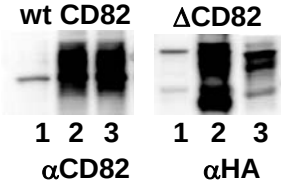


Figure 1: PC3 cells were stably transfected with wild type (wt CD82) or HA-tagged CD82 deletion mutant (ΔCD82). Levels of CD82 expression were monitored by immunoblotting with anti -CD82 (αCD82) or anti-HA antibodies (αHA). Lane 1: empty vector; Lanes 2 + 3: wt or ΔCD82 expressing cells.

The next step is to determine whether the CD82 mutants are still able to associate with $\alpha6\beta1$ and $\alpha3\beta1$ integrins or other tetraspanins that interact with integrins.

As a first step towards that goal we further validated the ability of wild type CD82 to specifically interact with α3 or α6 integrins and two other tetraspanins, CD9 and CD151 (Fig 2). We found that CD82 specifically associates with α6β1 in a tetraspanin complex containing CD151, but not CD9. Interestingly, CD9 seems to be displaced from the α6β1/CD151 complex in the presence of CD82 (Fig 2A). We failed to detect an association between CD82 and α3β1, although both CD9 and CD151 could associate with α3β1 (Fig 2B). Interestingly, the presence of CD82 diminished the amount of CD151 in the α3β1 complex (Fig 2B), in keeping with the strong association of CD151 with CD82 in α6 IPs (Fig 2A). Thus in CD82 expressing cells, there is an α6β1 integrin complex containing CD82 and CD151, and the complex between α3β1 and CD9 seems to be dissociated. Reciprocal IPs confirmed these associations, except that in CD9 IPs, there was a strong association with CD82, but no integrins (not shown). Activation of c-Met with HGF slightly increased the amount of CD9 in the CD151/integrin complexes in the absence of CD82, but had no effect in the CD82-expressing cells, in keeping with the idea that CD82 suppresses c-Met functions. Thus CD82 prevents HGF-induced changes in CD9 association with integrin/tetraspanin complexes. We then investigated whether c-Met was present in any of these complexes. c-Met was present only in the CD82/CD151/α6β1 complex (Fig 2A). Thus, CD82 seems to favor the formation of a complex between α6β1/CD151/c-Met, at the expense of breaking apart of complex between CD9/CD151/α3β1 integrin. CD82 also associates with CD9 outside of the CD151/α3β1 integrin complex. Because of our technical difficulties with the mutants we have not tested their effects on integrin/tetraspanin/c-Met complex formation yet.

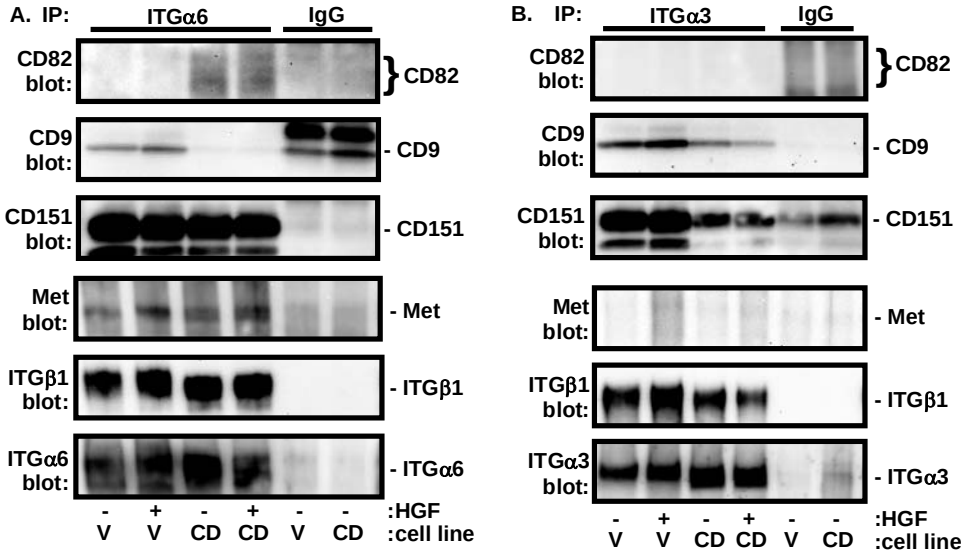
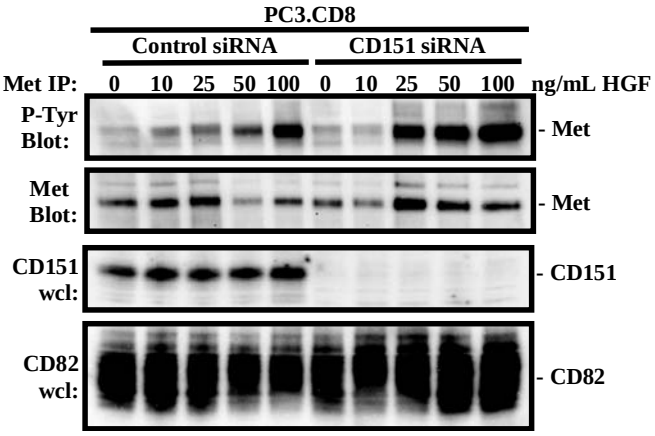


