ROLE OF CD82 TETRASPANINS IN REGULATION OF EXTRACELLULAR SIGNALS
WITHIN THE TUMOR MICROENVIRONMENT DURING TRANSENDOTHelial
MIGRATION

A Dissertation in
Bioengineering
by
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ABSTRACT

Melanoma cancer arises in approximately 70,000 new patients each year and nearly 1 in 63 of patients will have in an invasive form of the cancer, which spreads to the heart and lungs (King 2006; Pennacchioli, Tosti et al. 2012). Once melanoma has spread to distant organs, the disease is increasingly difficult to treat and few therapeutic approaches exist to inhibit melanoma metastasis. An important event during cancer metastasis is tumor cell extravasation through blood vessels into neighboring tissues in which the cancer cell adheres to endothelial substrates, degrades and migrates through the underlying extracellular matrix, and then forms a new colony in the invaded tissue. Understanding the underlying mechanisms that facilitate these events will lead to the development of effective cancer therapies. The goal of this work is to investigate the underlying mechanisms by which cancer cells activate endothelial signaling networks to form gaps in endothelial substrates and facilitate the migration of cancer cells into the blood stream.

Endothelial gap formation is triggered by adhesion molecules (such as \( \alpha_4\beta_1 \) integrins) and inflammatory cytokines (such as interleukin (IL)-8, IL-6, and IL-1\( \beta \)) from the tumor microenvironment. The binding of these molecules to endothelial cells allows Rac GTPase to exchange a GTP molecule for GDP and subsequent activation of PAK and p38 mitogen activated protein kinases (MAPKs), which regulate events leading to gaps in endothelial cells. Gaps in the endothelium form through two primary mechanisms: (i) the dissociation of vascular endothelial (VE)-cadherin homodimers and (ii) contractility of endothelial cells involving the phosphorylation of myosin light chain and actin polymerization.
In normal physiology, duffy antigen receptors/groups (DARC) on endothelial cells DARC binds to chemokines, in particular, IL-8. DARC receptors on endothelial cells undergo transcytosis of IL-8 to the lumen surface where they are presented on the cell surface to direct recruitment and chemotaxis leukocytes through endothelial monolayers (Middleton, Patterson et al. 2002). During tumor induced inflammation, excess secretion of IL-8 within the tumor microenvironment disrupts homeostasis and IL-8 on endothelial surfaces recruits PMN to sites of melanoma cell extravasation. In melanoma cells, IL-8 induced effects promote adhesion of melanoma cells to endothelial cells, facilitating formation of gaps and transendothelial migration of melanoma cells. Similarly, overexpression of VLA-4 adhesion molecules on melanoma cell surfaces result in abnormal activation of signaling pathways in endothelial cells and an increase in invasiveness of melanoma cells.

The mutated form of B-Raf is found in nearly 50% of patients with malignant melanomas and is a known target for treating melanoma cancer (Flaherty, Robert et al. 2012). Results have shown that targeting mutant V600EB-Raf reduces melanoma metastasis through reductions in active melanoma cell extravasation through endothelial cells. Mechanistically, reduced melanoma extravasation following inhibition of mutant V600EB-Raf is due to the disruption of downstream NF-κB signaling, which inhibits IL-8 production. While previous data show knocking down V600EB-Raf decreases melanoma cell extravasation, our current data shows that inhibiting V600E or wild-type B-Raf genes in early stage melanoma cancers increases melanoma cell extravasation. B-Raf is upstream of many proteins in the cell membrane, specifically tetraspanins known to have varying functions.
Tetraspanins (including CD82) play an important function in regulating adhesion molecules (VLA-4) and secretion of cytokines (IL-8) from melanoma cells. CD82 tetraspanin is one tumor suppressor gene which is expressed in the early stages of melanoma cancer, but disappears as melanoma cancer increases invasive capabilities of melanoma cancer.

In this work we show that re-introducing CD82 expression in late stage melanoma cancer suppresses melanoma cell invasion through human blood vessels. We show that CD82 binding to DARC receptors interrupts IL-8 binding to endothelial substrates. While knocking down B-Raf in late stage melanoma cells suppresses metastasis, CD82 expression is still present in early stage melanoma cancers. As a result, inhibiting B-Raf (an upstream mediator of CD82) could result in a decrease in CD82 expression and an increase in melanoma cell invasion.

Furthermore, CD82 expression plays a role in suppressing IL-8 secretion and maturation of the $\beta_1$ subunit of VLA-4 integrins and their binding affinities. Previous work has shown that these two factors within the tumor microenvironment play a key role in cancer invasiveness. Consistent with previous work, we find that IL-8 secretion and VLA-4 binding affinities trigger signaling proteins (such as p38 and PAK) that play a key role in regulating VE-cadherin disassembly and contractility of endothelial cells.
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<td>ABTS</td>
<td>2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Albumin Serum</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>CS</td>
<td>Calf Serum</td>
</tr>
<tr>
<td>CAM</td>
<td>Chick Chorioallantoic Membrane assay</td>
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<tr>
<td>CXCR1</td>
<td>CXC chemokine receptor I</td>
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<tr>
<td>CXCR2</td>
<td>CXC chemokine receptor II</td>
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<tr>
<td>DARC</td>
<td>Duffy antigen/chemokine receptor group</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s Phosphate Buffer Saline</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>ELISA</td>
<td>Enzyme Linked Immunosorbent Assay</td>
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<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FRET</td>
<td>Fluorescence Resonance Energy Transfer</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GS</td>
<td>Goat Serum</td>
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<tr>
<td>HPMEC</td>
<td>Human Pulmonary Microvascular Cells</td>
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<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial cells</td>
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<tr>
<td>Gro-α</td>
<td>Growth related oncogene - α</td>
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<td>IL-8</td>
<td>Interleukin-8</td>
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<td>IL-1β</td>
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<td>JNK</td>
<td>c-Jun N-terminal Kinase</td>
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<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
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<td>MAPK</td>
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Chapter 1

INTRODUCTION

1.1 Cancer Invasion and Metastasis. Cancer arises from defects in genes leading to the unregulated cell growth and survival within the body. A healthy cell will grow and destroy itself if the cell becomes unhealthy, but cancer cells evade this process to divide uncontrollably. As cancer cells divide they will eventually form solid tumors.

Secondary lesions form when cancer cells migrate away from the primary tumor into the bloodstream where they travel to distant organs. Once melanoma cells metastasize to distant organs the survival of patients dramatically decreases from 95% to 15.7% over a period of 5 years (Pollack, Li et al. 2011). When solid tumors grow larger they undergo angiogenesis and may then supply the solid tumor with nutrients and intravasate into the normal vasculature to travel through the bloodstream to vital organs. While the primary solid tumors could be removed by surgery, detecting circulating melanoma cells that are traveling in blood vessels is increasingly difficult because there are approximately 0.0001% of circulating melanoma cells in whole blood (Ghossein, Scher et al. 1995). Once melanoma cells travel through the vasculature, the melanoma cells must extravasate through the endothelial barrier and degrade the underlying extracellular matrix to invade vital organs.

There are three important steps in the process of melanoma cell extravasation: (i) rolling, tethering, and firm adhesion of the tumor cell to the endothelial substrate (ii) the retraction of the endothelial substrate (iii) the migration and invasion into the tissues. Fig. 1-1 shows a general schematic of tumor extravasation through vessels and formation of secondary lesions.
While in vivo studies have been able to show changes in metastasis in tissues (lungs, heart, etc.), quantifying melanoma cell extravasation through vascular blood vessels remains a challenge. Previous studies have been able to label actin filaments and Rap1 to capture melanoma cell protrusion through a single capillary in the cremaster muscle using intravital microscopy (Freeman, McLeod et al. 2010). These images were able to show that inhibition of Rap1 in the protrusion of single melanoma cells through capillaries. Similar studies by Weis were able to show extravasation of a single cancer cell using electron microscopy after 4 hours (Weis, Cui et al. 2004); however, these methods does not allow real time quantification of melanoma cells that have extravasated through the vessel versus cells that have divided to form secondary metastatic lesions. This issue is further complicated by the dramatic differences in the

*Figure 1-1: Schematic of melanoma cell extravasation through blood vessels.* One important event during melanoma metastasis is transendothelial migration of melanoma cells from their primary growth site to distant organs. As shown, solid tumors are detectable compared to small, newly formed lesions at distant sites, warranting the need for a underlying understanding of the metastatic process.
size of mice and human vasculature that could dramatically alter these events in circulation.

Finally, the extremely low numbers of circulating tumor cells in the blood flow make it difficult to realistically assess whether inhibiting proteins such as Rap1 may alter melanoma cell extravasation. As a result, quantitative in vitro models characterizing transendothelial migration in response to cancer therapies are vital to understanding the underlying mechanisms involved in melanoma cell extravasation.

1.1.1 Genes in Cancer As shown in previously, mutations in genes induce inappropriate MAPK signaling increasing proliferation and migration of cancer cells. In melanoma cells, the most common mutations occur in the B-Raf gene ((Nazarian, Shi et al. 2010)). About 50% of patients with metastatic melanomas have been reported as expressing the mutant form of the B-Raf gene. While previous studies have found that B-Raf regulates tumor suppressor genes such as CD82 tetraspanins, how B-Raf can regulate changes in CD82 expression is not well known. Furthermore, the epigenetics that regulate changes in CD82 expression has not been investigated. For instance, B-Raf may modulate CD82 expression through functional modifications to the gene without changing the nucleotide sequence. Examples of epigenetic changes include DNA methylation and histone modification, both of which regulate gene expression without changing the underlying DNA sequence.

1.1.2 Tetraspanins in Melanoma Cancer Tetraspanins are membrane proteins that span the cell membrane 4 times and are expressed in all mammals and associate with integrins, cytosolic, and membrane bound proteins. Tetraspanins play a role in regulating a multitude of cellular functions including endocytosis, secretion of soluble proteins, and cell division (Zoller 2009). Specifically tetraspanins bind integrin subunits
and one another to form tetraspanins webs holding molecules in microdomains within the cell (Miranti 2009; Zoller 2009).

The association of CD82 molecules with integrins plays a role in maturation of $\beta_1$ subunit (Aplin and Juliano 1999; Jee, Lee et al. 2007), which stabilizes the adhesion molecule and regulates the spatial distribution of integrins on the cell surface during melanoma cell adhesion to endothelial receptors. While endothelial cells, peripheral nerves, cardiac tissue, platelets, and lymphocytes cells express tetraspanins ubiquitously, aggressive forms of cancer lose expression of tetraspanins molecules (including CD9, CD82, CD151, and tetraspanin 8) leading to inappropriate cell signaling as shown in Fig. 1-2 (Miranti 2009; Zoller 2009). Specifically, highly aggressive forms of melanoma cancer seem to lose expression of CD82 tetraspanins (Miranti 2009; Zoller 2009). Reintroducing CD82 expression into highly metastatic cancer cells allows binding of CD82 to DARC receptors expressed on endothelial cells suppressing cancer metastasis. While in vivo models have found that CD82 expression suppresses overall metastasis, the effects of CD82 expression on transendothelial migration in blood vessels has not been shown.
Inflammatory cytokines from the tumor microenvironment play a vital role in regulating changes in endothelial permeability (Khanna et-al., 2010). Previously, using a raybiotech blot, our studies showed that melanoma/endothelial contact co-cultures increase levels of IL-8, IL-6, IL-1β, and growth-regulated oncogene (Gro)-α (data not shown). Melanoma cells with high metastatic potential have been shown to secrete higher amounts of IL-8, specifically (Singh and Lokeshwar 2009). Further studies indicated that melanoma cells over expressing and secreting higher levels of IL-8 show an increase in penetration of the extracellular matrix modeled using matrigel substrates (Luca, Huang et al. 1997). These results were further supported by in vivo studies showing an overall decrease in tumorigenicity and metastasis when mice lacking CXCR2 (the receptor for IL-8) were injected with melanoma cells (Miranti 2009; Zoller 2009).

**Figure 1-2:** Association of tetraspanins with one another regulates intracellular signaling. Tetraspanins associate with one another and integrins to regulate intracellular signaling. Reintroducing CD82 expression in highly metastatic melanoma cells can suppress inappropriate activation of signaling molecules, which regulate melanoma metastasis. For instance, CD82 holds an inhibitor molecule in place, preventing Src and Met interactions (left panel). Without CD82 holding inhibitor molecule in place Src and Met interact inducing inappropriate cell signaling within melanoma cells (right panel).
The unusually high amounts of IL-8 secreted from melanoma cells were a result of mutations in the BRAF gene that regulate the transcription of IL-8 (Liang, Sharma et al. 2007). Interestingly, normal levels of IL-8 are regulated by their binding to DARC receptors expressed on endothelial cells, this homeostasis is disrupted when unusually high levels of IL-8 are secreted from melanoma cells leading to endothelial junction disassembly through activation of p21 activated kinases (PAK). While the role of CD82 in regulating integrin adhesion has been well-documented, the role of CD82 in regulating cytokine activity has not been well studied.

1.1.4 The Role of Integrins in Cancer Integrins and cadherins are adhesion molecules that function to bind cells to one another, the extracellular matrix or to surfaces (Aplin and Juliano 1999). Integrins function to bind melanoma cells firmly to endothelial substrates under flow conditions (Liang and Dong 2008) and modulate MAPK signaling of soluble mitogens through binding of the mitogen receptor, controlling the receptor expression, or binding mitogen ligands to localize the cell’s responses (Aplin and Juliano 1999). In melanoma cancers, blocking specific integrin molecules such as α4β1 (very late antigen (VLA)-4) have been shown to decrease the number of tumor foci from 130 colonies down to 17 colonies after 6 weeks (Garofalo, Chirivi et al. 1995; Aplin and Juliano 1999; Klemke, Weschenfelder et al. 2007). Furthermore, overexpression of VLA molecules on the cell surface has been linked to a dramatic increase in the levels of metastatic potentials in melanoma cells(Garofalo, Chirivi et al. 1995; Garmy-Susini, Avraamides et al. 2010). VLA-4 integrins have been found to bind vascular cell adhesion molecule (VCAM)-1 on endothelial cell surfaces and induce dissociation of cadherin junctions in endothelial cells(van Wetering, van den Berk et al. 2003). Since VCAM-1 receptors can have more than one ligand, further studies are needed to assess how
direct VLA-4/VCAM-1 binding can activate intracellular networks to induce endothelial gap formation.

While in the above section we describe cancer genes and the role of tetraspanins in regulating adhesion molecules and soluble proteins within the tumor microenvironment, the next section describes how these signals regulate endothelial cells and some of our own approaches/ key findings in this thesis.

1.2 Mechanisms of Endothelial Gap Formation. CD82 expression in melanoma cells regulates VLA-4 adhesion and cytokines in the tumor microenvironment both of which facilitate gap formation in endothelial cells (Fig. 1-3; (Khanna, Yunkunis et al. 2010). Formation of gaps in endothelial cells requires two important events (i) disassembly of VE-cadherin proteins localized to endothelial borders (Garofalo, Chirivi et al. 1995; Dejana, Bazzoni et al. 1999; Hendrix, Seftor et al. 2001); and (ii) contractile forces induced by changes in intracellular signaling proteins (Liu, Tan et al. 2010). Cadherins are transmembrane proteins that function to hold cells together and are dependent on Ca^{2+} ions that act as bridges to hold cadherin repeats together (Fig. 1-3).

There are 4 classes of cadherin molecules including classical, desmosomal, protocadherins, and unconventional (Harris and Nelson 2010). VE-cadherin is the classical form of cadherin junctions that undergo homophilic binding (meaning binding only to other VE-cadherin molecules) and do not bind to other types of adhesion molecules or ligands. Instead, VE-cadherin attaches to the actin cytoskeleton through its catenins (mainly, β-catenin, α-catenin, and p120) and disassociation of these molecules from the cytoplasmic tails promote VE-cadherin disassembly. The tight regulation of the spatial expression of VE-cadherin molecules plays a key role in the passage of soluble proteins, cells, and fluids through the endothelial barrier(Dejana, Bazzoni et al. 1999; Harris and Nelson 2010).
Studies by Harris et-al. have shown that VE-cadherin is a class of molecules on endothelial cells that not only facilitate transendothelial migration of melanoma cells across endothelial borders, but over time VE-cadherin plays a role in tumor angiogenesis (Hendrix, Seftor et al. 2001). While these studies show that downregulation of VE-cadherin expression reduces tumor metastasis, targeting VE-cadherin without disrupting normal endothelial function is not feasible. As a result, this work focuses on VE-cadherin’s role in regulating the endothelial barrier during the initial stages of tumor cell extravasation. Recent studies by Sluis et-al. and Weis et-al. have shown that although there are several classes of cadherin molecules, spatial expression of VE-cadherin is vital to barrier function. Sphingosine-1-phosphate receptors have been shown to enhance endothelial barrier function by regulating VE-cadherin expression.

Figure 1-3: Endothelial signaling during VLA-4/VCAM-1 adhesion has not been well studied. CD82 expression regulates VLA-4 integrin adhesion and secretion of cytokines such as IL-8 both of which activate signaling networks in endothelial cells to induce gap formation.
near endothelial borders. Recent studies show that using a direct agonist of sphingosine-1-phosphate receptors dramatically reduced cancer cell extravasation (Van Sluis, Niers et al. 2009). Similar studies have found that blocking VE-cadherin junctions using BV13 antibodies results in an increase in cancer cell extravasation through endothelial barriers (Weis, Cui et al. 2004).

While VE-cadherin junctions localized to endothelial borders have been termed as the “glue” (Dejana, Bazzoni et al. 1999) that hold endothelial cells in tact, the notion that contractile forces initiate the disassembly of VE-cadherin and play a predominate role in gap formation is remains unknown ((Gavard 2009).

Our current results show that the melanoma cell extravasation induces changes in the cell cytoskeleton during gap formation. Furthermore, our current studies show inhibiting proteins that directly regulate contractile forces (such as myosin light chain kinases) within cells dramatically reduce gap formation. To substantiate the hypothesis that contractile forces disassemble VE-cadherin junctions, direct forces exerted on endothelial junctions by the cells were measured in studies conducted by Liu et-al. In these studies the deflection of a microneedle placed near endothelial junctions was used to measure the displacement of endothelial substrates seeded on micropatterned surfaces. Using the displacement data over time and the known spring constant of the microneedle, the force exerted by endothelial cells was measured. Specifically these measurements show that the traction forces increased by nearly 4 nN as a result of microinjection of active Rho GTPases into the endothelial cells, but not inactive forms of Rho GTPases (Liu, Tan et al. 2010). Furthermore, Liu shows that the traction forces exerted on the sides of the cell tug on fingerlike projections of endothelial junctions and trigger changes in localization of VE-cadherin junctions (Fig. 1-4). Similar studies in real time have found that forces on endothelial substrates are prevalent in regions of cell
adhesion, which could activate focal adhesion kinase and Src proteins (Na, Collin et al. 2008), which respond to high mechanical forces. The forces near adhesion sites increased up to 5.5 nN/μm² (Riveline, Zamir et al. 2001). Proteins such as Src and FAK are localized to adhesion sites, but are activated independent of Rac GTPases (Poh, Na et al. 2009).

These studies show that forces are regulated by intracellular networks, which not only result in rearrangement of the cytoskeleton, but also regulate changes in the traction forces produced by endothelial cells and disassociation of VE-cadherin proteins from endothelial borders. Both soluble proteins and receptor/ligand binding events led to contraction of endothelial cells and breakdown of endothelial junctions (VE-cadherin

Figure 1-4: VE-cadherin disassembly is triggered by traction forces that are triggered by intracellular signaling. Studies show that microinjection of active Rho GTPases induced changes in traction forces produced by HUVECs (human umbilical vein endothelial cells) compared to wild-type Rho proteins (Liu et-al.).
junctons) to facilitate tumor extravasation. The goal of these studies was to show the roles of extracellular inputs on the tumor microenvironment on VE-cadherin disassembly.

1.2.1 Regulation of MAPK Proteins during Endothelial Gap Formation (Fig. 1-5)

MAPK signaling networks regulate disassembly of VE-cadherin junctions and changes in endothelial contractility. MAPK signaling proteins are part of a group of proteins that respond to mitogens, osmotic stress, inflammatory cytokines, and heat shock (Fig.1-5).
These signals are further modulated by signals from adhesion molecules such as integrins (Aplin and Juliano 1999).

MAPK signals are divided into 6 groups of proteins: p38 MAPKs, c-jun N-terminal kinases (JNKs), ERK1/2, ERK5, ERK3/4, and ERK7/8 (Hagan, Garcia et al. 2006). Specifically, MAPK pathways responding to stresses in the environment include p38 MAPK and JNK MAPKs, which respond to inflammatory cytokines and uv radiation (Denes, Jednakovits et al. 2002; van Wetering, van den Berk et al. 2003).

Receptor/ligand binding on the cell surface transduce signals to intracellular serine/threonine kinases (such as BRaf) or GTPases to phosphorylate MAPK proteins. Activation of p38 MAPK is dependent on activation of Rac GTPases and formation of reactive oxygen species (ROS), which regulate the changes in VE-cadherin (van Wetering, van den Berk et al. 2003; Khanna, Yunkunis et al. 2010). Downstream effectors of p38 MAPK such as MKK6 proteins regulate parallel signaling proteins that regulate the release of intracellular calcium (Andrews, Ho et al. 2003). Cardiac muscle cells transfected with active MKK6 expression results in prolonged increase in intracellular Ca\(^{2+}\) compared to cells transfected with GFP (Andrews, Ho et al. 2003).

Current in vitro methodologies have not been able to relate changes in adhesion with changes in protein kinase activation over time in endothelial cells. In addition, the complexity of inter and intracellular signaling between tumor and endothelial cells is complicated by secretion of soluble proteins into the extracellular environment (inflammatory cytokines IL-8, IL-1\(\beta\), growth factors, etc.). Complexities of intracellular signaling events in response to multiple extracellular signals within the tumor microenvironment warrant the need for combined computational and experimental methodologies that identify specific signaling proteins that play a key role in contractility.
or VE-cadherin disassembly. As a result we modeled endothelial networks using described methods.

1.2.2 Modeling Endothelial Networks

Network assembly and decomposition: Results from experimental data and literature were used to determine which signaling pathways significantly affected endothelial gap formation. Identified pathways were used to assemble the signaling map and connections (within a module or across modules). Parameterization of the full signaling map was a difficult and challenging computational task due to the large number of parameters to be estimated (~100). We addressed this problem by exploiting the modular organizations of the signaling network and decomposing the large network into stand-alone sub-modules (i.e., individual pathways) following the ideas from (Saez-Rodriguez, Kremling et al. 2004; Koh, Teong et al. 2006).

Translating the signaling map into kinetic expressions: The sub-modules obtained in Fig. 1-6 were translated into a set of ODEs as this framework has been widely used to describe the dynamics of these and other biological networks (Schoeberl, Eichler-Jonsson et al. 2002; Bhalla 2004). We assigned each process depicted in the signaling map with a kinetic expression to describe the rate at which it occurs. We assumed Michaelis-Menten kinetics for enzymatic reactions to reduce the number of species in the model (i.e., the complex substrate-enzyme is omitted). Some of the Michaelis-Menten kinetics were replaced with mass action kinetics of the individual steps of the enzymatic reaction. Subsequently, a conservation equation (i.e., ODE) for each pathway components was set up according to the signaling map. The set of ODEs for the mathematical model was used to describe the dynamics of the signaling pathways. To illustrate the procedure as an example, here we consider the cleavage of $\text{PIP}_2$ by the $\text{PLC} - G$ complex within the calcium pathway. The rate of formation and dissociation of
the complex $PLC - G - PIP_2$ and the rate of PIP$_2$ cleavage can be written as:

$$r_3 = k_3 C_{PLC-G} C_{PIP},$$

$$r_4 = k_4 C_{PLC-G-PIP},$$

$$r_5 = k_5 C_{PLC-G-PIP},$$

where $C_{PLC-G}$, $C_{PIP}$ and $C_{PLC-G-PIP}$ are the concentrations of $PLC - G$, $PIP_2$ and $PLC - G - PIP_2$ complex and $k_3$, $k_4$ and $k_5$ are the corresponding rate constants. The conservation equation for $PLC - G - PIP_2$ is:

$$\frac{dC_{PLC-G-PIP}}{dt} = r_3 - r_4 - r_5.$$

Following the same procedure for every reaction and species in the sub-module, the mathematical model can be written as:

$$\frac{dC}{dt} = S \cdot r(C, k),$$

where $C$ is the vector of the temporal concentrations of all the species included in the sub-module (e.g., $C_{PLC-G}$ is the concentration of $PLC - G$ at time $t$), $S$ is a matrix specifying the stoichiometry of the reactions, $r$ is the vector of reaction rates and $k$ is the vector of kinetic parameters (e.g., $k_3$, $k_4$, etc). The initial concentrations were defined by the moment of the onset stimulation (e.g., melanoma cells or soluble factors) of the endothelial cells and will be determined from experimental measurements.

**Determination of parameter values and ranges:** Initial parameter-value ranges and initial component concentrations were based on measurements using western blots or flow cytometry, or from the literature. Data from the literature was collected from databases and model repositories such as BioModels Database (Le Novere, Bornstein et al. 2006), DOQCS database (Sivakumaran, Hariharaputran et al. 2003) and CellML Model
Repository (Lloyd, Lawson et al. 2008). Preliminary western blots and flow cytometry were used to provide quantitative analysis of protein concentrations over time to estimate model parameters that cannot be found in literature.

**Global sensitivity analysis:** Once the initial parameter values were determined, we performed global sensitivity analysis to pinpoint the processes and the corresponding kinetic parameters that were most influential to the sub-module outputs for parameter estimation purposes (e.g., Ca\(^{2+}\), p38 phosphorylation, etc.).

We employed two methods, namely, steady state sensitivity analysis, (Cho, Shin et al. 2003) and Sobol’s method (Sobol 2001).

![Diagram](image)

**Figure 1-6:** Sub-modules for signaling networks including in the network model of ODEs. Several signaling networks were modeled to assess which proteins played a key role in VE-cadherin disassembly and/or contractility of endothelial cells.
For Sorbol’s method, a scoring function based on the deviation of the model prediction from the nominal values was evaluated for every parameter set. Then each parameter set was classified as acceptable or unacceptable by comparing its score with the average of all the parameter set scores. Subsequently, for every parameter, the cumulative frequency of each case for increasing values of the parameter was calculated. We used Kolmogorov-Smirnov (KS) statistics to compare the cumulative frequencies to contrast the sensitivity of all parameters. The Sobol’s method is a variance based method where the measure of the sensitivity of a parameter is given by the ratio of the model output variance caused by the parameter to the overall variance of the model output.

We had previously used multiparametric sensitivity analysis (MPSA) to analyze the Ca^{2+} signaling pathway (Fig. 1-6) (Peng 2007). In brief, the scoring function used was defined for the intracellular calcium concentration:

\[ f(k_p) = \sum_{i=1}^{n} (C_{Ca}(i,k_p) - C_{Ca}(i,k_0))^2, \]

where \( k_0 \) is the vector of nominal parameter values, \( k_p \) is the vector of parameters values sampled from the parameter space, \( C_{Ca}(i) \) is the intracellular calcium concentration at time point \( i \) and \( n \) is the number of time points. The scoring function was evaluated for different parameter sets \( k_p \). The cumulative frequency for cases where the scoring function is greater or less than the average is calculated for increasing values of each parameter. Finally the cumulative frequency distributions for each parameter were compared using their correlation coefficient. The smaller the correlation coefficient, the more influence the parameter has on the output of the sub-module. MPSA results for the Ca^{2+} signaling sub-module suggested that the binding of PIP_{2} by the complex PLC-G (\( k_1 \)) was the most determinant process for Ca^{2+} signaling.
(Peng 2007). This result was in agreement with experiment results that show that the PLC inhibitor U73122 inhibits endothelial Ca\(^{2+}\) response following contact with melanoma cells (Peng, Hodgson et al. 2005). The outcome of this step provided a ranked list of the targets for designing experiments to obtain time series data for parameter estimation.

In summary, several types of sensitivity analysis exist. For the purposes of validating our model against experimental data, changes in protein concentrations over time were plotted. Local sensitivity analysis would not be appropriate since the model is non-linear (large variations in time scales) between signaling pathways and local sensitivity analysis is limited to first order taylor series approximation. Here we have multiple levels of proteins all with different changes in protein concentrations at different times. In our model we tuned the model parameters to a set range and used a steady state sensitivity analysis to assess which proteins had biological significance in regulating gap formation. Here, we wanted to see which proteins influence gap formation so we used phosphorylation of VE-cadherin as one “measurable marker” of gap formation. The sensitivity of each MAPK protein would be measured as the ratio of the change in VE-cadherin phosphorylation divided by the change in MAPK protein phosphorylation.

Sub-modules integration and model validation: The individual sub-modules were integrated and the parameters corresponding to the interactions between modules (e.g., cross-talk) were estimated as described previously. The assembled model was validated against time series data from experiments in which multiple pathways were stimulated simultaneously. The performance of the model was analyzed and the discrepancies between model predictions and the experimental data were analyzed to determine if it was necessary to update the signaling map, to carry out
more experiments and/or re-estimate the parameters. Overall, we found that model simulations matched overall changes in p38 and PAK phosphorylation over time. That is, Rac mediated PAK phosphorylation decreased over time at a slower rate (up to 90 minutes) when activating VCAM-1 receptors while p38 phosphorylation kinetics were much faster with p38 phosphorylation reaching a peak after 10 minutes and decreasing rapidly over 45 and 90 minutes (Khanna, Yunkunis et al. 2010). Since the model has over 100 parameters, further experimental studies are needed to validate the model simulations of protein activation over time to realistically assess the robustness of the model.

1.3 Experimental Models for Studying Transendothelial migration

Cell Lines and their metastatic potentials were qualitatively assessed based on the listed references (Table 1-1).

<table>
<thead>
<tr>
<th>Cells</th>
<th>Metastatic potential</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A375M</td>
<td>+++</td>
<td>(Huh, Liang et al. 2010)</td>
</tr>
<tr>
<td>UACC 903M</td>
<td>+++</td>
<td>(Huh, Liang et al. 2010)</td>
</tr>
<tr>
<td>Lu1205</td>
<td>+++</td>
<td>(Sharma, Tran et al. 2006)</td>
</tr>
<tr>
<td>A2058</td>
<td>++</td>
<td>(Slattery, Liang et al. 2005)</td>
</tr>
<tr>
<td>WM35</td>
<td>-</td>
<td>(Okamoto, Liu et al. 2010)</td>
</tr>
<tr>
<td>SBC12</td>
<td>-</td>
<td>(Ryu, Kim et al. 2007)</td>
</tr>
<tr>
<td>WM793</td>
<td>-</td>
<td>(Ryu, Kim et al. 2007)</td>
</tr>
</tbody>
</table>

Flow migration assays for micrometastasis studies: As shown in Figure 1-1, in vivo studies show a limited understanding of the metastatic process and detecting micrometastasis in mice models remains a challenge. Furthermore, flow dynamics vary in capillaries, venules, and lymphatics due to the ranges of sizes in the vasculature. Exploring transendothelial migration in specific physiological regions of the body warrants the need for in vitro model to simulate these flow conditions. To create these
conditions, a flow migration chamber was created by Dong and Slattery et-al. (Fig. 1-7)
The flow migration model is a powerful tool that can be used to predict *in vivo* phenomenon as shown by numerous studies (Sharma, Tran et al. 2006; Liang, Sharma et al. 2007; Huh, Liang et al. 2010). It is composed of a top plate, a gasket, a polycarbonate filter (seeded with endothelial cells), and a bottom plate with wells for the chemoattractant. In our previous studies, collagen IV was used as a specific chemoattractant for melanoma cells (Hodgson et-al., 2003). The assembled apparatus is then fastened to a pulsatile pump through an inlet and outlet drilled on both ends of the flow migration chamber. This basic design allows the user to simulate flow conditions and detect migration of as little as 25 cells, which is useful in assessing the effects of inhibiting cancer genes on formation of micrometastasis once the cancer cell migrates away from its primary location. The flow through the flow channel is laminar flow and the shear stress is described by the following equation:

**Figure 1-7: Flow chamber apparatus.** Flow migration assays were used to detect tumor cell extravasation, *in vitro*. These results were validated using *in vivo* mouse models and study tumor cell extravasation, a key event during cancer metastasis.
\[ T_w = \frac{6Q\mu}{wh^2} \]

The flow through the chamber is dependent on the wall shear stress \((T_w)\), the volumetric flowrate \((Q)\), viscosity of the liquid \((\mu)\), width of the channel \((w)\), and height of the channel \((h)\).

**Vascular models for cancer invasion studies:** While flow migration studies allow us to study migration of melanoma cells through an endothelial monolayer, a blood vessel has an underlying extracellular matrix. To observe invasion of melanoma cells through human vasculature, normal human veins were obtained from discarded surgical tissue. Vessels were clamped on each end and melanoma cells were injected into the vessels. Injected venules were maintained in DMEM (Invitrogen) supplemented with 10% FBS (HyClone) and 1X Penicillin/Streptomycin (Invitrogen) for 11 days. Cross sections of vessels using confocal microscopy revealed invasion of melanoma cells up to 50 μm and provided a method of quantitatively assessing melanoma cell invasion over time.

**1.4 Novelty and Significance**

While previous *in vivo* studies have been able to show endpoint metastasis, a further understanding of the metastatic process is vital to develop new therapies to treat different stages of cancer. For instance, in early stages of melanoma cancer, high levels of CD82 expression are present and knocking down CD82 expression increases the invasive potential of melanoma cancer. As cancer progresses to later stages the CD82 expression is no longer detected due to the accumulation of genetic mutations. As a result, cancer therapies have to progress to target cancer as it progresses from early to later stages.

One of the important steps in melanoma metastasis at both early and late stages of melanoma cancer is the transendothelial migration of cancer cells from primary sites.
into the blood stream and lymphatics. CD82 is a tumor suppressor gene that has been shown to interact with endothelial receptors (duffy antigen group receptors (DARC)) to prevent transendothelial migration; however, the role of IL-8 in this process has not been investigated. Furthermore, while previous studies have shown that binding of CD82 to DARC receptors prevent melanoma metastasis; studies have not shown that these interactions play a major role in vascular invasion. Our present work has led to the development of a novel assay to observe and quantify the effects of CD82 expression on melanoma cancer invasion through real human blood vessels.

In this study we find that CD82 tetraspanin binds DARC receptors to prevent transendothelial migration of melanoma cells and prevent binding of IL-8 to these receptors. While IL-8 binding to DARC receptors has not been shown to induce changes in signaling pathways within endothelial cells, it has been shown that DARC receptors regulate presentation of chemokines such as IL-8 on endothelial cell surfaces. The recruitment of chemokines to the surface of the endothelial cells act as a chemoattractant to neutrophils and increase the infiltration of neutrophils to inflammatory sites (Zarbock et-al.), which may exacerbate the problem of melanoma cell extravasation through blood vessels.

CD82 has been shown to regulate integrin adhesion affinities by associating with β1 integrins (Gee et-al.). In our present work we found that IL-8 in the extracellular environment and overexpression of VLA-4 integrins on the melanoma cell each induce gap formation. The complexity of signaling networks in endothelial cells that respond to both soluble proteins and ligands on the tumor cell surface warranted the need for computational analysis to identify key intracellular proteins that regulate endothelial gap formation during tumor metastasis. Using matlab software, a network model was developed based on preliminary data and literary searches and grouped into several
sub-modules (Fig. 1-4). Several key proteins including PAK, Src, and p38 were identified as components that play a role regulating VE-cadherin junctions and contractility.

Consistent with previous studies, model simulations revealed that VCAM-1 mediated activation of p38 MAPK and PAK phosphorylation are both important during gap formation. Endothelial p38 MAPKs are activated specifically by Rac GTPases; however, Rac GTPases also regulate PAK by binding its p21 binding domain (PBD) to regulate endothelial contractility (van Wetering, van den Berk et al. 2003; Liu, Tan et al. 2010). While previous studies and model simulations have found that VCAM-1 can regulate these key proteins, the direct binding of VLA-4 to VCAM-1 on endothelial cells has not been shown to regulate these events using current experimental methods. Current in vitro assays are limited in that intracellular signaling events cannot be captured upon the exact time of melanoma-to-endothelial cell adhesion. In the current thesis work, we combine micropipette and FRET imaging to capture spatial and temporal changes in Rac and PAK interactions upon direct contact of melanoma cells with endothelial substrates. We present a novel approach that allows direct visualization of protein interactions that can be used to extract kinetic rates of protein dissociation, and which can be used to validate advanced models with spatial components and derive kinetic rates to be fed directly into the model. Furthermore, gas driven micropipette can be used to control the force of tumor and endothelial cell contact.

In summary, the thesis work presents several key findings and approaches that can be used to investigate melanoma cell extravasation on macroscopic and microscopic levels:

Key Findings and Hypotheses:
1. We find that inhibiting mutant and/or wild-type B-Raf expression in early stage melanoma cancers decreases CD82 expression through epigenetic changes such as
methylation of the CD82 gene leading to highly invasive phenotype of melanoma cancer. On the other hand, CD82 expression is no longer evident in late stages of cancer and thus targeting B-Raf in these cancers reduces their invasive capabilities. Since early stage melanoma cells have very low invasive characteristics, it is difficult to detect micrometastatic lesions using mice models. To address these issues, in vitro flow chamber experiments mimicking physiological flow were used to link changes in CD82 expression to melanoma cell extravasation. Results from flow migration show targeting B-Raf gene in early stage melanomas increases their invasive capabilities because of down-regulation of CD82 expression. This phenomenon is a plausible explanation as to why patients treated with the B-Raf inhibitor develop drug-resistance to these treatments.

2. In late stage melanoma cells, we find that re-expressing CD82 allows binding to endothelial DARC receptors, which prevents IL-8 binding to DARC. Furthermore, CD82 expression in these melanoma cells reduces IL-8 secretion. We found that expression of α and β subunit composing the structure of integrin molecules does not change when re-expressing CD82 in melanoma cells. However, since IL-8 has been shown to regulate calcium signaling and integrin affinities, our current hypothesis is that CD82 expression could indirectly reduce integrin binding affinities and endothelial signaling.

3. Previous studies have been controversial regarding the role of CD82 expression on cancer metastasis. Furthermore, current assays have not shown the role of CD82 expression in melanoma cell transendothelial migration. We use a novel assay to quantify invasion of melanoma cells through human blood vessels and show that CD82 expression in melanoma cells completely diminishes VE-cadherin disassembly possibly through suppression of IL-8 secretion. These results make CD82 an attractive target for reducing cancer metastasis.
4. While previous studies have found that stimulating VCAM-1 receptors on endothelial cells promote endothelial permeability, studies have not shown that direct binding of VLA-4 and VCAM-1 induce endothelial gap formation. Our current work shows that cells exclusively expressing VLA-4 integrins result in endothelial gap formation.

5. The role of VE-cadherin disassembly and endothelial contractility in gap formation is not well understood. While experimentally it is difficult to separate VE-cadherin junctions from the actin cytoskeleton, sensitivity analysis can be used to assess the importance of proteins regulating contractility, migration, or production of proteases in gap formation. In the current model, proteins identified by sensitivity analysis are those that specifically regulate cell contractility.

6. While current technologies using FRET can show changes in the spatial distribution of proteins, challenges remain in capturing temporal and spatial aspects of protein kinase activity upon the moment of adhesion between two cells. In this work we show a novel approach combining micropipette manipulation of melanoma cells with FRET imaging of endothelial cells seeded onto substrates. This approach allows one to control the physical collision force while monitoring spatial and temporal changes in intracellular kinase activity due to cell to cell adhesion.
Chapter 2

ROLE OF BRAF IN CANCER INVASION AND CD82 GENE EXPRESSION

Forward

The following chapter contains exerpts from the manuscript entitled “Targeted Drugs Promote Epigenetic Changes leading to more Aggressive Cancer” (Chung et-al., 2012). Experiments with CAM and western blots were done by ChinYing Chung. In vitro migration assays were done by Payal Khanna

2.1 Introduction

Tetraspanins attach to integrin subunits (α or β) and one another to form tetraspanins webs to hold biomolecules in microdomains within the cell as shown in Fig. 2-1(Miranti 2009; Zoller 2009).