Figure 2: A) Integrin α6 (ITGα6) or **B)** integrin α3 (ITGα3) was immunoprecipitated (IP) from adherent vector-transfected (V) or CD82 expressing (CD) PC3 cells which were untreated (-) or treated (+) with 25ng/ml HGF. Immunoprecipitates (IP) were monitored for the presence of CD9, CD82, CD151, Met, α6, α3 and β1 integrin by immunoblotting. Mouse immunoglobulin (IgG) was used as a negative IP control.

Our **second task** is to identify the region on CD82 that is responsible for suppressing c-Met activity. The same mutants described and generated above will be tested for their ability to suppress c-Met activity. Thus far we have tested only the EC2 domain deletion mutant, and found that it failed to suppress c-Met activity as reported last year. We are waiting on the cell lines expressing the palmitoylation mutant so we can test their ability to suppress c-Met activity also. Thus, at the moment we can conclude that minimally the extracellular domain of CD82 is required for its ability to suppress c-Met activity.

Our **third task** is to determine if CD82 interaction with integrins is responsible for suppressing c-Met activity. Based on the immunoprecipitation data in figure 2, if the association of CD82 with CD151 and/or α6β1 is important for its ability to suppress c-Met activity, then loss of CD151 or α6β1 in the cell should prevent CD82 from suppressing c-Met. To test this hypothesis, we generated siRNA sequences specific for CD151 and CD9. Transfection of PC3



cells expressing wild type CD82 with CD151 or CD9 specific siRNA, but not scrambled siRNA restored c-Met activation (Fig 3 and 4). Thus, both tetraspanins are required for efficient suppression of c-Met.

Figure 3: PC3 cells expressing CD82 were transfected with CD151 siRNA or a scrambled (control) sequence and then stimulated with increasing concentrations of HGF. The level of c-Met activation was measured by immunoblotting of immunoprecipitates with anti-phosphotyrosine antibody (P-tyr Blot). The levels of CD9 and CD82 in the lysates (wcl) were monitored by immunoblotting.

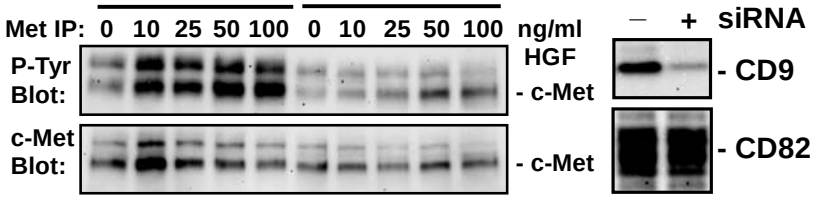


Figure 4: PC3 cells expressing CD82 were transfected with CD9 siRNA or a scrambled (scrm) sequence and then stimulated with increasing concentrations of HGF. The level of c-Met activation was measured by immunoblotting of immuno-precipitates with anti-phosphotyrosine antibody (P-tyr Blot). The levels of CD9 and CD82 were monitored by blotting.

While both CD9 and CD151 seem to be important for CD82 activity, their presence in different complexes suggests they may do so by different mechanisms. If integrins are important, then we would predict that the function of the CD151/α6β1/CD82 complex is to inhibit α6 activity, while dissociation of CD151 and CD9 from α3β1 inhibits its activity. To determine the extent to which integrins are important for CD28 suppression of c-Met activity, we blocked α3 or α6 integrin expression in CD82-expressing PC3 cells with siRNA. Loss of either α3 or α6 had no effect on the ability of CD82 to suppress c-Met activation (Fig 5A,B), nor did it affect how c-Met was activated in non-CD82 expressing cells (not shown). Thus, integrins are not required for c-Met activation, and CD82 suppresses c-Met activation independent of integrins.

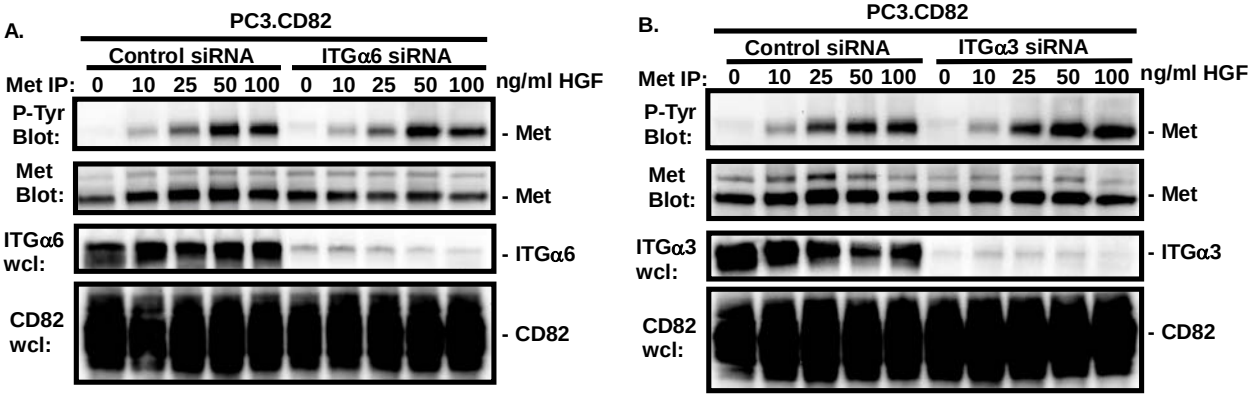


Figure 5: PC3 cells expressing CD82 were transfected with **A)** α6 siRNA (ITGα6), **B)** α3 siRNA (ITGα3), or a scrambled (control) sequence and then stimulated with increasing concentrations of HGF. The level of c-Met activation was measured by immunoblotting of immunoprecipitates with anti-phosphotyrosine antibody (P-tyr Blot). The levels of α3, α6 integrins and CD82 were monitored by blotting.

If CD9 and CD151 are required for CD82 to suppress c-Met activity, and CD82 suppresses invasion by blocking c-Met activity, then CD9 and CD151 should also be important

for CD82-induced suppression of invasion. To test this, CD9 or CD151 expression was down-regulated by siRNA in vector PC3 cells or PC3 cells expressing CD82 and the ability of the cells to invade Matrigel monitored. Similarly, the role of $\alpha 3$ or $\alpha 6$ integrin in Matrigel invasion was also measured. Loss of CD151, $\alpha 6\beta 1$ or $\alpha 3\beta 1$ all had a negative impact on cell invasion in normal PC3 cells, indicating these molecules all contribute to PC3 invasiveness. Furthermore, the ability of CD82 to suppress invasion was not reversed by loss of any of these molecules, but rather the loss of CD151 and $\alpha 3$ appeared to act cooperatively with CD82 to inhibit invasion. Loss of CD9 had no impact. These data suggest that the effect of CD82 on c-Met suppression mediated through CD151 and CD9 (see Fig 3 and 4) are not likely to be responsible for the effect of CD82 on invasion. The invasion data suggest that CD82 could either be working independently of CD151 and integrins to suppress invasion, or that is may partially inactivate their function by generating a CD82/CD151/$\alpha 6\beta 1$ complex and/or inactivating $\alpha 3\beta 1$. We will use the mutants to distinguish between these two possible models. If the mutants don't change the tetraspanin/integrin complexes, yet no longer suppress invasion or vice versa, then we can conclude that CD82 functions independently of tetraspanins/integrins to suppress invasion.