Figure 2-1: Tetraspanins associate with other tetraspanins and integrins to regulate cell function. Studies have shown that tetraspanins associate with integrins and other molecules to regulate intracellular signaling. In melanoma cells binding of CD82 to endothelial receptors induce cell senescence (Zoller et-al.).
CD82, also known as KAI-1, is a member of the tetraspanin family of metastasis suppressor genes ((Dong, Lamb et al. 1995)). CD82 is localized on the cell membrane, interacting with other tetraspanins, integrins and chemokines inhibiting cell migration, adhesion and signaling (Hemler 2005). Decreased CD82 expression has been reported to have a role in development of a metastatic invasive phenotype and re-expression in cancer cells that have lost the protein can suppresses the invasive phenotype (Phillips, White et al. 1998; Hemler 2005; Bandyopadhyay, Zhan et al. 2006; Takeda, Hattori et al. 2007).

While CD82 can regulate the spatial distribution of signaling proteins within the cell membrane, mutations in serine/threonine kinase proteins such as B-Raf can induce MAPK signaling in the absence of extracellular signals (Liang, Sharma et al. 2007). As shown in chapter 1, intracellular proteins play an important role in transcription of genes that regulate expression of CD82 proteins within cells.

Activation of MAPK pathway through B-Raf mutation occurs in nearly 7% of human carcinomas with ~60% of melanomas having active B-Raf mutations(Pohl, Ho et al. 2005; Flaherty, Puzanov et al. 2010). B-Raf mutations are acquired through somatic, post-zygotic events, not inherited in families. The predominant mutation of B-Raf in 90% of melanoma tumors is a single-base missense T to A substitution (at nucleotide 1799), which changes valine to glutamic acid at codon 600 (V600E) in exon 15. Mutated V600EB-RAF is 10.7-fold more active than wild type protein due to a conformational change in protein structure, where glutamic acid acts as a phosphomimetic between the Thr598 and Ser601 phosphorylation sites (Davies, Bignell et al. 2002). A novel V600EB-RAF selective inhibitor, called PLX4032 or clinically known as Venmurfenib, has been developed and evaluated in patients having mutant protein with an ~80% partial or complete anti-tumor response rate during the first 2 month treatment cycle (Flaherty,
Puzanov et al. 2010). However, as observed with molecularly-targeted agents in other malignancies, tumors were initially responsive to PLX4032, had an average regression period of 2 to 18 months and 6.2 months progression-free survival (Flaherty, Puzanov et al. 2010; Rubinstein, Sznol et al. 2010). These patients developed drug resistance and invasive tumors after initial responses due in part to secondary B-Raf mutations, alternate pathways of MAPK reactivation (Emery, Vijayendran et al. 2009; Johannessen, Boehm et al. 2010; Nazarian, Shi et al. 2010), or activation of compensating alternative survival pathways (Shao and Aplin 2010; Paraiso, Xiang et al. 2011). All PLX4032 treated patients eventually relapsed after a period of progression-free survival with drug resistant invasive disease (Flaherty, Puzanov et al. 2010; Wagle, Emery et al. 2011). Similarly, treating wild type B-Raf melanoma cells with PLX4032 promoted growth and invasiveness leading to the development of more invasive disease (Nazarian, Shi et al. 2010). These observations underscore the plasticity of melanoma in acquiring resistance to targeted chemotherapeutic agents and need to identify the various mechanism cancer cells utilize leading to the development of resistant invasive disease in order to design better approaches to circumvent this process.

The mechanisms leading to the development of more invasive cancer as a consequence of PLX4032 treatment is an important question that remains to be completely unraveled. This study demonstrates that an acquired more invasive resistant phenotype can result from PLX4032 treatment as a result of increased methyltransferase activity leading to methylation of the invasion suppressor CD82 promoter. Decreased CD82 protein expression increased the invasive potential of melanoma cells leading to the development of more metastatic disease, which could be reversed using 5-aza-2'-deoxycytidine, a DNA demethylating agent (Drucker, Tohami et al. 2006).
2.2 Materials and Methods

2.2.1 Cell Lines and treatment conditions

Low passage cell lines established from early primary melanoma tumors at the radial (WM35 and SbCl2), vertical (WM1646, WM3248, WM98 and WM793) and metastatic (1206Lu, UACC 903M, A375M, C8161 cl.9 and MelJuSo) stages of growth were used in this study57. WM35, UACC 903M, and A375M cell lines have also been genetically engineered to express GFP making detection and quantitation within collagen, human vessels, and mice lungs possible using fluorescence microscopy. Metastatic melanoma cell lines, 1205Lu, UACC 903M, C8161 cl.9, MelJuSo and A375M, were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) (Chung et-al., 2011). Melanocytes (NHEM) and primary melanoma cell lines WM35, SbCl2, WM1646, WM3248, WM98, and WM793 were maintained in MCDB 153 medium containing 20% Leibovitz L-15 medium, 2% fetal bovine serum (FBS), 0.2% sodium bicarbonate, and 5 μg/mL insulin. In all cases, cells were grown at 37°C with 5% CO₂; (Chawla-Sarkar, Leaman et al. 2002; Elmore, Jain et al. 2007; Abel and Aplin 2010).

2.2.2 Allele Specific PCR (AS-PCR)

Melanoma tumors or cell lines genomic DNA (GDNA) was extracted using Qiagen DNeasy kit (Qiagen). Allele specific PCR for the detection of V600EB-Raf and PCR reaction positive control GADPH were performed using published primers (Pollock et-al., 2003). Primer sequences for the AS-PCR are listed below: V600EB-Raf forward: 5’-CTAAACTCTTCTATAATGCTTGCTC-3’, V600EB-Raf reverse: 5’GGACCCACTCCATCGAGATTTC-3’ yielding a 152bp product; GADPH forward: 5’-GAAATGTGCTTTGGGAGCA-3’ and GADPH reverse: 5’-TTTGCAGGGCTGAGTCAGCTTC-3’ yielding a 247bp product. The 50 μL PCR reactions contained 50 ng GDNA, 20pmol primers and 25μL
Qiagen Taq Master Mix. The following PCR conditions were performed: initial
denaturation at 95 °C for 5 min followed by 40 cycles of 94°C for 30 sec, 56°C for 1 min,
and 72°C for 1 min followed by a final extension 72°C for 5 min. PCR products were
separated on 2% agarose gel and visualized by ethidium bromide staining.

2.2.3 PLX4032 and siRNA targeting of V600EB-Raf or CD82

SiRNA (100 pmol) was introduced into 1.0X10^6 WM35, SbCl2, WM793 and 1205 Lu
cells via nucleofection by using an Amaza Nucleofector (Koeln) with Solution R/program
U-20(WM35), T-20(SbCl2), T-16(WM793) or K-17(1205Lu), respectively(Huh, Liang et
al. 2010). After siRNA introduction, cells were allowed to recover for 2 days and then
cell lysates harvested for Western blotting analysis. Duplexed Stealth siRNA (Invitrogen)
were used for these studies. The following siRNA sequences were used: V600EB-Raf:
5′-GGUCUAGCUACAGAAGCUCCGAU-3′; WtB-Raf: CD82: 5′-UCUCGAAGUGAGCU
CAGUCACGAG-3′; scrambled siRNA, 5′-AAUUCUCGCCAGUGGGAACGUGAGA-3′ (Sharma, Tran et al. 2006).

In vitro PLX4032 treatment and lysate collection for western blotting analysis: 1.5 × 10^6
melanoma cells WM35, SbCl2, WM793, WM1646, WM3248, WM98, MelJuSo or 1205
Lu were grown in culture dish for approximately 36 hours. Exponentially growing cells
were treated with 1.0 and 5.0 μmol/L PLX4032 in culture medium for 24 to 48 hours.
Total cell lysates were collected from DMSO vehicle control and PLX4032 treated plates.
Cells were washed twice with PBS and incubated with 100 μL protein lysis buffer for 30
min on ice. Lysate concentration in the samples was quantified using the bicinchoninic
acid (BCA) assay (Thermo Scientific) for Western blots analysis.

2.2.4 Western blotting

Western blots analysis was undertaken as described previously (Chung 2011). Primary
antibodies used at present study: DNMT1, SP1 and H3K9 from Upstate Inc. (Lake
Placid), B-Raf, CD82 and α-enolase, from Santa Cruz Biotechnology (Santa Cruz) and DNMT 3A, DNMT3B, total Erk1/2, pErk1/2(T202/Y204) from Cell Signaling Technologies. Secondary antibodies conjugated with horseradish peroxidase were obtained from Santa Cruz Biotechnology. Immunoblots were developed by using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Blots were normalized to α-enolase or ERK1/2 and quantified by using ImageJ software (Klapper, MacKay et al. 1992). Briefly, western blots were probed for proteins in an experimental case and then reprobed with a control to ensure equal loading. From a single band each lane was selected and the band intensity for each experimental (i.e. CD82, B-Raf, etc.) and control case (ERK1/2 and α-enolase) was represented as a bell shaped curve. The total area under the curve for each experimental and control case was calculated using Image J. The ratio of the area for each experimental to control case was calculated for each lane. These values are shown directly above each band in the results section.

2.2.5 Collagen invasion assays
Radial growth phase (RGP) melanoma WM35 tagged with GFP was used for CD82 function study using collagen cell invasion assay. Cell invasiveness was evaluated as detailed earlier. Briefly, 3.75 x10^5 human fibroblast primary cells FF2441 were suspended in 1.5ml type collagen on ice in 24-well culture plate. 1 x10^6 RGP cells were nucleofected with scramble, V600EB-Raf, CD82 siRNAs as mentioned above. After recovery for 48h, cells were suspended in 100 μl collagen and transferred into collagen-fibroblast mix using micro loading tip to form primary tumor nest. Fluorescence images were taken 6 days after primary tumor construction to measure cell invasiveness using Nikon SMZ 1500 fluorescent microscope (Nikon Instruments). To measure the area of melanoma tumor nodules expressing GFP, we used IPLab software (BD Biosciences) to
quantify migrated cells that escaped from the primary tumor nest. Scrambled siRNA nucleofection served as a negative control for CD82 siRNA nucleofected cells. Data from 3 independent experiments were pooled and analyzed. The experiment was performed in triplicate and repeated 3 three times to assure data consistency.

2.2.6 CAM invasion assays

1 X 10^6 WM35 GFP cells nucleofected with 100 pmol of scrambled or V600EB-Raf or CD82 siRNA were seeded onto chick chorioallantoic membrane (CAM) as described previously with slight modification (Zijlstra and Quigley 2006; Huh, Liang et al. 2010). Briefly, eggs were sterilized with 70% ethanol spray and kept at 37°C incubator with 60% humidity. To descend the CAM membrane, 2 ml of albumen was removed from the pointed end of each egg by using a 21 G syringe-needle on day 3. On day 5, a 1.0 × 1.0 cm square window was cut through the shell and sealed with transparent tape, and embryos were incubated to enable development of a full vasculature. On day 10, CAM membranes were scrapped gently using sterile cotton Q-tips before seeding tumor cells. 1x10^6 cells nucleofected with scrambled or V600EB-Raf siRNA in 100ul of culture media were seeded on scrapped wounds. 3 and 7 days after cell administration, CAM membranes were fixed by injecting 2 ml 4% paraformaldehyde into the eggs. The next day, the CAM membranes were harvested and GFP pictures were taken to observe cell dissemination. Each siRNA knock down condition was applied to 3 eggs and was repeated twice.

2.2.7 Flow chamber invasion assay

Flow invasion assay was measured in a modified 48-well chemotactic Boyden chamber consisting of a top and bottom plate separated by a gasket (Slattery, Liang et al. 2005). Before each experiment a monolayer of HUVEC cells was grown on sterile polyvinylpyrrolidone-free polycarbonate filters (8 μm pore size; NeuroProbe) pre-coated
with 2% gelatin diluted in PBS, for 2 hours; Sigma. The center 12 wells of the bottom plate were filled with soluble chemo-attractant type IV collagen (100 μg/mL in RPMI 1640/0.1% bovine serum albumin; BD Biosciences) and surrounding control wells were filled with medium (RPMI 1640/0.1% BSA). Studies have shown that melanoma cells migrate toward the collagen IV chemo-attractant. The apparatus was assembled by placing a filter on the bottom plate followed by addition of a sealing gasket and top plate. The chamber was primed with 37°C medium to eliminate bubbles from the system. For the migration assay, 2 x 10^6 melanoma cells were placed in the chamber under shear flow conditions (2 dyn/cm² or 0.625 dyn/cm²) for 4 hours in a 37°C incubator. The total number of cells migrated over 4 hours were counted by capturing 12 fields of view using a NIKON inverted microscope with a 10X objective lens and 1X magnification. Images were converted to high resolution tiffs using NIKON’s Imaging Software (NIS elements).

2.2.8 Methylation specific PCR (MSP) to detect CD82 promoter methylation

Genomic DNA (GDNA) from Melanoma tumors or cell lines was extracted using DNeasy (Qiagen). Bisulfite treated GDNA (BSDNA) conversion was performed using the EZDNA MethylDirect kit (Zymo research) following manufacturer’s instructions. MSP was performed with published primers specific to amplify methylated CD82 promoter (Drucker et-al., 2006). The MSP CD82 promoter primer sequence: CD82 methylated modified forward primer: 5′-ATAGAGGAGAGATTTGAGC-3′, CD82 methylated modified reverse primer: 5′-CCGAAACTCAATCACTCCTC-3′. The 50 μL MSP reactions contained 50 ng BSDNA, 20pmol primers and 25μL QiagenTaqMastermix. The MSP conditions consisted of initial denaturation at 95 °C for 5 min followed by 40 cycles of 94°C for 30 sec, 56°C for 1 min, and 72°C for 1 min followed by a final extension 72°C for 5 min. PCR products were separated on 2% agarose gel and visualized by ethidium bromide staining.
Epigenetic promoter regulation studies following 5-AzaC treatment:

5-Aza-2'-deoxycytidine (Sigma) was dissolved in H2O to make 1mM stock solution. The PLX4032 treated, V600EB-Raf siRNA nucleofected primary melanoma or metastatic melanoma cells were treated with 2μM 5-AzaC in culture medium for 72 h. Afterward, GDNA and protein lysates were harvested for further analysis.

2.2.9 Ectopic expression of CD82 constructs and cell line selection

5μg of pcDNA3.1 hemagglutinin-tagged CD82 or pcDNA3.1 vector control constructs was nucleofected into metastatic melanoma cells lacking CD82 protein expression using the Amaxa Nucleofector. Transfection efficiencies were determined to be ~40% by using pMaxGFP plasmid (Koeln) as a green fluorescent protein expression plasmid control. Following nucleofection, cells were plated in 100mm dish and grown to 60–70% confluence before selection in medium containing 500 ng/ml puromycin). ~75% cell death was observed 3 days after puromycin addition and cell were selected under these conditions for 1 month. Puromycin-resistant cells overexpressing CD82 were confirmed by western blotting.

2.2.10 Statistical analysis

Statistical analysis was undertaken using the one-way ANOVA. Results represent at least 3 independent experiments and are shown as averages ± SEM. Results were considered significant at P < 0.05.

2.3 Results

2.3.1 B-Raf Mutation status and invasiveness regulated by PLX4032

Prior to treatment with B-Raf inhibiting agents, melanoma cell lines isolated from tumors at the various stages of melanoma development were characterized by allele specific PCR for detection of V600EB-Raf. The majority contained the T to A substitution at
nucleotide 1799 with the exceptions of SbCl2, which was isolated from a radial growth melanoma as well as C8161.Cl9 and MelJuSo derived from metastatic melanomas (Fig. 2-2A) (Kath, Jambrosic et al. 1991).

Figure 2-2: Treating melanoma cells with B-Raf (V600E) inhibitors result in highly invasive phenotypes. A. Allele specific PCR shows that the B-Raf mutation (V600E) is expressed in several early and late stage melanoma cell types, resulting in uncontrolled MAPK signaling. B. Treatment of melanoma cells with the B-Raf inhibitor, PLX4032 results in a more invasive phenotype. However, combining PLX4032 with 5AzaC (a DNA demethylating agent) reduces in vitro melanoma cell extravasation. (PCR data was kindly provided by Chin-Ying Chung).
Representative cell lines containing mutant V600EB-Raf protein (WM793) or wild type B-Raf (SbCl2) were treated with PLX4032, (a well known V600EB-Raf specific pharmacological inhibitor), and the invasive potential was measured using a flow migration chamber model, in which invasion of melanoma cells across endothelial cells was measured under flow conditions (Slattery, Liang et al. 2005). Irrespective of B-Raf mutational status, PLX4032 treatment led to a 3-fold increase in the cellular invasive potential, which could be reversed by combining PLX4032 treatment with a DNA demethylating agent 5-AzaC. Reversion of the invasive phenotype using a DNA demethylating agent suggested that PLX4032 was epigenetically promoting an invasive phenotype (Fig. 2-2B).

2.3.2 PLX4032 or siRNA targeting V600E B-Raf leads to silencing of CD82 expression

To elucidate the mechanism by which genetic modifications promote the invasive phenotype following PLX4032 treatment, siRNA targeting wild type or V600EB-Raf was introduced into melanoma cells. Protein expression decreased consistently through day 8 leading to a decrease in expression of CD82 (Fig. 2-3A-B).

Decreased CD82 expression has been reported to have a role in development of a metastatic invasive phenotype and re-expression in cancer cells that have lost the protein can suppresses the invasive phenotype. To test this hypothesis, we used in vitro flow assays to measure cellular invasiveness using the flow migration assay (Fig. 2-3C).
Results were validated against collagen IV invasion assays and CAM assays, increases melanoma cell invasion when knocking down CD82 or B-Raf (Fig. 2-4A-B)

Figure 2-3: Silencing wild-type and mutant B-Raf reduces CD82 expression in primary melanoma cells. A. Results show that knocking down B-Raf using siRNA inhibits CD82 expression in WM793 cells after 8 days of nucleofection. B. Similar results were obtained for SbCl2 cells that express wild-type B-Raf. C. *In vitro* flow migration results show that knockdown of B-Raf and CD82 genes result in a dramatic increase in invasion of primary melanoma cells. (Western blot data was kindly provided by Chin-Ying Chung)

Results were validated against collagen IV invasion assays and CAM assays, increases melanoma cell invasion when knocking down CD82 or B-Raf (Fig. 2-4A-B)
2.3.3 Treatment of melanoma cells with 5-AzaC leads to expression of CD82 protein.

To confirm that CD82 promoter methylation can cause protein silencing in late stage melanoma cells, two cell lines derived from patients with advanced tumors that...
lacked detectable protein and were not exposed to B-Raf targeting agents, were treated with 5-AzaC to determine whether this demethylating agent would lead to protein expression (Fig. 2-5). 5-AzaC treatment for 48 to 72 hours led to CD82 expression at physiological levels similar to those in control WM793 cell in both cell lines suggesting that methylation of the promoter region was silencing gene expression. Deletion of the gene evidenced through loss of genetic material has also been reported to be a mechanism leading to loss of CD82 expression in tumors but epigenetic silencing also appears to play a significant role in this process (Drucker et-al., 2006; Kim et-al., 2009; Kim et-al., 2005).

Expression of CD82 protein mediated by 5-AzaC demethylation supports methylation as being an important mechanism silencing expression of this protein in melanoma cells even when cells were not exposed to agents targeting MAPK signaling.

Figure 2-5: Demethylation of CD82 promoter induces CD82 expression in late stage melanoma cells. While knocking down CD82 expression in early stages of melanoma cancer promotes invasive phenotypes, CD82 expression is not present in late stages of cancer. Treatment of these melanoma cells with the demethylating agent, 5aZaC restores CD82 expression comparable to WM3211. (Western blots provided by Chin-Ying Chung).
2.4 Discussion

Late stage melanoma patients have very few effective treatment options, which make the development and recent FDA approval of Vermurafenib a major breakthrough for individuals having $^{V600E}$B-Raf (Bollag, Hirth et al. 2010; Flaherty, Puzanov et al. 2010; Rubinstein, Sznol et al. 2010; Yang, Higgins et al. 2010). No other agent available to melanoma patients’ gives response rates of 80-90% but in all cases more invasive resistant disease eventually recurs (Bollag, Hirth et al. 2010; Flaherty, Puzanov et al. 2010; Solit and Sawyers 2010). For patients with wild type B-Raf, no drug is currently available. In fact, treatment of patients with wild type B-Raf promotes disease progression and disease invasion (Hatzivassiliou, Song et al. 2010). Since most of the patients treated with Vermurafenib develop resistant invasive disease, melanoma remains deadly (Flaherty, Puzanov et al. 2010). Mechanisms leading to Vermurafenib-mediated resistance need to be unraveled in order to develop more effective treatment approaches that limits melanoma cell invasion into vital organs and tissues.

This study shows that irrespective of mutational status, targeting B-Raf can enhance the activity of DNA methyltransferases. These events lead to promoter methylation and silencing of genes that aid the development of more invasive disease (Maddodi, Bhat et al. 2010). Use of siRNA targeting B-Raf or Vermurafenib led to CD82 promoter methylation, which decreased protein expression and promoted the development of invasive metastatic disease. Mechanistically, targeting B-Raf decreased levels of the MGMT/AGT inhibitor, which in turn increased DNA methyltransferase activity thereby causing CD82 promoter methylation to assist invasion through vessels and the development of metastases. CD82 acts as an inhibitor of EGFR signaling in several human cancers to prevent metastasis (Wang, Yan et al. 2007; Danglot,
Chaineau et al. 2010). Loss of CD82 can promote the development of more invasive disease through hyperactivation of EGFR signaling (Wang, Yan et al. 2007; Danglot, Chaineau et al. 2010). Vermurafenib resistant cell lines derived from melanoma patients have increased EGFR expression (Nazarian, Shi et al. 2010).

Epigenetic silencing of the CD82 promoter mediated by Vermurafenib could be reversed irrespective of B-Raf mutational status through concurrent treatment with the demethylating agent 5-AzaC (Drucker, Tohami et al. 2006). Restored CD82 protein expression, reduced cellular invasiveness and decreased lung metastasis development in mice. While siRNA-mediated targeting of B-Raf led to consistent increases in DNA methyltransferase activity in all cell lines irrespective of B-Raf mutational status, certain cell lines treated with Vermurafenib did not exhibit increased activity. For these cells, treatment with Vermurafenib instead promoted CD82 protein expression, likely due to off-target effects mediated by the drug, which occurred with Sorafenib (Chen, Kerkela et al. 2008; Whittaker, Kirk et al. 2010). Sorafenib is a non-specific Raf kinase inhibitor that has off-target effects decreasing the activity of VEGF receptor (VEGFR)-1, VEGFR2, VEGFR3, platelet-derived growth factor receptor-β, Flt-3, p38, c-Kit, and fibroblast growth factor receptor 1 VEGF, which gives the agent efficacy as a regulator of angiogenesis rather than an inhibitor of cellular proliferation (Madhunapantula and Robertson 2008).

The differential effect mediated by Vermurafenib on DNA methyltransferase activity could be used as a strategy to stratify patients who develop invasive resistant disease when treated with this drug based on the altered activity in the cells. Prior to treatment with Vermurafenib, characterization of patients’ DNA methyltransferase
response to the drug might be useful in selecting those whose activity does not increase, which a useful predictor of patients who would not develop aggressive invasive disease through this mechanism. For those in which methyltransferase activity does increase, concurrent treatment with 5-AzaC might be an approach to retard the effect on the promoters of genes such as CD82 and prevent the development of invasive recurrent disease through this mechanisms.

Increased DNA methylation might not only lead to CD82 methylation but could silence other genes such as PTEN, and concurrent treatment of Vermurafenib with 5-AzaC might be a solution to combat PTEN-mediated resistance (Mirmohammadsadegh, Marini et al. 2006). PTEN, is a phosphatase that act as a tumor suppressor by regulating the levels of the second messenger PIP$_3$ to modulate the activity of Akt3 in melanoma cells and thereby decrease cellular apoptosis (Stahl, Cheung et al. 2003). PTEN promoter methylation occurs in up to 62% patients with metastatic melanomas (Mirmohammadsadegh, Marini et al. 2006) and loss of activity has been reported to cooperate with $V^{600E}$-B-Raf in melanoma development (Dankort, Curley et al. 2009). Methylation mediated by Vermurafenib might enhance PTEN promoter methylation to activate Akt3 pathway signaling and prevent the activation of a normal cell’s apoptotic machinery (Stahl, Cheung et al. 2003; Mirmohammadsadegh, Marini et al. 2006). Increased Akt3 activity has been reported as a mechanism of developing Vermurafenib mediated resistance (Shao and Aplin 2010; Paraiso, Xiang et al. 2011). Combining 5-AzaC and Vermurafenib treatment might be used to restore PTEN protein expression to reactivate Akt3 survival pathways signaling to prevent this route of resistance development and make cell more sensitive to agents triggering cellular apoptosis.
(Mirmohammadsadegh, Marini et al. 2006; Dankort, Curley et al. 2009; Paraiso, Xiang et al. 2011).

Our discoveries suggest that epigenetic modulation of gene expression plays an important role activating compensating alternative survival pathways following Vermurafenib treatment. Therefore, combining Vermurafenib with demethylating agents such as 5-AzaC might retard epigenetic silencing of genes promoting resistance by this mechanism and extend progression-free patient survival.
Chapter 3

REINTRODUCING CD82 EXPRESSION IN LATE STAGE MELANOMA CELLS PREVENTS IL-8 MEDIATED ENDOTHELIAL GAP FORMATION

Forward

The following chapter was taken from the manuscript entitled “CD82/KAI expression Prevents IL-8 mediated endothelial gap formation” (Khanna et-al., 2012). Mice experiments were performed by ChinYing Chung.

3.1 Introduction

One important event during cancer metastasis is melanoma cell invasion through the endothelial vessels, which increases through mutations in the B-Raf gene in late stages of cancer. Western blot results show that patients with late stages of melanoma cancer have non-existent levels of CD82 protein expressed in the melanoma cells (Fig. 3-1). While we have shown that knocking down wild/type or mutant forms of the B-Raf gene inhibits CD82 expression and subsequent invasion in early stages of cancer, less is known about how CD82 effects adhesion molecules and soluble proteins within the tumor microenvironment during the later stages of cancer (Fig. 3-1).

Figure 3-1: Western blot analysis shows that CD82 expression disappears in 7 out of 8 patients with late stages of melanoma cancer. (Data by ChinYing Chung).
As discussed previously, tetraspanins are a family of membrane proteins present in all tissue types that play a vital role in a wide spectrum of biological functions. In cancers, tetraspanins (including CD82) play a vital role in cellular invasion, particularly during cell movement (Singethan and Schneider-Schaulies 2008; Zoller 2009). Studies have found that expression levels of CD82 tetraspanins decrease in highly metastatic cancers, increasing tumor cell extravasation. While the metastatic-suppressive effects of CD82 have been demonstrated in animal models; (Takaoka, Hinoda et al. 1998; Lombardi, Geradts et al. 1999; Zoller 2009), the molecular mechanism by which CD82 expression suppresses tumor cell extravasation remains unclear. Previous work has shown that CD82 binds to Duffy antigen receptor/chemokine group (DARC) and furthermore, that DARC expression in endothelial cells is necessary for CD82 mediated tumor suppression (Bandyopadhyay, Zhan et al. 2006). Binding of CD82 on tumor cells to DARC receptors on endothelial cells prevents invasion of CD82 positive tumor cells by inducing senescence in CD82+ cells. DARC also binds C-X-C and C-C cytokine receptors (including interleukin (IL)-8) secreted by highly metastatic melanoma cells (Bandyopadhyay, Zhan et al. 2006).

IL-8 has been shown to be an important of tumor extravasation through endothelial cells and underlying extracellular matrix (ECM) (Gabellini, Trisciuglio et al. 2009; Singh, Varney et al. 2009; Huh, Liang et al. 2010). IL-8 binds with high affinity to CXCR2 and DARC receptors (Peiper, Wang et al. 1995; Leong, Lowman et al. 1997). Studies in fibroblasts have shown that knocking down DARC diminishes cell senescence and increases tumor cell extravasation (Bandyopadhyay, Zhan et al. 2006; Zijlstra and Quigley 2006). In normal physiology, DARC on endothelial cells modulates IL-8 signaling by acting as a “chemokine sink” where DARC binds to chemokines, but does not activate downstream signaling pathways (Zarbock, Bishop et al. 2010). During tumor
induced inflammation, excess secretion of IL-8 within the tumor microenvironment disrupts this homeostasis. In melanoma cells, these effects are exacerbated by synergistic secretion of IL-8 from endothelial cells bound to melanoma cells, facilitating migration of melanoma cells through the blood vessels (Li, Dubey et al. 2003; Dong, Slattery et al. 2005; Liang, Sharma et al. 2007; Peng 2007; Mantovani, Allavena et al. 2008; Waugh and Wilson 2008). Upon IL-8 binding to CXCR2 receptors, signaling pathways such as phosphatidylinositol-3 kinase (PI3K) and/or p38 mitogen-activated protein kinase (MAPK) are activated. This signaling cascade inhibits tumor cell senescence and trigger internalization of VE-cadherin junctions in the endothelial cells forming gaps in endothelial monolayers (Dejana, Bazzoni et al. 1999; Murdoch, Monk et al. 1999; Criscuoli, Nguyen et al. 2005; Peng, Hodgson et al. 2005; Gavard, Hou et al. 2009; Khanna, Yunkunis et al. 2010). While several studies have focused on CD82 and DARC interactions, the role of CD82 in regulating IL-8 signaling remains unclear.

In melanoma cells, IL-8 binding to endothelial cells plays an important role regulating VE-cadherin disassembly via activation of CXCR2 receptors (Khanna, Yunkunis et al. 2010). However, previous literature shows that ectopic expression of CXCR2 in tumor cells results in tumor cell senescence (Acosta, O'Loghlen et al. 2008; Acosta and Gil 2009). These studies show that during oncogene-induced senescence there is an increase in IL-8 that binds CXCR2 receptors on tumor cells to reinforce tumor cell growth arrest and migration (Acosta, O'Loghlen et al. 2008). In cancer cells, cell senescence can be reversed by knocking down CXCR2 expression (Bandyopadhyay, Zhan et al. 2006; Acosta and Gil 2009). While several studies have found that CD82 binding DARC induces tumor cell senescence via a p21-dependent mechanism (Gartel, Serfas et al. 1996; Campisi 2001; Ishikawa 2003; Houben, Ortmann et al. 2009; Liu and
In the present work, we showed that ectopic CD82 expression suppresses IL-8 secretion and that binding of CD82 tetraspanins to DARC receptors interrupts IL-8 signaling in endothelial cells. The p21/Waf1 protein is a cyclin-dependent kinase (CDK) inhibitor, which binds active CDK2 or CDK4 complexes (Houben, Ortmann et al. 2009). Inhibition of CDK complexes prevents progression of cells through the G1 phase of the cell cycle resulting in growth arrest of cells or cellular senescence. Consistent with previous work, melanoma cells expressing physiological levels of CD82 bound DARC while inducing p21/Waf1 expression in melanoma cells. We extended previous findings to show that CD82 expression in highly invasive melanomas binds available DARC receptors on endothelial cells, interrupting IL-8 binding to the endothelial cell surface and subsequent intracellular signaling within endothelial cells. Furthermore, these binding events induce p21-induced cell senescence preventing melanoma cells with mutant BRAF (Roninson 2002) to migrate through blood vessels, *in vivo*. These results show that CD82 expression suppresses tumor effects not only via DARC binding, but via regulation of IL-8 secretion making CD82 an effective therapeutic target for late stage cancer metastasis.

3.2 Materials and Methods

3.2.1 Cell lines and culture conditions

HUVECs were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and were maintained in F12-K medium with 10% fetal bovine serum (FBS), 30 μg/ml of endothelial cell growth supplement, 50 μg/ml heparin (Mallinckrodt Baker, Inc), 100 units/ml of penicillin-streptomycin (Biofluids, Inc). A375M and UACC 903M were kindly provided by Dr. Gavin P. Robertson and cell lines were maintained in
Dulbecco’s modified eagle medium (DMEM) (Invitrogen, Carsbad, CA) supplemented with 10% FBS. WM35 and WM793 melanoma cells (provided by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA) were maintained in Roswell Memorial Park Institute (RPMI) supplemented with 10% FBS and 100 units/ml of penicillin-streptomycin. All cells were cultured in a humidified incubator at 37°C and 5% CO2.

3.2.2 Transfection of cDNAs and selection of stable clones

5μg of pcDNA3.1 hemagglutinin-tagged CD82 or pcDNA3.1 vector control constructs were nucleofected into A375M and UACC 903M melanoma cells. Transfection efficiencies were assessed using pMaxGFP plasmid (Koeln, Germany) as a green fluorescent protein expression plasmid control. Following nucleofection, the cells were plated in 100mm dish and grown to 60–70% confluency before selecting antibiotic resistant clones. Puromycin-resistant cells overexpressing CD82 were isolated and CD82 expression levels were confirmed using western blotting. Constitutively active CD82 levels in nucleofected A375M and UACC 903M melanoma cells were compared to endogenous CD82 expression in primary melanoma cell lines (WM35, WM793).

3.2.3 Small interfering RNA (siRNA) targeting CD82 and IL-8

SiRNA (100 pmol) was introduced into 1.0x10^6 A375M melanoma cells via nucleofection using an Amaxa Nucleofector using Solution R/program K-17 (15). Transfection efficiency was >95% with 80-90% cell viability (Huh et-al., 2010). Following siRNA introduction, cells were allowed to recover for 2 days in culture dishes before passaging cells into 96-well plates. Five days later, cell viability was measured using an MTS assay (Promega, Madison, WI) (Huh et-al., 2010). Duplexed Stealth siRNA (Invitrogen, Carlsbad, CA) were used for these studies. The following siRNA sequences were used. Scrambled: 5’-AAUUCUCCGAACGUGUCACGUGAGA-3’; IL-8#1: 5’ GCAGCUCUG
UGUGAAGGUGCAGUUU-3'; IL-8#2: 5’ CCAAGGAGUGCUA AAGAACUUAGAU-3’; and CD82: 5’- UCUCGAUUGAGCUCAGUCACGAUGC-3’.

3.2.4 Animal studies

Animal experimentation was performed according to protocols approved by the Institutional Animal Care at the Pennsylvania State University. Tumor formation was measured in athymic female nude mice purchased form Herlan Sprague Dawley (Indianapolis, IN). Briefly, 1.0x10⁶ A375M or UACC 903M melanoma cells nucleofected with empty vector (3.1 vector) or CD82 were collected in 0.2 ml of HBSS and then injected i.v. in 4-6 week old female mice. Mice were sacrificed 17 days after cell injection and the presence of fluorescent metastatic lesions was detected using a Nikon SMZ 1500 dissecting microscope. 15 images of random fields were photographed at a magnification of 40X from each lung and the number of fluorescent lesions counted. Each control or experimental group consisted of 6 mice.

3.2.5 In vitro flow migration assay

Tumor cell migration was measured using a modified 48-well chemotactic Boyden chamber consisting of a top and bottom plate separated by a gasket (Dong, Slattery et al. 2005). Before each experiment a monolayer of HUVECs were grown on sterile polyvinylpyrrolidone-free polycarbonate filters (8 μm pore size; NeuroProbe) precoated with 2% gelatin diluted in PBS, for 2 hours (Sigma Aldrich). The center 12 wells of bottom plate were filled with soluble chemoattractant type IV collagen (100 μg/ml diluted in RPMI 1640 with 0.1% bovine serum albumin (BSA); BD Biosciences) and surrounding control wells were filled with RPMI 1640 containing 0.1% BSA. Previous work has shown melanoma cells migrate toward collagen IV acting as a chemoattractant (Hodgson, Henderson et al. 2003). The apparatus was assembled by laying the filter on the bottom plate followed by a gasket and top plate. The chamber was primed with RPMI
1640 and 0.1% BSA to eliminate bubbles from the system. For the migration assay, 2 x 10^6 GFP labeled melanoma cells were placed in the chamber and subjected to a shear flow of 0.625 dyn/cm² for 4 hrs in a 37°C incubator. In some cases cells were used for gap analysis and Boyden chamber experiments simultaneously. In these set of experiments, we stained migrated cells with wright-geimson stain. The total number of cells migrated over 4 hours were counted by capturing 12 fields of view using a NIKON inverted microscope with a 10X objective lens and 1X magnification. Images were converted to high resolution tiffs using NIKON’s Imaging Software (NIS elements). Brightness of the GFP or brightfield images was adjusted until pores were barely visible and these images were converted to jpeg format. Software was used to divide small or large clumps of cells into even sections and each green or black dot was counted as one cell using Image J software.

3.2.6 In vivo imaging of human vessels

All patients gave informed consent. All experiments using human material were undertaken according to protocols approved by the Institutional Review Board Committee at The Pennsylvania State University. All procedures were performed according to protocols approved by the Penn State Human Subjects Protection Office. Human veins from human subjects were isolated from discarded patient surgery material remaining from routine surgical procedures and divided into 10 μm sections. Approximately 2 cm of human vessels were clamped at both ends before using 27 gauge needles were used to inject 1.0 X 10^6 A375M cells with stable pcDNA3.1 or CD82 expression. Human vessels containing GFP melanoma cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and Penicillin/Streptomycin (Invitrogen) for 11 days. Each section was stained using anti-human VE-cadherin antibodies (dilution 10:1000 in PBS/CS/GS solution containing
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phosphate buffer saline (PBS), 2% goat serum (GS), 5% calf serum (CS), and 0.3 triton-x). Sections were incubated in antibody overnight at 4°C and then washed twice with PBS. Sections were incubated using secondary 594 Alexa fluor goat anti-mouse antibodies (dilution 1:1000 in PBS/CS/GS) for 1 hour at room temperature before washing each section 2 times with PBS. Each 1 μm section of the vessels was captured ranging from the outside to towards the lumen of the vessel using an Olympus Fluoview 1000 confocal microscope with a PlanApo 60X/1.4 oil immersion lens and then reconstructed using 3-D Autoquant deconvolution software. The number of melanoma cells in each section of the reconstructed vessel was quantified and plotted using Sigma Plot software.

3.2.7 Fluorescence imaging and analysis

Prior to experiments, 25 mm coverslips were washed with PBS and then equal concentrations of HUVECs were grown to 95-99% confluency. HUVECs were co-cultured with melanoma cells for 45 minutes. Cells were then fixed with 5% formaldehyde in PBS for ten minutes. Cells were permeabilized with PBS/CS/GS solution for 20 minutes. Coverslips were incubated for 1 hour in primary VE-cadherin antibodies (10:1000 dilution in PBS/CS/GS solution) and washed twice with PBS. Finally, each of the coverslips was incubated with Alexa Fluor 488/520 goat anti-mouse IgG antibody (dilution of 1:1000). The coverslips were incubated at room temperature for 1 hour and rinsed with PBS three times before imaging using a NIKON TE-2000 microscope. For each experimental condition, one coverslip was viewed under a 40X objective and a series of six images were taken of randomized fields using an EZ coolsnap camera. Each image was then analyzed using Image J software version 1.32 (O'Neil, Mitchell et al. 1989)
3.2.8 Analysis of gaps and disruption of VE-cadherin

Disruption of VE-cadherin was identified by areas lacking green fluorescence at VE-cadherin junctions between HUVECs. Gap area within disrupted VE-cadherin junctions was determined from six images (Khanna, Yunkunis et al. 2010). Gap area was quantified as the ratio of pixels within all the gaps and the total number of pixels in one image (Khanna, Yunkunis et al. 2010). The average % of endothelial gaps was calculated from six images and plotted as a function of time.

3.2.9 Immunoprecipitation and western blots

For coimmunoprecipitation of CD82 and DARC, we mixed the CD82+ tumor cells (A375M or UACC melanoma cells) with HUVECs in the presence of the cell-impermeable cross-linker DTSSP for 30 min at 24°C. Whole-cell extracts were prepared by resuspending cells in 2 ml of lysis buffer (1% NP40, 10 mM Tris, pH 8.0, 150 mM NaCl, 3mM MgCl2, and 2 mM PMSF). Lysates were incubated on ice for 30 min followed by centrifugation at 16,000g for 5 min at 4°C. The pellet was discarded and the supernatant was immunoprecipitated with antibody to DARC in the presence of protein G agarose beads. After immunoprecipitation, bound proteins were analyzed by western blot using CD82 (1:1000), and IL-8 (1:1,000) antibodies. The lysate was mixed with 2 × SDS running buffer (0.2% bromophenol blue, 4% SDS, 100 mM Tris, 200 mM DTT, 20% glycerol) in a 1:1 ratio. 40 μl was loaded onto a 10% SDS-PAGE gel and proteins were transferred to 0.2 μm nitrocellulose membrane (Schleicher and Schuell, Keene, NH, USA) by electroblotting. Primary antibodies included anti-CD82 (mouse monoclonal IgG; Invitrogen, Carlsbad, CA, USA), anti-IL-8 (mouse monoclonal IgG; R and D Systems, Minneapolis, MN, USA), anti-β-actin IgG1 (Cell Signaling technologies), and anti-p21 (human polyclonal IgG; Santa Cruz Biotechnologies, Santa Cruz, CA, USA). Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG, goat anti-rabbit IgG.
Proteins were detected using the Enhanced Chemiluminescence Detection System (Amersham Pharmacia Biotech, Arlington Heights, IL, USA). Nitrocellulose membranes were subsequently stripped and reprobed using anti-β-actin as described above.