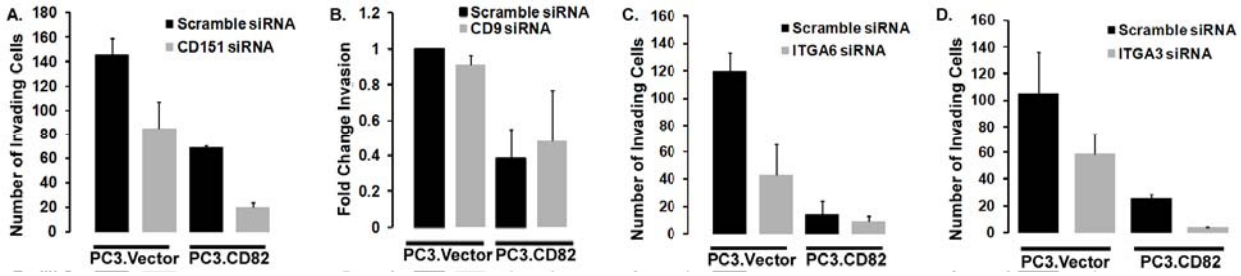


Figure 6: PC3 vector or PC3 cells expressing CD82 were transfected with **A)** CD151 or **B)** CD9, C) $\alpha 6$ integrin, or D) $\alpha 3$ integrin siRNA or a scrambled sequence and then allowed to invade Matrigel. The ability to invade through the Matrigel was quantified by counting the number of cells that successfully invaded through the Matrigel and appeared on the lower membrane.

In summary, we have found a disconnect between the mechanisms by which CD82 suppresses c-Met versus how it suppresses invasion. Tetraspanins CD151 and CD9, but not the integrins, are required for c-Met suppression. None are required to suppress invasion. On the other hand, CD151 and $\alpha 3\beta 1$ and $\alpha 6\beta 1$ all contribute to invasion and their loss cooperates with CD82 to suppress invasion. We have generated stable cell lines expressing a deletion mutant of CD82 and are working through the technical difficulties of generating the palmitoylation mutant. Once those are in hand we will be able to finish up the last set of experiments looking at the role of tetraspanins and integrins in CD82 suppression of c-Met activity and invasion.

Summary of Aim 2. The goal of aim 2 is to determine how loss of CD82 leads to metastatic prostate cancer. Our *working hypothesis* is that CD82 loss *in vivo* results in increased c-Met signaling, both of which are required for the development of metastatic disease. Our **first task** is to determine if CD82 expression inhibits metastasis in HGF transgenic mice. We have demonstrated that DU145 cells will only invade Matrigel in the presence of HGF and CD82 suppresses this HGF-dependent invasion (9), suggesting that inhibition of invasion is mediated by suppression of c-Met. We wished to test this in an *in vivo* xenograft model. Only human HGF will bind human c-Met, thus we have taken advantage of transgenic SCID mice which over express human HGF (14). In this model HGF interaction with c-Met on the metastatic prostate cancer cell line DU145 was predicted to induce HGF/c-Met-dependent metastasis. Indeed we found this to be the case. Orthotopic injection of metastatic DU145 cells failed to generate

metastases in normal SCID mice, while inducing metastasis in 60-95% of the HGF-SCID mice. CD82 expression suppressed metastasis, but not growth. Furthermore, while c-Met was present in the CD82-expressing tumors, it was not active, whereas it was active in the tumors not expressing CD82. These data were presented in last year's annual report. Thus HGF-/c-Met dependent metastasis of DU145 cells is inhibited by expression of CD82, and is accompanied by a loss in c-Met activation *in vivo*.

The next step is to test the mutants being generated in Aim 1 to determine if they still have the capacity to suppress metastasis and whether that is associated with a loss in c-Met activation. We have generated stable cell lines of DU145 expressing wild type and the CD82 deletion mutant (Fig 7). We are still in the process of generating DU145 cells expressing the palmitoylation mutant. Once generated, both mutants along with wild-type will be tested in the HGF-SCID mice for their ability to suppress metastasis.

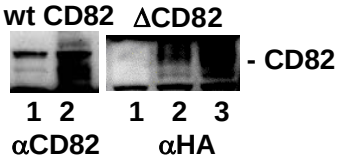


Figure 7: DU145 cells were stably transfected with wild type (wt CD82) or HA-tagged CD82 deletion mutant (ΔCD82). Levels of CD82 expression were monitored by immunoblotting with anti -CD82 (αCD82) or anti-HA antibodies (αHA). Lane 1: empty vector; Lanes 2 or 3: wt or ΔCD82 expressing cells.

Our **second task** is to determine if loss of CD82 expression in mice genetically manipulated to produce prostate tumors is required for the development of metastatic disease. We have generated floxed CD82 mice. CD82 floxed mice were crossed to CMV-CRE mice to generate a complete knock-out of CD82, or to Probasin-Cre mice to generate loss of CD82 only in prostate epithelial cells. RT-PCR and immunoblotting of either whole prostates or laser captured prostate epithelial cells indicate loss of the correct message and elimination of CD82 expression as reported last year. Loss of CD82 did not result in increased expression of other tetraspanins such as CD151 or CD9 (not shown). No tumors, no PIN, nor any consistent changes in the prostate epithelium was observed in the prostates of CD82 null mice. Thus, loss of CD82 in the prostate has no effect on normal prostate physiology.