3.2.10 Enzyme-linked immunosorbent assay (ELISA)

Cell free media from 3.1 or CD82 expressing A375M, and UACC cultures was collected after 24-hours. Medium was spun at x430g and supernatants were extracted from cell debris. Supernatants were stored at -20 ºC until undertaking sandwich ELISA for individual cytokines was performed at the Pennsylvania State University General Clinical Research Center using standard protocols. Briefly, each 48 well plate was coated with the appropriate mouse anti-human capture antibody diluted in 0.1 M NaHCO₃ [pH 8.2] at a final concentration of 2 µg/ml (R and D Systems, Minneapolis, MN, USA). Plates were incubated overnight at 4 ºC. Next day, each plate was blocked for 2 hours at room temperature using PBS with 1% BSA. Samples and standards were added at 100 µl per well and incubated overnight at 4 ºC (R and D Systems, Minneapolis, MN, USA). Finally, wells were incubated for two hours at room temperature in detection antibody (concentration: 5 µg/ml) (R and D Systems, Minneapolis, MN, USA). Plates were read on a Packard spectacount plate reader at a wavelength of 405 to 415 nm.

3.2.11 Adhesion assay

HUVECs were seeded into 24-well plates and grown to confluence. A375M and UACC 903M melanoma cells were trypsinized and resuspended in DMEM with 10% FBS. Following recovery, 10³ melanoma cells were incubated with the confluent bottom cell layers in the presence or absence of antibody against CD82 or DARC antibody. After 15 minutes or 1 hour, wells were washed with DMEM 3 times and then the cells were incubated for 12 hours at 37°C. The number of GFP labeled melanoma cells attached to the confluent HUVEC monolayers were counted using a NIKON fluorescence microscope.
microscope and the percentage of attached cells was calculated. For each well, 8 fields was counted and the number of cells was averaged.

3.2.12 Statistical analysis

Statistical significance for multiple comparisons between groups was conducted using ANOVA followed by appropriate post hoc tests. For comparisons between two samples, t-tests were used. Results were considered significant at a p-value of <0.05.

3.3 Results

3.3.1 CD82 expression in highly metastatic melanoma cells reduces invasion into lung tissues. Levels of CD82 expression in A375M and UACC 903M melanoma cells was analyzed using western blots (Fig. 3-2). These levels were comparable to endogenous CD82 expression in primary melanoma cell lines (WM35 and WM793 melanoma cells). To test the hypothesis that CD82 mediates invasive properties and formation of metastatic lesions, GFP-tagged UACC 903M or A375M cells were injected via tail-vein into nude mice.
Mice were sacrificed two weeks later. Compared to vector transfected cells (3.1 vector), in vivo results showed that CD82 expressing tumor cells significantly decreased the appearance of metastatic lesions in the lungs of nude mice (Fig. 3-3A).

In vitro flow experiments showed that CD82 expression in A375M and UACC 903M melanoma cells decreased extravasation under low shear flow compared to the control cases (Fig. 3-3B) suggesting that CD82 expression decreased tumor cell extravasation. Since cells without CD82 expression migrate under low shear stresses (0.625 dyne/cm²) rather than high shear stresses (2 dyne/cm²), these results show that CD82 mediates the motility and subsequent extravasation of tumor cells rather than adhesion of tumor cells.

**Figure 3-2: CD82 expression in highly metastatic melanoma cells dramatically reduces their invasion into lung tissues.**

A. **Left:** Shows prominent lung lesions when A375M tumor cells transfected with empty (3.1) vectors are injected into mice compared to CD82 A375M cells (middle). **Right:** Western blot analysis shows inducible CD82 expression in A375M melanoma cells is comparable to primary melanoma cells (WM35 cells).

B. **Left:** Shows prominent lung lesions when UACC 903M tumor cells with empty (3.1) vectors are injected into mice compared to CD82 expressing UACC 903M cells (middle). Images are representative of 6 separate experiments. (40X). **Right:** Western blot analysis shows inducible CD82 expression in UACC 903M melanoma cells is comparable to primary melanoma cells (WM793 cells).
However, the role of CD82 in tumor cell extravasation remains unknown.

3.3.2 CD82 expression in highly metastatic lines dramatically reduces in vitro gap formation. Fluorescence microscopy shows continuous green lines where VE-cadherin junctions were intact (Fig. 3-4). CD82 expressing A375M cells co-cultured with HUVECs had negligible gap formation compared to control cases (3.1 vector) where gap formation occurs in regions of tumor cell adhesion. Areas of gap are indicated by disruption of the green lines for the endothelial junctions (Fig. 3-4). Since VE-cadherin
junctions serve as the primary mediators of endothelial permeability (Weis, Cui et al. 2004; Khanna, Yunkunis et al. 2010), we hypothesized that CD82 was able to regulate these endothelial cell junctions.

HUVECs were grown to 98-99% confluency on coverslips and co-cultured with A375M melanoma cells transfected with 3.1 vector or CD82 cDNAs. Quantification of the total gap area shows a significant reduction in total gap area and gap sizes in cells transfected with CD82 (Fig. 3-5A) compared to the control cases (3.1 vector) where total gap area and sizes of the gaps are much larger. CD82 expression prevented gap formation. Knocking down CD82 expression using siRNA increases gap formation (Fig. 3-5A). Gap formation is one important step during tumor cell extravasation (Brodland and Zitelli 1992; Luzzi, MacDonald et al. 1998; Pang, Zhao et al. 2011). We found that reintroducing CD82 expression into melanoma cells decreased tumor cell extravasation.

Figure 3-4: CD82 expression decreases gap formation and subsequent invasion of blood vessels in late stage melanoma cells. Bars, 15 μm. HUVECs (0.3 X106 cells) without A375M melanoma cells show intact VE-cadherin junctions (left). Disassembly of VE-cadherin junctions (FITC) after HUVECs were in direct contact with A375M tumor cells transfected with CD82 cDNAs (middle) is significantly lower compared with HUVECs co-cultured with control A375M cells (3.1 vector; right). Views captured under bright field, shows tumor cells coinciding with regions of gap formation (white arrows).
through HUVEC monolayers after 4 hours (Fig. 3-5B). These results show that CD82 is an important mediator of VE-cadherin mediated gap formation.

### 3.3.3 CD82 expression in highly metastatic lines dramatically reduces invasion of blood vessels

To assess whether CD82 affected tumor cell invasion through blood vessels, $1 \times 10^6$ GFP-A375M melanoma cells were nucleofected with either empty vector (3.1 vector) or CD82 cDNAs and injected into the lumen of human blood vessels (Fig. 3-6A-B), which were then placed in DMEM with 10% FBS for 3 days. Harvested vessels...
were fixed and stained for VE-cadherin (red regions). Figure 3-6B (left panels) shows the disruption of VE-cadherin and invasion of blood vessels up to 40 μm where GFP labeled A375M and UACC 903M melanoma cells made contact with the vessels.

**Figure 3-6: CD82 expression decreases invasion of blood vessels in late stage melanoma cells.**

**A.** Shows that A375M melanoma cells with inducible CD82 expression have little invasion of blood vessels about 10 μm from the lumen (right) compared to the control cases where there is an increase in invasion of blood vessels up to 20 and 30 μm (left).  

**B.** Shows that UACC 903M melanoma cells with CD82 expression, there is little invasion of blood vessels about 10 μm from the lumen (right) compared to the control cases where there is an increase in invasion of blood vessel walls and invasion of blood vessels up to 20 and 30 μm (left).

Control A375M and UACC 903M melanoma cells attached and invaded through the vessel and moved to regions near the outer walls of the vessel. (Fig. 3-6A-B, left panels). Consistent with in vitro observations, GFP-A375M and UACC903M melanoma cells with CD82 expression failed to invade deeply through human blood vessels (Fig. 3-6A-B, right panels).
Quantification of the number of A375M and UACC 903M melanoma cell invasion through the vessel lumen showed that GFP-A375M melanoma expressing CD82 failed to invade human vessels beyond 10 μm compared to the control cells where the cells invaded blood vessels up to 30 and 40 μm distances from the lumen (Fig. 3-7A-B).

**3.3.4 CD82 expression reduces secretion of IL-8.** In A375M and UACC 903M melanoma cells, secretion of IL-8 in 0.3 x 10^6 cells was measured. Control melanoma cells secreted ~120 pg/ml of IL-8 compared to melanoma cells with CD82 expression where IL-8 secretion decreased by approximately 50% (Fig. 3-8A). SiRNA targeting CD82 and IL-8 mRNA were introduced to A375M melanoma cells.
After 24-48 hours incubation, cells were lysed and proteins were separated using SDS page and cell supernatants were collected for ELISA analysis to assess knockout efficiency (Fig. 3-9A-B). Compared to the buffer and scrambled cases, cells treated with siRNA targeting CD82 or IL-8 in CD82+ A375M melanoma cells resulted in a 20% decrease in endothelial gap formation (Fig. 3-9C).

To support our hypothesis that CD82 mediates tumor cell extravasation, a Boyden chamber was used to measure tumor cell extravasation when knocking down IL-8 or CD82 expression. We showed that A375M melanoma cells nucleofected with CD82 or IL-8 siRNA decreased melanoma cell extravasation by 60% (Fig. 3-9C).

Figure 3-8: CD82 expression decreased IL-8 secretion from melanoma cells. A. IL-8 protein levels were higher in 0.3 x 10^6 A375M melanoma cells, but decreased when melanoma cells were transfected with CD82 cDNAs. Levels of secreted IL-8 protein were compared in control and CD82 expressing cells. Higher metastatic cells with CD82 expression showed decreased levels of IL-8 (left). (*p < 0.05 compared to control melanoma cells (3.1 vector)). B. Significantly lower levels of IL-8 tend to be secreted from metastatic cells transfected with CD82 cDNAs while in co-culture with HUVECs (right). (*p < 0.05 compared to control melanoma cells (3.1 vector)).
3.3.5 CD82 expression inhibits IL-8 binding to HUVECs through interaction with DARC receptors. CD82 expressing A375M melanoma cells adhered to confluent HUVECs after 1 hour, the number of melanoma cells bound to confluent HUVECs decreased when A375M melanoma cell and HUVEC co-cultures were incubated with either anti-CD82 or anti-DARC antibodies. To assess whether CD82 directly binds DARC receptors on endothelial cells we measured cell adhesion to confluent HUVEC monolayers after 15 minutes in the presence of CD82 or DARC antibodies.

Figure 3-9: CD82 expression decreased IL-8-induced gap formation and extravasation. A. To assess knockout efficiencies for IL-8 and CD82 siRNAs, whole cell lysates were assessed using western blot analysis. B. To assess knockout efficiencies of IL-8 siRNAs, cell supernatants were collected after 24-48 hours and IL-8 concentrations were measured using ELISA. C. Decreasing IL-8 secretion or CD82 expression in late stage melanoma cells (A375M) resulted in a dramatic decrease in gap formation compared to the control cases (left) and subsequent tumor cell extravasation (right). (*p < 0.05 compared to control melanoma cells (scramble or 3.1 vector)) Data represents mean ± SEM.
While CD82 expressing A375M or UACC903M melanoma cells remained bound to HUVECs after 15 minutes, the number of cells adhered to HUVECs decreased by nearly 50% in the presence CD82 or DARC antibodies (Fig. 3-10 and 3-11).

The attachment of GFP-A375M and GFP-UACC903M melanoma cells transfected with empty vector (3.1 vector) showed little change in attachment to HUVEC monolayers when incubated with CD82 or DARC antibodies for either 15 minutes or 1 hour (Fig. 3-10 and Fig. 3-11). These results show that antibodies are acting specifically to inhibit DARC and CD82 interactions both over short and long time periods. To assess whether IL-8 also binds DARC receptor in the presence of CD82, we immunoprecipitated DARC receptors with CD82 and IL-8 after co-culturing GFP-A375M or UACC 903M melanoma cells with HUVECs for 24 hours (in the presence of cross-linkers, DTSSP).

Figure 3-10: CD82 expressing cells bind DARC receptors. Higher levels of CD82 expression in A375M melanoma cells results in adhesion to DARC receptors on confluent endothelial monolayers, these adhesion events decrease by nearly 50% after incubating co-cultures with anti-DARC or anti-CD82 antibodies (left) while these antibodies have no effects on wild-type melanoma cells without CD82 expression (right). (*p < 0.05 compared to CD82 cases). Data represents mean ± SEM.
These results suggest that in co-cultures with wild-type melanoma cells and HUVECs, DARC binds IL-8 (Fig. 3-12). However, in the case of CD82 expressing A375M and UACC 903M melanoma cells, the binding of DARC to CD82 activates p21/Waf1 expression, which has been shown to induce melanoma cell senescence and prevent

**Figure 3-11: CD82 expressing cells bind DARC receptors.** Higher levels of CD82 expression in UACC 903M melanoma cells results in adhesion to DARC receptors on confluent endothelial monolayers, these adhesion events decrease by nearly 50% after incubating co-cultures with anti-DARC or anti-CD82 antibodies (left) while these antibodies have no effects on wild-type melanoma cells without CD82 expression (right). (*p < 0.05 compared to CD82 cases). Data represents mean ± SEM.

**Figure 3-12: CD82 expression interrupts IL-8 binding to endothelial cells.** Immunoprecipitation blots show that DARC directly binds CD82 (left) to induce p21 expression (right; whole cell lysate). In the absence of CD82 expression, control melanoma cells cannot bind DARC receptors because there is little CD82 expression (left) and IL-8 binds DARC receptors (left).
tumor cell extravasation through endothelial monolayers (Fig. 3-12). These findings suggest that the direct binding between CD82 and DARC receptors interrupt binding of IL-8 to DARC receptors on HUVECs, preventing invasion through endothelial cells.

3.4 Discussion

The progression of melanoma cells from an early benign to malignant state is associated with increased cytokine secretion and diminishing CD82 expression. While studies have found that potent inflammatory cytokines influence melanoma development (Singh and Lokeshwar 2009; Singh, Varney et al. 2009), the exact mechanisms by which inflammatory cytokines are regulated are still being investigated. Furthermore, the role CD82 and other tetraspanins play in mediating chemokines remains unknown. In particular, no studies have shown how CD82 regulates IL-8 expression and secretion during tumor/endothelial interactions to promote tumor metastasis development. This is the first study to show that CD82 mediated IL-8 regulation with VE-cadherin junction disassembly to promote melanoma cell extravasation, and development of metastasis.

This study shows that during cancer invasion, metastatic melanoma cells secrete high levels of IL-8 and these levels promote metastatic potential of the cells (Huh, Liang et al. 2010), which can be modulated by CD82. Expression of CD82 in melanoma cells interrupted IL-8 mediated activation of endothelial cells and triggered tumor cell senescence. Thus, targeting intracellular CD82 in melanoma cells reduced secreted concentrations of IL-8, suggesting CD82 as a potential target for preventing the metastatic process.

The extravasation of melanoma cells was significantly reduced under low shear flow conditions following expression of CD82 in melanoma cells. These results were
confirmed using animal studies where CD82 expression diminished metastatic lesions in vivo, reducing the number of lesions by nearly 95%. Under low shear stresses (0.625 dynes/cm²), late stage melanoma cells could not extravasate through endothelial monolayers, suggesting that CD82 tetraspanins mediate the motility of melanoma cells during tumor/endothelial interactions. Previous studies have shown that VE-cadherin disassembly is an important event during tumor cell extravasation (Dejana, Bazzoni et al. 1999; Weis, Cui et al. 2004). We now show that p21 expression in melanoma cells increases after CD82 expressing melanoma cells bind to DARC receptors, indicating a potential mechanism for reduced extravasation under low shear stresses. This observation is supported by previous studies showing that extravasation is interrupted by p21-induced cell senescence (Roninson 2002; Fan, Jiang et al. 2011).

Consistent with previous studies, we found that knocking down CD82 dramatically increased gap formation and subsequent tumor extravasation. Previous studies have reported that an increase in tumor cell extravasation is primarily due to disruption of binding between DARC and CD82 that prevents tumor cell senescence (Gartel, Serfas et al. 1996). While DARC has been described as a "chemokine sink" in erythrocytes, in endothelial cells DARC binds to chemokines and translocates these chemokines to surface of endothelial cells (Zarbock, Bishop et al. 2010). In mice with DARC deficiency, vascular permeability dramatically decreased, but these effects were diminished upon the activation of CXCR2 receptors (Zarbock, Bishop et al. 2010).

These studies suggest inducible DARC expression may not be sufficient to modulate excess chemokines secretion into the tumor microenvironment. Thus, CD82 could provides a therapeutic target that would potentially decrease metastasis development by
reducing IL-8 secretion from melanoma cells. New technologies with the potential to target and reduce IL-8 levels in melanoma cells have also been developed to carry siRNAs (Tran, Gowda et al. 2008) or an antibody-based immunotherapy specifically targeting secreted IL-8 (Waugh and Wilson 2008). The obstacles for siRNA containing-liposomes for IL-8 or CD82 siRNAs would be efficiencies high enough for each cell to undergo endocytosis and uptake the siRNA to dramatically decrease IL-8 protein. Similar issues arise in late stage cancers where targeting tumor cells and inducing DARC expression has been suggested to reduce cancer metastasis (Bandyopadhyay, Zhan et al. 2006; Zijlstra and Quigley 2006). In targeting CD82, adverse side effects of antibody-based or liposome treatments would be diminished since CD82 is expressed in normal cells (Elghetany 2002; Iiizumi, Mohinta et al. 2007).

In conclusion, we have shown that targeting CD82 reduces melanoma metastasis, which is mediated through a reduction of melanoma cell extravasation through the endothelium. Mechanistically, the reduction in melanoma cell extravasation following expression of CD82 is due to the binding of CD82 to DARC receptors. These events disrupt IL-8 binding to DARC receptors and induce p21 expression in tumor cells. Therefore, our study suggests that targeting CD82 signaling may offer a potential mechanism for therapeutic inhibition of IL-8 function in melanoma cancer.
Chapter 4

p38 MAPK IS NECESSARY FOR MELANOMA-MEDIATED REGULATION OF VE-CADHERIN DISASSEMBLY

Forward

The following chapter contains excerpts from the manuscript entitled “p38 MAP Kinase is necessary for melanoma-mediated regulation of VE-cadherin disassembly” (Khanna et al., 2010).

4.1 Introduction

Retraction of the endothelium during tumor cell extravasation occurs due to the breakdown of intercellular channels or gap junctions that allow the passage of soluble proteins and cells (Voura, Sandig et al. 1998; Tremblay, Auger et al. 2006; Alcaide, Newton et al. 2008). Vascular endothelial (VE)-cadherin junction is one such class of transmembrane endothelial junctions involved in regulation of the endothelial barrier playing an important role in angiogenesis, tumor metastasis, cell and protein signaling, and overall endothelial survival (Liao, Doody et al. 2002; Sulkowska, Famulski et al. 2006; Harris and Nelson 2010). VE-cadherin localizes to the cell junctions, and associates with α-catenin, β-catenin, plakoglobin, and p120 catenins via its cytoplasmic tails. Cadherin-mediated endothelial integrity is disrupted by phosphorylation of VE-cadherin tyrosine residues that result in decoupling of the p120 and β-catenin complex from the cytoplasmic tails of VE-cadherin (Esser, Lampugnani et al. 1998; Alcaide, Newton et al. 2008; Lu, Payvandi et al. 2009). Previous studies show that breakdown of VE-cadherin is mediated by adhesion events (van Wetering, van den Berk et al. 2003; Vincent, Kermani et al. 2005).
High expression levels of α4β1 integrin associated with highly metastatic melanoma cells is correlated with a marked increase in melanoma extravasation through endothelial layers (Arroyo, Sanchez-Mateos et al. 1992; Garofalo, Chirivi et al. 1995; Klemke, Weschenfelder et al. 2007). While previous studies have focused on the effects of α4β1 and vascular adhesion molecule-1 (VCAM-1) interactions on metastasis and adhesion of melanoma cells to the endothelium (Klemke, Weschenfelder et al. 2007), we have found that these adhesion events lead to the breakdown of VE-cadherin which facilitates melanoma transendothelial migration (van Wetering, van den Berk et al. 2003; Klemke, Weschenfelder et al. 2007). However, melanoma cells themselves secrete large amounts of soluble proteins including interleukin (IL)-8, IL-6, IL-1β, and growth-regulated oncogene (Gro)-α. Melanoma cells with high metastatic potential have been shown to secrete higher amounts of IL-8 (Liang, Hoskins et al. 2008). Further studies showed that melanoma cells over expressing and secreting higher levels of IL-8 show an increase in penetration of matrigel filters (Luca, Huang et al. 1997). These results were further supported by in vivo studies showing an overall decrease in tumorigenicity and metastasis when mice lacking CXCR2 (the receptor for IL-8) were injected with melanoma cells (Gabellini, Trisciuoglio et al. 2009).

It is well established that p38 mitogen activated protein (MAP) kinase activation plays a key role in the initial break down of VE-cadherin junctions to facilitate cell migration through the endothelium (van Wetering, van den Berk et al. 2003; Khanna, Yunkunis et al. 2010). Van Wetering and colleagues have shown that the VCAM-1 receptor on the endothelium induces intercellular gap formation through the Rho-like GTPase Rac1 signaling that results in activation of p38 MAP kinase proteins further downstream of the Rac pathway. However, in these studies, human umbilical vein endothelial cell (HUVECs) monolayers were pre-stimulated with 10 ng/ml of IL-1β for 30
minutes prior to stimulation with anti-VCAM-1 antibodies and the study focused on leukocyte transendothelial migration. The question remains as to whether melanoma cells trigger VE-cadherin disassembly primarily through cell-cell contact mediated events or through soluble protein-receptor events. Furthermore, could these tumor-induced events modulate specific intracellular pathways in the endothelium leading to a breakdown of VE-cadherin junctions?

Previously we have shown that adhesion of sialyl-Lewis^x/a- negative melanoma cells to the endothelium is regulated by $\alpha_4\beta_1$/VCAM-1 interactions under low shear flow conditions (Liang and Dong 2008). It is therefore possible that melanoma-facilitated breakdown of VE-cadherin occurs through similar mechanisms as seen in leukocytes (Liang and Dong 2008; Liang, Hoskins et al. 2008)

Inhibition of phospholipase C in endothelium was previously shown to decrease melanoma cell induced VE-cadherin disassembly, indicating the importance of intracellular calcium pathways (Peng, Hodgson et al. 2005). However, it was also found that inhibiting phosphatidylinositol-3 (PI3)-kinase in HUVECs did not alter the breakdown of cadherin junctions when contacting melanoma (Peng, Hodgson et al. 2005). In light of these studies, we postulated that while activation of these signaling molecules plays some role in facilitating melanoma metastasis, other downstream signaling molecules might play a more significant role in tumor specific regulation of VE-cadherin junctions. Previous work has shown that activation of VCAM-1 receptors induced phosphorylation of p38 MAP kinase and VE-cadherin disassembly (van Wetering, van den Berk et al. 2003). We found that that inhibiting p38 MAP kinase reduces VE-cadherin disassembly and subsequently decreases melanoma extravasation. The effects of inhibiting p38 using SB220025 (a potent p38 inhibitor) on VE-cadherin disassembly and transendothelial migration was studied using immunofluorescence and Boyden chamber
experiments. We found that both soluble proteins, including IL-8, IL-6, IL-1β, and Gro-α released from melanoma cells, and the VCAM-1/α4β1 interactions, regulate p38 MAP kinase pathways which in turn regulate VE-cadherin junctions.

4.2 Materials and Methods

4.2.1 Cells and Reagents

HUVECs were obtained from American Type Culture Collection (ATCC) (Manassas, VA) and maintained in F12-K medium with 10% fetal bovine serum (FBS), 30 μg/ml of endothelial cell growth supplement, 50 μg/ml heparin (Mallinckrodt Baker, Inc), 100 units/ml of penicillin-streptomycin (Biofluids, Inc). The Lu1205 melanoma cell line (kindly provided by Dr. Gavin P. Robertson, Penn State Hershey Medical Center, Hershey, PA) and A2058 cells (obtained from ATCC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO) supplemented with 10% FBS and 100 units/ml of penicillin-streptomycin. WM35 melanoma cells (provided by Dr. Meenhard Herlyn, Wistar Institute, Philadelphia, PA) were maintained in Roswell Memorial Park Institute (RPMI) supplemented with 10% FBS and 100 units/ml of penicillin-streptomycin. All cells were maintained in a humidified incubator at 37°C and 5% CO2.

4.2.2 Tumor conditioned medium (TCM)

Tumor cells (WM35, A2058, and Lu1205) were cultured in 75 cm2 flasks under growth conditions described above to 90-95% confluency, after which medium was aspirated and replaced with fresh 5 ml of RPMI (for WM35 cells) or DMEM (for A2058 and Lu1205 cells) with 2% FBS. The medium was then removed after a 24-hour period of chosen tumor cell culture and centrifuged in 50 ml conical tubes at 1500 rpm at 4°C for 5 minutes to remove any remaining cells.
4.2.3 p38 inhibition studies

Prior to experiments, HUVECs were maintained in F-12K medium with 2% FBS without additional supplements mentioned above for 12 hours at 37°C and 5% CO2. All experiments were carried out in F-12K medium with 2% FBS without additional supplements to ensure that signaling was not influenced by additional growth factors. For inhibitor studies, HUVECs were treated with 1 μM concentration of 5-(2-Amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl)imidazole (SB220025) (Calbiochem; Gibbstown, NJ) for 30 minutes after which the inhibitor was washed out with F-12K medium containing 2% FBS and then the HUVEC monolayer was stimulated by TCM, soluble recombinant proteins, or direct contact with A2058 melanoma cells.

4.2.4 Fluorescence imaging and analysis

Prior to experiments, 25 mm coverslips were washed with phosphate buffer solution (PBS) and then coated with fibronectin (1 μg/ml) and incubated at room temperature under sterile conditions overnight. Equal amounts of HUVECs were then grown to 95-99% confluency and in some cases treated with SB220025 (a potent p38 MAP kinase inhibitor) for 30 minutes. HUVECs were co-cultured with TCM, recombinant proteins, or A2058 melanoma cells in the presence or absence of SB220025. Cells were then washed twice with PBS and fixed with 5% formaldehyde in PBS for ten minutes. Following fixation, coverslips were washed twice with PBS and cells permeabilized with 0.3% Triton-X 100 in PBS with 5% calf serum (CS) and 2% goat serum (GS). Coverslips were then incubated for 1 hour and washed twice with PBS/CS/GS. Finally, each of the coverslips were incubated with VE-cadherin antibody in 0.3% Triton-X 100/PBS/CS/GS (dilution of 10:1000) overnight at 4°C. Coverslips were then washed with PBS/5% CS/2% GS and treated with Alexa Fluor 488/520 goat anti-mouse IgG antibody (dilution of 1:1000). The coverslips were then incubated at room temperature in the dark for 1
hour and rinsed with PBS/5 %CS/2%GS three times before imaging under a Nikon fluorescence microscope. For each experimental condition, one coverslip was viewed under a 100x objective and a series of six images were taken of randomized fields of view. Each image was then analyzed using Image J software version 1.32 (O'Neill, Mitchell et al. 1989).

4.2.5 Analysis of gaps and disruption of VE-cadherin
Disruption of VE-cadherin was identified from analysis of discontinuity of green fluorescence at VE-cadherin junctions between HUVECs. Gap area within disrupted VE-cadherin junctions was determined from six images. Gap area was quantified as the ratio of pixels within all the gaps and the total number of pixels in one image. The average % endothelial gaps was calculated from six images and plotted as a function of time as shown in the results section.

4.2.6 Western blots
Equal amounts of whole cell lysates of HUVECs were prepared and each condition was resuspended in cold lysis buffer (1 M Tris-hydrochloric acid (HCl) [pH 7.4], 5 M sodium chloride, 500 mM ethylenediaminetetraacetic acid (EDTA) [pH 8.0], 1 M sodium vanadate, 1 M sodium fluoride, 1 M sodium pyrophosphate, 1.0% Nonidet P (NP)-40, 1.0 mM phenylmethylsulphonyl fluoride (PMSF), 1% pepstatin, and phosphatase inhibitor) and incubated in ice for 30 minutes. Following incubation, cell lysates were centrifuged at 14,000x g for 5 minutes and the supernatant was mixed with 1M Dithiothreitol (DTT) and sodium dodecyl sulfate (SDS) buffer (4% SDS, 20% glycerol, 0.2% Bromophenol blue, and 100 mM Tris base). Each well was loaded with an equivalent amount (3μg/μL) of cell lysate. Western blot analysis on the samples was conducted following procedures previously described (19). Briefly, protein was transferred onto a 0.2 μm nitrocellulose membrane (Millipore Co., Billerica, MA). All western blots were probed with primary
antibodies against p38 MAP kinase (Cell Signaling Technologies), p-p38 MAP kinase (Cell Signaling Technologies), or β actin (Santa Cruz Biotechnologies). All blots were re-probed with β actin (Cell Signaling Technologies) to ensure equal loading of proteins.

4.2.7 Enzyme-linked immunosorbent assay (ELISA)

TCM collected from a 24-hour period of the respective tumor cell (WM9, A2058, Lu1205) culture were stored at -20 ºC until ELISA for individual cytokines performed at the Pennsylvania State University General Clinical Research Center. Each 48 well plate was coated with the appropriate mouse anti-human capture antibody diluted in 0.1 M NaHCO3 [pH 8.2] at a final concentration of 2 µg/ml. The plates were incubated overnight at 4 ºC. The next day, each plate was washed three times in phosphate buffer solution containing 20% Tween 20 (PBS-T) and blocked for 2 hours at room temperature using PBS with 1% BSA. Samples and standards were added at 100 µL per well and incubated overnight at 4 ºC. After washing plates with PBS-T, wells were incubated for two hours at room temperature in detection antibody (concentration: 5 µg/ml). Each plate was washed with PBS-T and then conjugated with streptavidin peroxidase (concentration: 1 µg/ml) for 30 minutes at room temperature. Finally, each plate was subject to colorimetric analysis after incubating the plate at room temperature for 60 to 90 minutes in ABTS (2,2’-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid, Sigma Aldrich) substrate with 30% hydrogen peroxide. The plates were read at a wavelength of 405 to 415 nm using a microtiter plate reader.

4.2.8 Transfection with cDNAs

Flag MKK6(glu) (Addgene plasmid 13518) and mRFP (Addgene plasmid 13032) plasmid was kindly provided by Dr. Roger Davis and Dr. Doug Golenbock (University of Massachusetts Medical School, Worcester, MA). Clones were selected with ampicillin and plasmid was extracted using a Qiagen Maxi Kit as per manufacturer’s instructions.
Following DNA purification, transfection complexes were formed by adding 3 μg of MKK6(glu) and mRFP DNA to 25 μL of virofect reagent, and 15 μL of targefect reagent (Targeting Systems, San Diego, CA). Transfection complexes were added to each well of HUVECs seeded on microslides in 1 ml of F12K medium with 10% FBS. HUVEC responses were assayed using fluorescence microscopy and Image J analysis 24 – 48 hours post transfection. HUVECs were tested for MKK6(glu)/mRFP expression using western blot analysis as described in previous sections.

4.2.9 Static cell migration assay

For the static cell migration study, HUVECs were grown to confluency on fibronectin coated polyvinylpyrrolidone-free polycarbonate filters (8 μm pore size; Neuroprobe). The wells on the bottom plate of the chamber were filled with HUVEC media with 2% FBS and the middle 12 wells were filled with collagen type IV (concentration: 100 μg/ml collagen IV in RPMI with 1% Bovine Serum Albumin (BSA)) to act as a chemoattractant to melanoma cells (Hodgson, Henderson et al. 2003; Slattery, Liang et al. 2005). The filter was loaded onto the top of the wells on the bottom chamber with HUVECs seeded on the opposite side and then a top plate was screwed tightly to the static migration chamber. Melanoma cells were then loaded into wells on the top plate of the migration chamber in the middle twelve wells. After incubation at room temperature for four hours, the number of melanoma cells migrating through the endothelial layer onto the bottom of the filter was counted by staining the cells with Protocol Brand Hema 3 solution (Fisher Scientific, Inc.) and counting stained cells using a inverted microscope (Diaphot 330, Nikon) with Image J software (O’Neill, Mitchell et al. 1989).

4.2.10 Statistical analysis

Standard deviations for the percentage of endothelial gaps in all microscopic fields of view and western blots represent three separate experiments that were performed in
triplicate. Levels of significance of all experimental values were determined using a student’s t-test (Sigma Plot 8.0).

4.3 Results

4.3.1 Melanoma cells induce VE-cadherin junction disassembly through cytokine and VCAM-1 mediated events

Fluorescence imaging of HUVECs stained for VE-cadherin showed disruption of VE-cadherin junctions when co-cultured with A2058 melanoma cells (Fig. 4-1 C,E,G). The breakdown of VE-cadherin was evident through the discontinuity of the green fluorescent line labeling the VE-cadherin junctions (Fig. 4-1A).

![Figure 4-1: Melanoma cells induce gap formation when in contact with endothelial cells. A-B. A continuous VE-cadherin staining pattern in endothelial cells. C-D. Loss of VE-cadherin expression near the membrane regions of the endothelial cells was caused by A2058 cells contact after 45 min (labeled using Alexa 546 cell tracker red dyes (Molecular Probes). Arrows indicate the sites of gap formation.](image)

The corresponding DIC image (Fig. 4-1B, D, F, H) shows that the A2058 melanoma cells were located within the sites of gap formation labeled in Fig. 4-1C, E, and G. These results show that highly metastatic melanoma cells induce breakdown of VE-cadherin junctions. To determine if VE-cadherin disassembly was primarily mediated by soluble factor or receptor/ligand binding signals, HUVECs were brought in contact with TCM or melanoma cells with increasing metastatic potential for 45 minutes. Melanoma cells with
increased metastatic potential, or TCM from those cells showed a significant increase in ability to induce gap formation, indicating an increase in VE-cadherin disassembly (Fig. 4-2A). Also, HUVECs stimulated with anti-VCAM-1 showed a significant increase in gap formation similar to that seen in the presence of TCM. However neither anti-VCAM-1 nor TCM induced the same degree of endothelial gaps as A2058 cells in co-culture with HUVECs (Fig. 4-2B).

Figure 4-2: Endothelial gaps increase with metastatic potential of melanoma cells, which are mediated by both soluble factors and anti-VCAM-1. A. HUVECs were co-cultured with TCM or anti-VCAM-1 (30 μg/ml), which significantly increase % gap formation compared to HUVECs alone but less gap formation than A2058 cells co-cultured with HUVECs. The values represent the mean SD of 3 different experiments. P values are indicated, comparing % endothelial gap of HUVEC+TCM and HUVEC+anti-VCAM-1 with % endothelial gap of HUVEC alone and HUVEC+A2058 cells (* P < 0.05). B. Melanoma induced VE-cadherin disassembly is mediated by both cell contact soluble mediated events. Here, 0.3 million HUVECs were stimulated with anti-VCAM-1 (30 μg/ml) for 45 minutes before adding 0.9 million A2058 melanoma cells. The values represent the mean SD of 3 different experiments. P values are indicated, comparing % endothelial gap of HUVEC+anti-VCAM-1 and HUVEC+anti-VCAM-1+A2058 cells with % endothelial gap of HUVEC alone (* P < 0.05).

Since both anti-VCAM-1 and TCM induced a gradual increase in the percentage endothelial gap formation over time (Fig. 4-3A), we determined how gap sizes were regulated.

In the case of TCM, the degree of VE-cadherin disassembly and gap sizes increased gradually to more than 100,000 pixels or a 10 μm diameter by the 90 minute time point (Fig. 4-3B).
Fig. 4-4A shows that anti-VCAM-1 induced larger gaps (sizes of 100,000 pixels or more) after 10 minutes, after which the gaps became smaller indicating a decrease in VE-cadherin disassembly or closure of these gaps. These results suggest that VCAM-1 induces a transient VE-cadherin disassembly, while soluble proteins show a prolonged effect enlarging existing gaps to allow the passage of melanoma cells (4-4B).
As shown by data in 4-4B, the gap size increased gradually to more than 100,000 pixels when treating HUVECs with TCM.

4.3.2 Secretion of IL-8 and IL-1\(\beta\) by A2058 melanoma regulates VE-cadherin junction disassembly

Cytokines present within TCM secreted by melanoma cells over 24 hours were analyzed using a Raybiotech cytokine screen (data not shown). We found that several cytokines were secreted by melanoma cell types at high concentrations including IL-8, IL-6, IL-1\(\beta\), and Gro-\(\alpha\). These cytokines were further quantified using ELISA (Table 4-1).

Table 4-1: Concentrations of cytokines secreted by melanoma over 24 hours

<table>
<thead>
<tr>
<th>Melanoma Cell Line</th>
<th>IL-8 Concentration (ng/ml)</th>
<th>IL-6 Concentration (ng/ml)</th>
<th>IL-1(\beta) Concentration (ng/ml)</th>
<th>Gro-(\alpha) Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lu1205</td>
<td>24</td>
<td>23.2</td>
<td>5</td>
<td>0.08</td>
</tr>
<tr>
<td>A2058</td>
<td>15</td>
<td>6</td>
<td>0.051</td>
<td>5.4</td>
</tr>
<tr>
<td>WM35</td>
<td>0.084</td>
<td>0.078</td>
<td>0.00</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Clearly, highly metastatic melanoma cells (e.g., Lu1205) produce higher soluble concentrations of these cytokines compared with those of lesser metastatic potential (e.g., WM35). Stimulating HUVECs with recombinant forms of individual cytokines showed no dramatic increase in the percentage of gap area (Fig. 4-5A).

Combining cytokines in TCM, specifically IL-8 and IL-1β only had additive effects on VE-cadherin disassembly rather than being synergistic (Fig. 4-5B).

Since concentrations of cytokines in TCM are simply the bulk concentrations, HUVECs in direct contact with melanoma cells may sense much higher or lower local concentrations of cytokines within the cell-cell contact region than that found in TCM. We therefore specifically addressed whether IL-8 or IL-1β is involved in this response by neutralizing these cytokines secreted from melanoma cells during co-culture with HUVECs.

**Figure 4-5:** IL-8 and IL-1β have additive rather than synergistic effects on VE-cadherin disassembly. **A.** The indicated cytokines induced VE-cadherin disassembly. P values are comparing % gap area of cytokines with % gap of HUVEC alone (* P < 0.05). **B.** Stimulation of HUVECs with recombinant forms of IL-8 and IL-1β or IL-6 and IL-1β induced additive effects on endothelial gap formation. Concentrations of cytokines were based on TCM concentrations measured using ELISA (Table 2). P values compare % gap area of combinations of cytokines with % gap of HUVEC+IL-8, HUVEC+IL-6, HUVEC+IL-1β.
Neutralization of either IL-8 or IL-1β decreased VE-cadherin disassembly; however, the endothelial gaps were still comparable to that induced by anti-VCAM-1 (Fig. 4-6A). However, simultaneous neutralization of IL-8 and IL-1β dramatically reduced the breakdown of VE-cadherin junctions (Fig. 4-6B). These results show the importance of these cytokines in melanoma induced VE-cadherin disassembly in the presence of VCAM-1 interactions. Furthermore, using neutralization antibodies we confirmed that IL-8 and IL-1β both play significant roles in the breakdown of VE-cadherin junctions.