The CD82/Probasin-Cre mice were crossed to Pten floxed mice to generate combined loss of both CD82 and Pten in the prostate. After analyzing 36 and 48 week old CD82-/-;Pten-/- mouse prostates, about half of which developed full adenocarcinoma; however, no metastases were observed. About the same number of mice developed tumors in the Pten conditional (Pten-/-) mice. Thus, loss of CD82 in this context had no effect on either tumor growth, number of tumors, or metastatic potential.

Genetic background can impact the outcome of tumor studies, and other tetraspanin mice develop distinctly different phenotypes dependent on background. Therefore, we have also backcrossed all three strains, $CD82^{-/-}$, Probasin-Cre, and $Pten^{flox/flox}$ mice, into both FVB/N and Balb/c backgrounds. The FVB/N backcrosses have recently been completed (assisted by speed congenic strain analysis), and the Balb/c backcrosses are still in progress. Crossing of the FVB/N strains together has begun. We currently have CD82-/-;$Pten^{flox/flox}$ mice in FVB, awaiting crossing into Probasin-Cre. In addition, we have also generated crosses of the CD82/Probasin-Cre mice into $APC^{flox/flox}$ mice, another strain that also develops only primary prostate cancer (3). As a third strategy we have also initiated crosses of CD82-/- mice to Pten+/- mice, to see if loss of CD82 in cell types other than those that express Probasin can be induced to metastasize. Finally, there remains the possibility that loss of more than one metastasis suppressor is required for metastasis. Therefore, in the future a major effort will be made to determine which other metastasis suppressor genes are absent in PC3/DU145 cells, determine which ones cooperate with CD82 to suppress invasion *in vitro*, and then to ultimately cross the CD82 mice to mice lacking one of the other metastasis suppressor genes.

While not directly relevant to prostate cancer per se, the CD82 mice are displaying some alterations in the functions of other organ systems. These phenotypes include increased ability

to initiate new blood vessel formation, increased numbers of platelets and enhanced clotting, increased urine/fecal water content output associated with microscopic changes in kidney architecture, and defects in TLR signaling in dendritic cells. The platelet and angiogenesis phenotypes are the exact opposite of those observed in CD151 null mice, suggesting a reciprocal phenotypic relationship between CD151 and CD82. Based on our *in vitro* studies, there is likely a connection between the functions of CD82 and CD151. This relationship needs to be investigated further to determine their relevance to metastasis.

KEY RESEARCH ACCOMPLISHMENTS

1. Generated stable transfectants of PC3 cells expressing a deletion mutant of CD82.
2. Demonstrated that the EC2 domain of CD82 is required for CD82-mediated suppression of c-Met activity.
3. Demonstrated that CD82 preferentially associates with a CD151/α6β1/c-Met complex and dissociates CD151 and CD9 from their association with α3β1. CD82 also independently associates with CD9.
4. Demonstrated that CD9 and CD151, but not integrins, are required for CD82-mediated suppression of c-Met activity.
5. None of these tetraspanins or integrins are required for CD82 suppression of invasion, but rather CD151, α3, and α6 loss cooperate with CD82 to suppress invasion.
6. Have generated DU145 cells expressing the deletion mutant of CD82.
7. Failed to generate metastatic lesions in mice in the context of Probasin-Cre/Pten -/- mice in a mixed background.
8. Generated FVB/N specific strains of CD82-/-, Probasin-Cre, and Pten$^{flox/flox}$ mice and initiated crosses between these strains.
9. Generated CD82/Probasin-Cre x APC$^{flox/flox}$ crosses and Pten-/+ x CD82 -/- crosses to investigate the effect of CD82 loss on metastasis in other backgrounds.

REPORTABLE OUTCOMES

The following items have been generated due to the research carried out in the last year.

1. Three posters were presented at scientific meetings. The abstracts are in the appendix.

Presented by Dr. Miranti:

Saari KM, Spotts S, Rajah G, Tesfay L, Schulz VS, and **Miranti CK** (2009) Mechanism of Metastasis Suppression by KAI1/CD82: Integrins, Tetraspanins, and Suppression of c-Met Activation. **Beatson International Cancer Conference: Microenvironment, Motility, and Metastasis**, Glasgow, Scotland, July 5-8.

Presented by Kristen Saari:

Saari KM, Edick MJ, Sian KR and **Miranti CK** (2009) Development of a total kai1/cd82 murine knockout. **8th Annual Symposium Michigan Prostate Research Colloquium**, Wayne State University, Detroit, MI, May 30.

Presented by Dr. Elly Park:

Park E and Miranti CK (2009) Tetraspanin KAI1/CD82 Regulation of Cell-Cell Adhesion: a Mechanism of Metastasis Suppression? **Gordon Research Conference: Cell Contact and Adhesion,** Waterville Valley, NH, June 28-July 3.

2. We have generated additional stable cell lines of PC3 cells and new DU145 cell lines expressing a deletion mutant of CD82. These will be useful for others interested in studying CD82 function.

3. We are the first lab to generate CD82-/-;Pten$^{flox/flox}$ in a homogeneous FVB/N background, and to generate CD82/Probasin-Cre;APC$^{flox/flox}$ and Pten+/-:CD82-/- strains. These strains will be immensely valuable in assessing the effects of genetic background on prostate cancer susceptibility and progression.

CONCLUSIONS

Prior to our studies the role of CD82 loss in regulating prostate tumor metastasis had not been determined. We have demonstrated that in tumor cells where c-Met expression is responsible for enhancing migration and invasion *in vitro*, re-expression of CD82 suppresses c-Met function. We have also shown this to be true *in vivo*. We generated a deletion mutant of CD82 that will allow us to access the relationship between CD82 loss and c-Met activation *in vitro* and *in vivo*. Our studies will also allow us to determine which of the many functions attributed to CD82 *in vitro* are required for its metastatic suppressive activity *in vivo*.

So What: Our findings have broad implications for the control of metastatic cancer. CD82 loss has been reported in many types of cancers. Likewise, c-Met over expression, mutation, or activation has also been reported for a wide range of cancers and its aberrant activity correlates with the development of metastasis (1, 2, 10). We propose that loss of CD82 may be required for the development of metastasis, by removing a control point for c-Met signaling. These studies will establish whether this is true in an *in vivo* setting. If this proves to be so, then the mouse models that we have generated in these studies will serve as excellent preclinical models for drug testing, and defining more precisely the molecular mechanisms involved.

Our studies will also advance the knowledge of how members of the tetraspanin family function. Many possible functions have been attributed to CD82, but it is not clear which ones are relevant to its metastasis suppressor functions. Interestingly, two other tetraspanins, CD151 and CO-029, appear to behave opposite to CD82, in that their levels of expression and activity are elevated in tumors (5, 11). Since tetraspanins are known to interact with each other, it is possible that loss of CD82 may act in part by enhancing the expression or activity of other tetraspanins to drive metastasis. Our studies will determine if this is a possible mechanism.

PERSONNEL SUPPORTED BY GRANT

Dr. Cynthia Miranti	20% effort
Kristen Saari	100% effort
Susan Spotts/Dr. Electa Park	50%/30% effort

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APPENDIX

Beatson International Cancer Conference: Microenvironment, Motility, and Metastasis, Glasgow, Scotland, July 5-8.

MECHANISM OF METASTASIS SUPPRESSION BY KAI1/CD82: INTEGRINS, TETRASPANINS, AND SUPPRESSION OF C-MET ACTIVATION
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The mechanism by which the metastasis suppressor gene, KAI1/CD82, suppresses metastasis remains unsolved. KAI1/CD82 is a member of the tetraspanin family, whose members work together to regulate integrins. Previous studies in our lab demonstrated that KAI1/CD82 suppresses the activity of the receptor tyrosine kinase c-Met, which is known to promote migration and invasion of tumor cells. Furthermore, expression of KAI1/CD82 is sufficient to completely suppress c-Met -dependent metastasis and activation in HGF-transgenic mice. The extent to which suppression of c-Met by KAI1/CD82 is regulated by tetraspanin-dependent interactions with integrins was investigated. The tetraspanin CD151 is known to bind directly to $\alpha3\beta1$ integrin and to promote integrin-dependent cell migration. CD151 also associates with other tetraspanins such as KAI1/CD82 and CD9. Thus, re-expression of CD82 in CD151-expressing migratory cells might negatively impact CD151. Therefore, our hypothesis was that interactions between CD151/$\alpha3\beta1$ and CD82 are required to suppress c-Met activity. We found that siRNA-mediated loss of tetraspanin CD9 and CD151 blocked the ability of CD82 to suppress c-Met activation, but not invasion. Loss of $\alpha3$ or $\alpha6$ integrin had no effect on the ability of CD82 to suppress c-Met. Loss of CD151, $\alpha3$, or $\alpha6$ cooperated with loss of CD82 to suppress invasion. In addition, the presence of CD82 in cells decreased the levels of CD9 and CD151 association with $\alpha3\beta1$, and promoted the association of CD151/$\alpha6\beta1$/c-Met. Thus, we propose that CD82-mediated suppression of c-Met requires CD151, which sequesters $\alpha6\beta1$ and c-Met, and dissociates $\alpha3\beta1$ from other tetraspanins and c-Met, to limit c-Met and integrin activation.