Figure 4-6: Anti-VCAM-1 and neutralization of both IL-8 and IL-1β dramatically reduces the breakdown of VE-cadherin. **A.** HUVECs were co-cultured with A2058 cells +anti-IL-8, A2058 cells +anti-IL-1β, or A2058 cells +anti-IL-8 +anti-IL-1β. P values are comparing each experimental condition with % endothelial gap areas during HUVEC alone (\(^* p < 0.05\)). **B.** Using the same controls as in panel A, data was graphed to make comparisons between the effects of neutralizing individual and pairs of cytokines. HUVECs were co-cultured with either A2058 cells, anti-VCAM-1, A2058 cells +anti-IL-8, A2058 cells +anti-IL-1β, or A2058 cells +anti-IL-8+anti-IL-1β. Neutralization of both IL-8 and IL-1β dramatically decreased % endothelial gaps compared to HUVECs stimulated with anti-VCAM-1 or anti-IL-8 and anti-IL-1β alone. P values are comparing each experimental condition with % endothelial gap areas for HUVEC+anti-VCAM-1, HUVEC+A2058 cells+anti-IL-8, and HUVEC+A2058 cells+anti-IL-1β (\(^* P < 0.05\)). Values for graphs and ELISA are means ± SD.
4.3.3 Soluble protein and anti-VCAM-1 induce phosphorylation of p38 MAP kinase regulates VE-cadherin disassembly

To determine whether the degree of VE-cadherin disassembly correlated with phosphorylation of p38 MAP kinase, soluble proteins from A2058 melanoma (TCM) were used to stimulate HUVECs for 10, 45, and 90 minutes. Lysates were subjected to SDS-PAGE to determine the levels of p38 phosphorylation. Stimulation of HUVECs with A2058 melanoma TCM upregulated p38 phosphorylation for up to 90 minutes (Fig. 4-7A-B). On the other hand, stimulation of the HUVECs with anti-VCAM-1 upregulated p38 phosphorylation for 10 minutes before decreasing dramatically at 45 and 90 minutes (Fig. 4-7C-D).

Figure 4-7: Western blots show p38 phosphorylation. A. When HUVECs are stimulated with A2058 TCM for 10 minutes, 45 minutes, and 90 minutes. The location of “S” and “SS” lanes have been moved for ease of interpretation, but are from the same western blot. B. Western blots in panel A were quantified. P values are comparing TCM stimulated case with the ss case. C. Effects of anti-VCAM-1 on p38 phosphorylation in HUVECs over 10, 45, and 90 minutes. D. Western blots in panel C were quantified. P values are comparing TCM stimulated case with the ss case. E. Effects of neutralizing cytokines in TCM from A2058 cells for 45 minutes. Neutralizing antibodies (indicated) were respectively added in TCM prior to co-culture with HUVECs. F. Western blots in panel E were quantified. P values are comparing TCM stimulated case with cases where IL-8, IL-1β, IL-6, or a combination of these were neutralized in TCM and then added to HUVECs. Western blots represent 3 different experiments. Values are means + SD.
Imaging HUVECs before and after exposure to TCM showed that total p38 was localized primarily in the cytosol, similar to that seen with HUVECs alone (Fig. 4-8A, C). However, phosphorylated p38 was localized to the nucleus of endothelial cells with small amounts present in the cytosol in the case of HUVECs alone, most likely at the cytoskeleton or endothelial junctions (Fig. 4-8D). Increased localization of phosphorylated p38 within the cytosol was observed when HUVECs were in contact with TCM or TNF-α (Fig. 4-8E, F). These results suggest that p38 may not regulate VE-cadherin junctions directly, but rather through interactions with cytoskeletal elements.

![Figure 4-8: p38 MAP kinase proteins translocate from the cytosol to the nucleus after phosphorylation. (A-F; Bars, 25 μm). HUVECs were fixed and stained with anti-p38 followed by Alexa 488 labeled secondary antibodies. The actin cytoskeleton was stained with phalloidin conjugated to Alexa 564. A. Fluorescent images of unstimulated HUVECs. B. Fluorescent images of HUVECs stimulated with recombinant TNF-α for 45 minutes. C. HUVECs in contact with A2058 TCM for 45 minutes. D. Fluorescent images of HUVECs fixed and stained with phospho-p38 followed by Alexa 488 secondary antibodies and phalloidin. E. Fluorescent images of HUVECs stimulated with recombinant TNF-α for 45 minutes. F. Fluorescent images of HUVECs in contact with A2058 TCM for 45 minutes. Images are representative of 3 separate experiments.](image)

Since p38 MAP kinase was activated in HUVECs via melanoma produced soluble mediators as well as anti-VCAM-1, we next determined whether constitutively active p38
affected VE-cadherin disassembly. Transfection of MKK6 (an upstream activator of p38 MAP kinase) that leads to constitutively active p38 induced a high degree of gaps between endothelial cells compared to transfection of HUVECs with RFP alone (Fig. 4-9 A,B).

We further tested whether inhibiting p38 would affect the homotypic contacts formed by VE-cadherin. HUVECs pretreated with a potent p38 inhibitor (SB220025) for 30 minutes and co-cultured with A2058 melanoma cells showed significant decrease in gap formation compared to the control (Fig. 4-10).

**Figure 4-9: Overexpression of p38 increases disassembly of VE-cadherin homodimers to form endothelial gaps.** A. Transfection efficiency of HUVECs with red fluorescent protein (RFP) or MKK6(glu)/RFP was assessed using western blots probed for either anti-p38 or anti-phospho-p38. Western blots are representative of three separate experiments. B. HUVECs were transfected with constitutively active MKK6(glu) fused with red fluorescent protein (RFP) to label transfected cells. Endothelial gap formation was assessed 36-48 hrs post-transfection. P values are comparing each experimental condition with % gap of HUVECs alone and HUVECs + RFP (* P < 0.05).
**4.3.4 p38 facilitates melanoma extravasation likely through the cytoskeleton**

The functional role of p38 in endothelial cells during melanoma extravasation was also examined using small interfering RNA (siRNA) approaches. siRNA mediated knockdown of p38 in HUVECs was confirmed using western blotting (Fig. 4-10A). When p38 expression was knocked down, Melanoma transendothelial migration after 4 hours decreased significantly compared to the control (Fig. 4-11 C,D).

In the controls, HUVECs did not migrate through the polycarbonate filters towards collagen IV (Fig. 4-11B). Melanoma extravasation through HUVECs decreased to nearly 20% when p38 was knocked out, compared to 98% of melanoma cells when p38 expression was at normal levels (Fig. 4-11E). These results show that p38 is not only important in the regulation of VE-cadherin junctions, but also in endothelial gap formation to facilitate overall tumor cell extravasation.

**Figure 4-10: Inhibiting p38 phosphorylation prevents the disassembly of VE-cadherin homodimers.** HUVECs were treated with SB220025 (a potent p38 inhibitor) for 30 minutes before they were co-cultured with A2058 melanoma cells for 45 minutes. Values are means SD. P values are comparing each experimental condition with % gap of HUVEC+A2058 cells (* P < 0.05).
4.4 Discussion

The interaction of blood-borne tumor cells with endothelial cells is a key step in facilitating melanoma metastasis (Luzzi, MacDonald et al. 1998; Ghislin, Obino et al. 2011; Pang, Zhao et al. 2011). However, research on drug therapies to treat such cancers has focused on single cell studies without considering the effects of tumor interactions with normal cell physiology. In these studies A2058 melanoma cells were used to study VE-cadherin disassembly since they are characterized by highly invasive capabilities and secrete high levels of soluble proteins, including growth factors and
moderate levels of IL-8, IL-6, IL-1β, and Gro-α, and serve as an ideal system in studying metastasis (Garcia de, Boyano et al. 1998; Huang, Ullrich et al. 1999; Singh and Varney 2000). Lu1205 cells have even higher metastatic potential than A2058 or WM35 cells and secrete higher levels of IL-8, IL-6, IL-1β, and Gro-α, while WM35 cells exhibit the lowest metastatic potential and secrete the lowest levels of these cytokines (Triozzi, Kim et al. 1995)(Boyano, Garcia-Vazquez et al. 1997). Melanoma cells with higher metastatic capabilities induce larger sized gap areas that increase over time corresponding to a higher degree of VE-cadherin disassembly. For instance, HUVECs in contact with Lu1205 cells for 10 to 45 minutes form gaps of average sizes of approximately 100,000 pixels; whereas, WM35 cells induce the formation of smaller gaps with an average size of about 1000 pixels. We show that anti-VCAM-1 initially induces the breakdown of VE-cadherin gap formation, with a dramatic increase in the number of gaps with a size of 100,000 pixels or greater after 10 minutes. However, after 45 and more dramatically 90 minutes, there is a decrease in the number of larger gaps (> 100,000 pixels), which shows that the gaps formed by anti-VCAM-1 are closing after 10 minutes. On the other hand, the release of soluble cytokines including IL-8, and IL-1β prolong the time over which gaps remain open, which correlates with greater VE-cadherin disassembly. This phenomenon is shown by the gradual increase in the number of large gaps (size of 100,000 pixels or greater) over 90 minutes. The changes in gap area correlate with phosphorylation of p38 MAP Kinase where greater phosphorylation levels of p38 correlate with an increase in the sizes of gaps.

In this study, we focused on the specific soluble proteins that regulate the breakdown of VE-cadherin junctions to facilitate melanoma metastasis. In vivo studies have illustrated that VE-cadherin junction breakdown is an important event during melanoma metastasis (Weis, Cui et al. 2004). These results show that the injection of nude mice with BV13
(anti-VE-cadherin antibody, which induces VE-cadherin disassembly) results in a four-fold increase in tumor metastasis and an increase in overall permeability of the endothelial layer (Weis, Cui et al. 2004). While these studies have focused on the in vivo aspects of VE-cadherin roles, our present in vitro studies provide the first evidence that shows the importance of soluble cytokines released from melanoma in regulating VE-cadherin junctions. In particular, we found IL-8 and IL-1β play a prominent role in soluble factor mediated breakdown of VE-cadherin junctions. These results are consistent with previous in vivo studies that show that CXCR2/- nude mice injected with melanoma cells result in a dramatic decrease in melanoma metastasis (Muller, Homey et al. 2001). Furthermore, we used TCM to show that soluble proteins alone are capable of facilitating the breakdown of these junctions showing that melanoma metastasis is not primarily mediated by adhesion events, but rather that both VCAM-1 and soluble proteins control the temporal disassembly of VE-cadherin. We further tested whether adhesion events were capable of inducing the phosphorylation of p38 MAP kinase proteins by stimulating the HUVEC layer with anti-VCAM-1 functional antibodies. Previous studies have shown that the β1 subunit of αβ1 mediates binding to its ligand, VCAM-1 (Arroyo, Garcia-Pardo et al. 1993; Zetter 1993). Furthermore, studies with A2058 melanoma cells have illustrated a dramatic decrease in invasiveness, adhesion, and migration of melanoma when the beta 1 subunit of αβ1 integrin of A2058 melanoma is blocked using functional antibodies (Melchiori, Mortarini et al. 1995). These results indicate the importance of αβ1 and VCAM-1 interactions in aggressive forms of melanoma metastasis (Arroyo, Sanchez-Mateos et al. 1992; Arroyo, Garcia-Pardo et al. 1993). Our results show that VCAM-1 on endothelial cells induce the transient phosphorylation of p38 correlating with dramatic but early formation of large gaps (sizes
of 100,000 pixels or more) in HUVECs. A size of 100,000 pixels corresponds to the size of A2058 and Lu1205 melanoma cells, which are about 14 μm in diameter.

In this paper, we primarily focused on the importance of IL-8 and IL-1β on VE-cadherin disassembly; however, our results warrant further studies as to the exact role of IL-6 and Gro-α in melanoma metastasis since these cytokines are upregulated during co-culture of melanoma cells and HUVECs. Since the stimulation of HUVECs with these TCM induced increasing breakdown of endothelial junctions over a period of 45 to 90 minutes while anti-VCAM-1 only induced dramatic breakdown of VE-cadherin for short periods of time, secretion of soluble proteins induce VE-cadherin disassembly to a greater degree to induce larger gaps in the endothelium so that more metastatic melanoma cells may rapidly transmigrate between the endothelial cells before they are dislodged from the endothelium under physiological flow.

We investigated the effects of soluble proteins on endothelial signaling, by elucidating the molecular events that regulate VE-cadherin junctions. Previous studies (Tremblay, Auger et al. 2006) have showed that the activation of Src kinases and VE-cadherin mediated disassembly are required for the process of transendothelial migration in colon carcinoma (Tremblay, Auger et al. 2006). In particular, endothelial transmigration mediated by p38 MAP kinase cannot take place without phosphorylation-mediated formation of stress fibers of myosin light chains, in concert (Borbiev, Birukova et al. 2004). The molecular mechanisms by which melanoma and colon cancer induce metastasis could be via similar signaling events. Consistent with previous findings (Pedersen and Hoffmann 2002) we find that inhibiting p38 MAP kinase dramatically decreases the degree of phosphorylation of VE-cadherin (data not shown). Since the phosphorylation state of VE-cadherin regulates the disassembly and assembly of these junctions, p38 MAP kinase must play a major role in regulation of the endothelial barrier.
(Borbiev, Birukova et al. 2004). The importance of p38 MAP kinase in melanoma metastasis is strengthened by our studies where knockdown of p38 MAP kinase in HUVECs resulted in a 60% decrease in melanoma migration through the HUVEC layer. Our results provide the first evidence that p38 MAP kinase activation plays a predominant role in overall endothelial permeability and tumor extravasation. Furthermore, we find that melanoma produced soluble proteins dramatically affects the degree of p38 phosphorylation and VE-cadherin disassembly in HUVECs. Interestingly enough, neutralizing both IL-8 and IL-1β or IL-6 and IL-1β decreases p38 phosphorylation and VE-cadherin disassembly while melanoma cells contact HUVECs, suggesting the importance of these cytokines in breakdown of the endothelial barrier. In agreement with this conclusion, patients with highly aggressive melanomas have a marked increase in the secretion of IL-8, IL-6, and IL-1β (Boyano, Garcia-Vazquez et al. 1997). While IL-1β is normally secreted by white blood cells, a higher amount of IL-8, IL-6 and IL-1β is secreted by melanomas when in co-culture with neutrophils (Liang, Hoskins et al. 2008; Huh, Liang et al. 2010). However, the role of these cytokines in tumor extravasation has yet to be elucidated. We have shown the importance of soluble proteins in p38 MAP kinase activation and that neutralizing these cytokines leads to dramatic decrease in disassembly of VE-cadherin. However, since neutralization of IL-8, IL-6 and IL-1β did not completely abolish p38 phosphorylation, there may be other growth factors or cytokines released from melanoma that are capable of activating p38. In conclusion, our results support a role for cytokines and VCAM-1 binding in regulating p38 MAP kinase phosphorylation and the breakdown of VE-cadherin junctions.
Chapter 5

MODEL SIMULATIONS REVEAL VCAM-1 ACTIVATION AUGMENTS PAK ACTIVATION, AMPLIFYING p38 PHOSPHORYLATION

Forward

The following chapter contains excerpts from the manuscript entitled “Model Simulations Reveal VCAM-1 Augment PAK Activation Rates to Amplify p38 MAPK and VE-cadherin Phosphorylation” (Khanna and Weidert et-al., 2011). Both Payal Khanna and Eric Weidert contributed equally to this work.

5.1 Introduction

Our results show that CD82 can suppress IL-8 secretion and maturation of the $\beta_1$ subunit of the very late antigen (VLA)-4 molecule adhesion to vascular cell adhesion molecules (VCAM)-1 on endothelial cells (Jee, Lee et al. 2007). High expression levels of VLA-4 integrin are associated with a marked increase in melanoma extravasation through endothelial layers (Zetter 1993; Klemke, Weschenfelder et al. 2007), in accordance with the observation of VLA-4 overexpression in highly metastatic cells. The binding of VLA-4 to VCAM-1 induces the activation of downstream mitogen activated protein kinase (MAPK) signaling cascades, which regulate the vascular endothelial (VE)-cadherin junctions that hold together endothelial cells (van Wetering, van den Berk et al. 2003).

VE-cadherin junctions serve as barriers that regulate the passage across the endothelium of soluble proteins, immune cells, and tumor cells. In vivo studies have found that breaking down of VE-cadherin junctions lead to a dramatic increase in the
levels of tumor cell extravasation (Voura, Sandig et al. 1998; Weis, Cui et al. 2004). When localized to the cell junctions, VE-cadherin associates with several proteins via its cytoplasmic tails including α-catenin, β-catenin, plakoglobin, and p120 catenins (Alcaide, Newton et al. 2008). Phosphorylation of VE-cadherin on its tyrosine residues results in decoupling of the p120 and β-catenin complex, disrupting the integrity of the endothelium (Weis, Cui et al. 2004; Tremblay, Auger et al. 2006).

The complex signaling mechanism of MAPK pathways depends on several factors including kinetics of receptor activation and spatial localization of membrane proteins and receptors (Poh, Na et al. 2009; Ouyang, Huang et al. 2010). This mechanism is further complicated by amplification of MAPK signaling downstream of membrane bound proteins (Schoeberl, Eichler-Jonsson et al. 2002). For example studies by Shoeberl et al. (18) showed that the receptor activation rate was more determinant for the activation of extracellular signal-regulated kinase (ERK) than variations in the concentration of endothelial growth factor (EGF), the ligand for the EGF receptor.

In endothelial cells, VCAM-1 binding mediates the rate of cell contractility via Rac GTPases, which has been shown to regulate the autophosphorylation of p21 activated kinases (PAK) (Stockton, Schaefer et al. 2004; Lozano, Frasa et al. 2008). Activated PAK, a downstream target of activated Rac has also been shown to cause retraction of endothelial cells via phosphorylation of downstream p38 MAPK, myosin light chain kinases (MLCK), and calcium pathways to regulate VE-cadherin disassembly (3,25,26). Specifically, PAK has been shown to regulate contractility by binding specific residues on MLCK protein to increase and decrease contractility (Stockton, Schaefer et al. 2004).
Previous reports also showed that chemokines, in particular interleukin (IL)-8 also alter Rac-GTPase dynamics to regulate the formation of polymerized actin when binding to its receptors CXCR1 and CXCR2 (Schraufstatter, Chung et al. 2001). IL-8 generally has a higher binding affinity for CXCR2 than CXCR1 though both receptors are able to activate signaling pathways (Schraufstatter, Chung et al. 2001; Fuhler, Knol et al. 2005). However, studies by Schraufstatter et-al. show that IL-8 initially activates actin stress fiber formation via IL-8 binding to CXCR1 while Rac is activated in a CXCR2-dependent fashion at later time points leading to gap formation. These studies suggest that endothelial signaling via CXCR1 and CXCR2 may be modulated by different signaling cascades (Schraufstatter, Chung et al. 2001). Consistent with these reports, it has been found that IL-8 mediates both calcium and phosphatidylinositol-3 (PI3)-kinases signaling (Knall, Worthen et al. 1997; Lomakina and Waugh 2010).

Studies have also found that IL-1β phosphorylates p38 MAP kinase and SAPK/JNK since SB220025, a very selective inhibitor of p38 MAP kinase, completely inhibits IL-1β-induced effect (Jackson, Bolognese et al. 1998). Kinetic studies of p38 phosphorylation have shown that p38 phosphorylation increases over 30-40 minutes and then decreased when stimulated with lipopolyssacharide (LPS) at various concentrations. Similar kinetics are observed when endothelial cells are stimulated with chemokines (in particular IL-1β) (Heit, Tavener et al. 2002). While studies have shown the kinetics of p38 phosphorylation in response to IL-1β and the importance of calcium pathways in regulating IL-1β desensitization, less is known about how VCAM-1 regulates p38 phosphorylation kinetics and in turn, VE-cadherin phosphorylation resulting in endothelial permeability. One of the drawbacks of
experimental methods is the difficulty to characterize signaling dynamics in response to several stimuli.

In the present study, we elucidated the relative roles of VCAM-1 on p38 phosphorylation. The model simulations show that VCAM-1 increases p38 phosphorylation kinetics via PAK activation. VCAM-1, but not IL-1β was able to modulate Rac and PAK temporal activation, which resulted in a rapid increase or decrease in p38 phosphorylation.

5.2 Materials and Methods

5.2.1 Cell culture. The A2058 melanoma cells were cultured in DMEM/F-12 (Invitrogen). Media was supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Before each experiment, melanoma cells were detached when confluent using 0.05% trypsin-EDTA (Invitrogen) and washed twice with fresh medium. The cells were then suspended in fresh media and allowed to recover for 1 hour at 37°C. Human pulmonary microvascular endothelial cell line HPMEC ST1.6R (kindly provided by Dr. Kirkpatrick, Institute of Pathology, Johannes-Gutenberg University, Germany) were grown on coverslips for all experimental purposes and maintained as described elsewhere (Khanna, Yunkunis et al. 2010).