8th Annual Symposium Michigan Prostate Research Colloquium, Wayne State University, Detroit, MI, May 30.

DEVELOPMENT OF A TOTAL KAI1/CD82 MURINE KNOCKOUT
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KAI1/CD82, a nearly ubiquitous transmembrane tetraspanin protein in mammals, was first identified as a metastasis suppressor in a rat prostate cancer model and has since been shown to play the same role in many cancers and cell lines. Our study, which investigates the effects of CD82 elimination in normal cells, is the counterpart to most experiments, which reintroduce CD82 into invasive cancer cells where its expression has been lost. The objective of our project is to develop a total murine knockout, in which expression of CD82 is deleted in all tissues. Using the Cre/LoxP system, we targeted exons IV and V to be deleted and included a neo cassette for positive selection and a viral thymidine kinase cassette for negative selection of homologous recombinants. Our construct was transfected into embryonic stem cells by electroporation with the surviving clones being screened for the correct homologous

recombination by PCR, sequencing, and Southern Blotting. After two electroporations, two out of five positive homologous recombinants were injected into mice blastocysts, which in turn produced four male chimeric offspring. Two chimeras backcrossed to C57/BL6 wild type females went germline and produced 100% agouti mice. Male homozygous CD82 floxed mice crossed with female CMV-Cre mice produced a conventional CD82 total knockout mouse. We have successfully deleted Exon IV and V (DNA and mRNA), and cannot detect functional CD82 protein in the liver, kidney or prostate. There was not a compensatory increase in tetraspanins CD9, CD81 or CD151 in these tissues. The CD82 null mice are not embryonic lethal, show no breeding phenotype, nor have any other visual phenotypic defects. We are currently investigating the total CD82 knockout mice for any blood or blood vessel defects via a tail bleeding assay, Matrigel plug angiogenesis and oxygen-induced retinopathy. We are also interested in looking at CD82 knockout in the context of Pten or APC prostate-specific knockout for a potential metastasis model.

Gordon Research Conference: Cell Contact and Adhesion, Waterville Valley, NH, June 28-July 3.

TETRASPANIN KAI1/CD82 REGULATION OF CELL-CELL ADHESION: A MECHANISM OF METASTASIS SUPPRESSION?

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One of the molecular features of metastatic disease is the loss of cell-cell adhesion. Adhesion of cells to themselves, as well as to the tissue, prevents their dissociation and maintains tissue integrity. However, during metastasis, cells lose this tissue association and become free to move about the body where they can set up secondary tumors at distant sites. Classical cadherins, including E-cadherin (Epithelial cadherin), N-cadherin (Neuronal cadherin), and P-cadherin (Placental cadherin) are a family of transmembrane proteins that are critical for cell-cell adhesion. In cancers of epithelial origin, loss of E-cadherin mediated cell-cell contact due to alterations in cadherin function or expression effectively frees cells of their neighbors, potentially allowing migration out of the primary tumor. The tetraspanin protein KAI1/CD82 was first characterized as a metastasis suppressor in prostate cancer; loss of CD82 is observed in many different human cancers, including prostate, breast, colon, lung, ovarian, and pancreatic malignancies, where it correlates with poor prognosis. Re-expression of CD82 in metastatic tumor cells lines has been demonstrated to increase cell-cell adhesion of tumor cells. The mechanism by which CD82 enhances cell adhesion is unknown. Thus, one possibility is that loss of CD82 promotes the reduction in E-cadherin based cell-cell adhesion in metastatic tumor cells, which may explain how loss of CD82 promotes cancer metastasis. The prostate cancer cell lines PC-3 and DU145 were isolated from bone and brain metastases respectively, and model aggressive, invasive prostate cancer. While PC-3 cells have lost expression of E-cadherin and its complex partner α-catenin, they do express N-cadherin, and the non-classical, osteoblast-specific cadherin-11. DU145 cells retain some expression of E-cadherin, as well as its downstream binding partners, α- and β-catenin. We hypothesize that CD82 re-expression in metastatic prostate cancer cell lines will lead to increased N-and E-cadherin mediated cell-cell adhesion, resulting in decreased motility and invasion *in vitro* and decreased metastasis *in vivo*. To investigate this hypothesis, we will generate PC-3 and DU145 cell lines that stably express wild-type and mutant CD82 and examine the effect on cell-cell adhesion, adherens junction and actin organization, *in vitro* migration and invasion, and *in vivo* metastasis.

REPORT OF INVENTIONS AND SUBCONTRACTS

(Pursuant to "Patent Rights" Contract Clause) (See Instructions on back)

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The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing the burden, to the Department of Defense, Executive Services Directorate (9000-0095). Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

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1.a. NAME OF CONTRACTOR/SUBCONTRACTOR	c. CONTRACT NUMBER	2.a. NAME OF GOVERNMENT PRIME CONTRACTOR	c. CONTRACT NUMBER	3. TYPE OF REPORT (X one)
Van Andel Research Institute	Same as 2.c.	USAMRMC	W81XWH-08-1-0053	X a. INTERIM ☐ b. FINAL

b. ADDRESS (Include ZIP Code)	d. AWARD DATE (YYYYMMDD)	b. ADDRESS (Include ZIP Code)	d. AWARD DATE (YYYYMMDD)	4. REPORTING PERIOD (YYYYMMDD)
333 Bostwick Ave NE Grand Rapids, MI 49503	20080201	1077 Patchel Street Fort Detrick, MD 21702	20080201	a. FROM 20090201 b. TO 20100131

SECTION I - SUBJECT INVENTIONS

5. "SUBJECT INVENTIONS" REQUIRED TO BE REPORTED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME(S) OF INVENTOR(S) (Last, First, Middle Initial) a.	TITLE OF INVENTION(S) b.	DISCLOSURE NUMBER, PATENT APPLICATION SERIAL NUMBER OR PATENT NUMBER c.	ELECTION TO FILE PATENT APPLICATIONS (X) d.				CONFIRMATORY INSTRUMENT OR ASSIGNMENT FORWARDED TO CONTRACTING OFFICER (X) e.	
			(1) UNITED STATES		(2) FOREIGN			
			(a) YES	(b) NO	(a) YES	(b) NO	(a) YES	(b) NO
None								

f. EMPLOYER OF INVENTOR(S) NOT EMPLOYED BY CONTRACTOR/SUBCONTRACTOR		g. ELECTED FOREIGN COUNTRIES IN WHICH A PATENT APPLICATION WILL BE FILED	
(1) (a) NAME OF INVENTOR (Last, First, Middle Initial)	(2) (a) NAME OF INVENTOR (Last, First, Middle Initial)	(1) TITLE OF INVENTI...	(2) FOREIGN COUNTRIES OF PATENT APPLICATION
(b) NAME OF EMPLOYER	(b) NAME OF EMPLOYER		
(c) ADDRESS OF EMPLOYER (Include ZIP Code)	(c) ADDRESS OF EMPLOYER (Include ZIP Code)		

SECTION II - SUBCONTRACTS *(Containing a "Patent Rights" clause)*

6. SUBCONTRACTS AWARDED BY CONTRACTOR/SUBCONTRACTOR *(If "None," so state)*

NAME OF SUBCONTRACTOR(S) a.	ADDRESS (Include ZIP Code) b.	SUBCONTRACT NUMBER(S) c.	FAR "PATENT RIGHTS" d.		DESCRIPTION OF WORK TO BE PERFORMED UNDER SUBCONTRACT(S) e.	SUBCONTRACT DATES (YYYYMMDD) f.	
			(1) CLAUSE NUMBER	(2) DATE (YYYYMM)		(1) AWARD	(2) ESTIMATED COMPLETION
None							

SECTION III - CERTIFICATION

7. CERTIFICATION OF REPORT BY CONTRACTOR/SUBCONTRACTOR *(Not required if: (X as appropriate))* ☐ SMALL BUSINESS or ☐ NONPROFIT ORGANIZATION

I certify that the reporting party has procedures for prompt identification and timely disclosure of "Subject Inventions," that such procedures have been followed and that all "Subject Inventions" have been reported.

a. NAME OF AUTHORIZED CONTRACTOR/SUBCONTRACTOR OFFICIAL (Last, First, Middle Initial)	b. TITLE	c. SIGNATURE	d. DATE SIGNED