5.2.2 Constructs for FRET imaging. cDNAs for FRET imaging were obtained from Addgene and were kindly provided by Dr. Adam D. Hoppe (Hoppe and Swanson 2004). Briefly, Polymerase chain reaction was first used to amplify regions of PAK1 protein (PBD), BglII, and HindIII restriction sites. The newly synthesized cDNA with these regions was cloned into pmCFP-N1 to form the entire construct for CFP-PBD. A similar procedure was done to synthesize Rac-YFP cDNAs. Rac and PBD constructs are shown in Fig. 5-1.
5.2.3 Live-cell FRET imaging and immunofluorescence microscopy. For live-cell fluorescence microscopy, HPMEC cells were transfected on 25-mm round glass coverslips. After 24 h, cells were placed in an imaging chamber in medium at room temperature. Both Rac and p21 binding domains (PBD) labeled with cyan and yellow fluorescent proteins (CFP and YFP, respectively) were captured using 3 separate channel for donor/donor (Dd) emission/excitation, acceptor (Aa) emission/excitation, and donor/acceptor (Da) emission/excitation channels were captured 30 seconds apart for 10 minutes at 200 millisecond exposure time and with 2x2 binning using an EZ Coolsnap camera from NIKON. Subtraction for filter bleed through and background was conducted using methods described 9. Briefly, background from each image was subtracted. Images were then fractionally subtracted from raw FRET images on the basis of measurements for CFP bleedthrough (approximately 0.51) and YFP bleedthrough.
(0.025). This fractional subtraction generated corrected FRET images that showed co-localization of these proteins. For measurements at endothelial junction, regions of interest chosen were measured for each time frame at the same localization over 10 minutes. For visualization of VE-cadherin junctions, HPMEC were grown on cover slips. Cells were washed in PBS and fixed with 5% paraformaldehyde in phosphate buffered saline (PBS) for 15 minutes permeabilized for 20 minutes in 0.3% Triton/PBS with 2% goat serum (GS) and 5% calf serum (CS). Coverslips were incubated overnight at 4°C human anti-VE-cadherin (10 μg/ml) diluted in PBS/0.3% triton-X/2%GS/5%CS. After washing in PBS, coverslips were incubated for 1 hour with Alexa fluor 188 goat anti-mouse antibodies (Molecular Probes) diluted 1:1000 as per manufacturer's instructions and images using NIS elements software.

5.2.4 Analysis of VE-cadherin disassembly. Gap area was quantified as the ratio of pixels within all the gaps and the total number of pixels in one image captured using a 40X lens. The average % endothelial gaps was calculated from six images and plotted as shown in the results section.

5.2.5 Modeling of signaling pathways. Inputs to the model were included based on experimental data showing stimulation of endothelial cells with anti-VCAM-1 resulted in gap formation (Fig. 5-1). Further studies showed that neutralization of IL-8, IL-1β also reduced gap formation (Fig. 5-1). The model was constructed using a MATLAB based systems biology (SB) toolbox developed by Schmidt et-al. (Schmidt and Jirstrand 2006) (Fig. 5-3). A detailed schematic of experimental and literary inputs used to define model parameters and nodes is illustrated in Fig. 5-3. Briefly, all reaction rates describing the behavior of signaling cascades were based on mass action kinetics or Michaelis-Menten like kinetics (Fig. 5-4). The change of each species concentration over time was defined by the following general equation:
\[
\frac{dC}{dt} = \sum_i r_i - \sum_j r_j, \quad \text{(eq. 1)}
\]

where \( C \) represents the species concentration and the first and second summations in the right-hand side represent the overall production or consumption rates of the species, respectively. Reactions described by Michaelis-Menten kinetics are indicated in Fig. 5-4 (parameters marked \( k_m \)) and parameters and initial conditions are listed in Table 5-1 and 5-2 in appendix A. For example, binding \((k_{s1})\) and dissociation \((k_{s2})\) of IL-1\(\beta\) to its receptor (IL-1\(\beta\)R) is described by mass action kinetics whereas the activation of PAK by GTP-bound Rac (Rac\(^*\)) is described by Michaelis-Menten kinetics as follows:

\[
\text{IL-1}\beta + \text{IL-1}\beta\text{R} \rightleftharpoons \text{IL-1}\beta : \text{R} \quad r_{s1} = k_{s1}C_{\text{IL-1}\beta}C_{\text{IL-1}\beta\text{R}}, \quad r_{s2} = k_{s2}C_{\text{IL-1}\beta:\text{IL-1}\beta\text{R}} \quad \text{(eq. 2)}
\]

The changes in concentrations of the above proteins over time is given by the following:

\[
\frac{dC_{\text{IL-1}\beta}}{dt} = r_{s2} - r_{s1}; \quad \text{(eq. 3)}
\]

\[
\frac{dC_{\text{IL-1}\beta:\text{IL-1}\beta\text{R}}}{dt} = r_{s1} - r_{s2} \quad \text{(eq. 4)}
\]

\[
\frac{dC_{\text{IL-1}\beta-R}}{dt} = r_{s2} - r_{s1} \quad \text{(eq. 5)}
\]

In equations 2-5, \( r_{s1} \) represents the production rate of the IL-1B:IL1BR complex and the consumption rate of IL-1B and IL1BR while \( r_{s2} \) represents the production rate of IL-1B and IL1BR and the consumption rate of the IL-1B:IL-1BR complex. Kinetic values for equations 2-5 are as follows: \( k_{s1} = 0.001 \text{ uM-1s}^{-1} \), \( k_{s2} = 0.002 \text{ s}^{-1} \). The initial conditions
for IL-1β, IL-1βR, and IL-1β:IL-1βR are $C_{IL-1β}(0) = 1$ uM and $C_{IL-1βR}(0) = 1.25$ uM, $C_{IL-1β:IL-1βR}(0) = 0$, respectively.

PAK molecules are transformed in the following processes:

$$P_{PAK}^{k_{18},k_{38}} \rightarrow P_{PAK}^{*}, \quad r_{38} = k_{38}C_{Rac}\cdot C_{PAK}/(k_{M39} + C_{PAK}) \quad (eq. 6)$$

$$P_{PAK}^{*} \rightarrow P_{PAK} \quad (eq. 7)$$

The concentrations of PAK molecules over time is given by the following:

$$\frac{dC_{PAK}}{dt} = -r_{38} + r_{45} \quad (eq. 8)$$

$$\frac{dC_{PAK}^{*}}{dt} = r_{38} - r_{45} \quad (eq. 9)$$

In equations 6-9, $r_{38}$ represents the production rate of activated PAK ($P_{PAK}^{*}$) and the consumption rate of PAK. The kinetic rates for equations 6-8 are as follows: $k_{38} = 0.091$ s$^{-1}$, $k_{M39} = 0.25$, $k_{45} = 0.25$ The initial concentrations for PAK and activated PAK are $C_{PAK}(0) = 0.10$ uM, $C_{PAK}^{*}(0) = 0.0$ uM, respectively. Similar equations were written for p38 complexes in the cytosol and the nucleus described in later sections.

The model was built using the following assumptions:

1. Viscous effects of the cytosol and spatial localization of proteins were not considered in the model, but rather the model was defined based on ordinary differential equations (ODEs) and included appropriate kinetic parameters for protein fluxes localized to various cell compartments (i.e., cytoplasm and nucleus).

2. Initial conditions were pulled from literature, previous experimental data or initially set to zero $^{2,8,14,15}$. We stimulated the system with concentrations of cytokines and receptors based on measurements from tumor conditioned medium (TCM)
and/or activation antibodies (methods for collecting TCM are as described previously\textsuperscript{10}).

3. It was assumed that the total concentration of proteins (i.e., active and inactive forms) in the system were constant over the experimental period of time, which may not necessarily reflect the true behavior of all proteins in a cell.

\textbf{5.2.6 Modeling changes in p38 MAPK and VE-cadherin disassembly.} In previous work it has been found that VE-cadherin disassembly is mediated by phosphorylation of cadherin junctions on their residues. For this reason, we used VE-cadherin phosphorylation\textsuperscript{(Alcaide, Newton et al. 2008; Chabot, Spring et al. 2009)} as a marker of gap formation. Previous studies found that cytosolic p38 MAPK translocates to the nucleus upon phosphorylation to activate downstream heat shock proteins (HSP)\textsuperscript{27}. Activated HSP27 regulates contractility or formation of actin/myosin cross-bridges. Contractility of the endothelial cells results in VE-cadherin phosphorylation to increase gap formation\textsuperscript{(Alcaide, Newton et al. 2008)}. To incorporate the local compartmental changes in cells, we multiplied the flux of the protein by the volume of the cell compartment. In this case we assumed the size of the cell to be 1 pL and that the nucleus was 1/10 the size of the entire cell\textsuperscript{6}. Cytosolic p38 MAPK can translocate to the nucleus in a reversible manner depending on its phosphorylation level. The dephosphorylated form (p38) favors localization in the cytoplasm whereas the phosphorylated form (p38P) favors localization to the nucleus. The phosphorylated form of p38 can also leave the nucleus when complexed with MK2. The reactions for p38 MAPK entering and leaving the nucleus (nuc) are as follows (Fig. 5-4):
We can write expressions describing consumption and production of p38 concentration in the cytosol using similar expressions as those shown in equations 3-5 and 7-8. The kinetic parameters and initial concentrations in equation 10 are as follows: $k_{77} = 0.40 \text{ s}^{-1}$, $k_{81} = 0.0028 \text{ s}^{-1}$, $C_{p38P}(0) = 0.0 \text{ uM}$, and $C_{p38Pnuc}(0) = 0.0 \text{ uM}$. The change in p38 concentrations over time are given by eq. 11.

$$p38 \leftrightarrow p38_{\text{nuc}}, r_{82} = k_{82}C_{p38}, r_{83} = k_{83}C_{p38_{\text{nuc}}} \quad (eq. 11)$$

Kinetic parameters for eq. 11 are as follows: $k_{82} = 0.00076 \text{ s}^{-1}$, $k_{83} = 0.00031 \text{ s}^{-1}$. The initial concentrations of p38 in the cytosol and nucleus were given as 6.5 uM and 0.0 uM, respectively (Hendriks, Hua et al. 2008) The concentration of phosphorylated p38 in complex with phosphorylated MK2 is given by eq. 12.

$$p38 : MK2P_{\text{nuc}} \leftrightarrow p38P : MK2P, r_{87} = k_{87}C_{p38P:MK2P_{\text{nuc}}}, r_{88} = k_{88}C_{p38P:MK2P} \quad (eq. 12)$$

Kinetic parameters and initial concentrations for eq. 12 are as follows: $k_{87} = 0.0045 \text{ s}^{-1}$, $k_{88} = 0.0018 \text{ s}^{-1}$, $C_{p38P:MK2P_{\text{nuc}}}(0) = 0.0$, $C_{p38P:MK2P}(0) = 0.0$.

The amount of p38 MAPK in the nucleus is also dependent on the rate of p38 dephosphorylation by p38 phosphatases ($p38P_{\text{Pase}}$) that occurs in the nucleus ($k_{80}$ is the rate at which $p38P_{\text{Pase}}$ complex dissociates) as follows:

$$p38_{\text{nuc}} + p38P_{\text{Pase}}_{\text{nuc}} \leftrightarrow p38P : Pase_{\text{nuc}}, r_{78} = k_{78}C_{p38P_{\text{nuc}}}C_{p38P_{\text{Pase}}}, r_{79} = k_{79}C_{p38P:Pase_{\text{nuc}}} \quad (eq. 13)$$
The kinetic parameters and initial concentrations for eq. 13 are as follows: $k_{78} = 0.69 \text{ uM}^{-1} \text{s}^{-1}$, $k_{79} = 0.011 \text{ s}^{-1}$, $C_{p38P\text{nuc}(0)} = 0.0 \text{ uM}$, $C_{p38P\text{Ppasenuc}(0)} = 0.0033 \text{ uM}$, $C_{p38P:P\text{pasenuc}(0)} = 0.0 \text{ uM}$. The concentration of phosphorylated p38 bound to p38 phosphatase in the nucleus is given by eq. 14.

$$p38P : \text{P} \xrightarrow{k_{80}} p38_{\text{nuc}} + p38P_{\text{P} \text{nuc}}$$  \hspace{1cm} (eq. 14)

The kinetic parameters and initial concentrations for eq. 14 are as follows: $k_{80} = 0.056 \text{ s}^{-1}$, $C_{p38P:P\text{pasenuc}(0)} = 0.0 \text{ uM}$, $C_{p38\text{nuc}(0)} = 0.0 \text{ uM}$, $C_{p38\text{Ppasenuc}(0)} = 0.0033 \text{ uM}$

Here, the initial concentration of p38 was measured experimentally and reflects total p38 in the cell (data not shown). Previous work on endothelial gap formation has been shown to be regulated by molecules that mediate contractility (Verin, Patterson et al. 1995; Wojciak-Stothard, Potempa et al. 2001; Yamanishi, Katsumura et al. 2006; Liu, Guevara et al. 2009). In our model, VE-cadherin phosphorylation (VE-cadherinP) was catalyzed by myosin light chain (MLC) phosphorylation ($k_{33}$) and HSP27 phosphorylation ($k_{101}$), which are downstream of calcium and p38 MAPK pathways, respectively, and have been cited in the literature as proteins that regulate cell contractility (Liu, Guevara et al. 2009; Liu, Guevara et al. 2010). The reactions are given below.

$$\text{VE} - \text{cadherin} \xrightarrow{k_{33}} \text{VE} - \text{cadherinP}$$  \hspace{1cm} (eq. 15)

The kinetic parameters and initial concentrations for eq. 15 are as follows: $k_{33} = 0.00027 \text{ uM}^{-1} \text{s}^{-1}$, $C_{\text{MLCP}(0)} = 0.0 \text{ uM}$, $C_{\text{VE-cadherin}(0)} = 1.0 \text{ uM}$, $C_{\text{VE-cadherinP}(0)} = 0.0 \text{ uM}$. The other
major event regulating cadherin junctions is VE-cadherin phosphorylation catalyzed by hsp27. The change in VE-cadherin phosphorylation is shown in eq. 16.

\[
\text{VE-cadherin}^{\text{P}} \xrightarrow{k_{101}} \text{VE-cadherin} \quad , \quad r_{101} = k_{101} C_{\text{Hsp27P}} C_{\text{VE-cadherinP}}
\]  

(eq. 16)

The kinetic parameters and initial concentrations for eq. 16 are as follows: \( k_{101} = 1 \text{ uM}^{-1} \text{s}^{-1} \), \( C_{\text{Hsp27P}}(0) = 0.0 \text{ uM} \), \( C_{\text{VE-cadherin}}(0) = 1.0 \text{ uM} \), \( C_{\text{VE-cadherinP}}(0) = 0.0 \text{ uM} \)

VE-cadherin phosphorylation can be reversed through binding its phosphatase (\( k_{102} \)) shown by the following expression.

\[
\text{VE-cadherinP} + \text{VE-cadherinP}_{\text{pase}} \xrightarrow{k_{102}} \text{VE-cadherin} \quad , \quad r_{102} = k_{102} C_{\text{VE-cadherinP}} C_{\text{VE-cadherinP}_{\text{pase}}}
\]  

(eq. 17)

The kinetic parameters and initial concentrations for eq. 17 are as follows: \( k_{102} = 1 \text{ uM}^{-1} \text{s}^{-1} \), \( C_{\text{VE-cadherinP}}(0) = 0.0 \text{ uM} \), \( C_{\text{VE-cadherinP}_{\text{pase}}}(0) = 0.1 \text{ uM} \), \( C_{\text{VE-cadherin}}(0) = 1.0 \text{ uM} \)

5.2.7 Statistical analysis.
All results are means ± SEM. Pairwise t-tests were used for analysis and one way ANOVA was used for multiple comparisons. \( p < 0.05 \) was considered statistically significant.

5.3 RESULTS
5.3.1 Tumor induced gap formation occurs upon VCAM-1 binding. In our previous work we found that tumor cells induce a dramatic breakdown of endothelial junctions upon contact. Co-culture of A2058 tumor cells with 99% confluent HPMEC monolayers for 45 minutes resulted in a disruption of the continuous green fluorescent signal from
the endothelial junctions. The disruption of VE-cadherin led to large gaps in regions where tumor cells reside. Separate brightfield images showed clusters of tumor cells in regions of gap formation (Fig. 5-2C-D) compared to the control cases where the continuous green fluorescence showed intact endothelial junctions (Fig. 5-2A-B).

Quantitative analysis of gap formation showed a 32% increase in gap area during tumor cell and endothelial cells co-cultures for 45 minutes (Fig. 5-2E). Several factors mediated endothelial gap formation including VCAM-1 receptor binding (Fig. 5-2E) and chemokines such as IL-8 and IL-1β (Fig. 5-3A). The neutralization of both chemokines dramatically reduced gap formation close to control levels (around 10%) (Fig.5-3B). In the present model, we focused on how VCAM-1 regulated downstream p38 MAPK kinetics. Stimulation of HPMECs resulted in a 22% increase in gap formation compared to the control case (Fig. 5-2E). Previous work has shown that both p38 and calcium

**Figure 5-2:** Gap formation in human pulmonary endothelial cells is comparable to HUVECs. (A-B) A continuous VE-cadherin staining pattern in endothelial cells. (C-D) Loss of VE-cadherin expression near the membrane regions of the endothelial cells was caused by A2058 cells contact after 45 min (labeled using Alexa 546 cell tracker red dyes (Molecular Probes). Arrows indicate the sites of gap formation. (E) Quantification of endothelial junction disassembly. Endothelial gap formation dramatically increases during melanoma cell/endothelial cell co-cultures after 45 minutes and cross-linking of anti-VCAM-1 antibodies using Fab2 fragments.
pathways regulate endothelial permeability via IL-8 and IL-1β (Ohkawa, Ikeda et al. 1995; Hashimoto, Matsumoto et al. 1999; Murdoch, Monk et al. 1999).

While the effects of IL-8 and IL-1β on downstream p38 phosphorylation have been well studied, less is known about how VCAM-1 mediates p38 kinetics. Based on previous reports, we hypothesized that VCAM-1 regulated p38 MAPK via activation of PAK, a direct target of Rac GTPases.

5.3.2 Anti-VCAM-1 mediates activation of PAK via binding of Rac-GTPases. In the current work, we found that VCAM-1 stimulation of HPMECs induced Rac/PAK interactions at the endothelial junctions. In the case of VCAM-1 stimulation, Rac/PAK interactions first increased slightly (Fig. 5-4 A-B) at endothelial junctions and then decreased and dissociated over 3 minutes (Fig. 5-4 D-E). These interactions were identified by red and blue regions indicating high levels of FRET signal (5-4 B and E). Quantification of FRET signals found that there was an overall decrease in Rac/PAK localization at the endothelial junctions when stimulating HPMEC with anti-VCAM-1 (Fig. 5-4 G-H). The colocalization of these proteins at endothelial junctions formed by VE-

**Figure 5-3: Neutralizing IL-8 and IL-1β reduces endothelial gap formation. (A)** Results show that neutralization of either IL-8 or IL-1β significantly decreases the degree of gap formation from 33% to 20%, (B) Results show that neutralization of both IL-8 and IL-1β (“mAb”) results in a decrease in gap formation from 33% to nearly 12%. Scale bar = 15 μm Results show the mean ± SEM; n = 6–19.
cadherin (Fig. 5-4 C and F) show the importance of Rac/PAK proteins during gap formation. We hypothesized that VCAM-1 regulates p38 phosphorylation via Rac/PAK activation during melanoma/endothelial interactions.

To elucidate the role of VCAM-1 on Rac/PAK kinetics and downstream kinases (p38 MAPK), we constructed a model of the signaling network involved in gap formation. The model included external stimuli that regulated endothelial gap formation (IL-8, IL-1β, VCAM-1), calcium, and p38 MAPK signaling pathways. Construction of the model was based on our studies that showed that inhibiting proteins in these signaling cascades affected gap formation in endothelial cells (Verin, Patterson et al. 1995; Khanna,

Figure 5-4: FRET imaging shows dissociation of Rac/PAK molecules from endothelial junctions. FRET imaging shows the initial localization of Rac/PAK signals at the endothelial junctions (A-B) where VE-cadherin also resides (C). As cells are stimulated, the molecules dissociate from these areas within the endothelial cells (D-E). Fluorescent images show that VE-cadherin also dissociates from endothelial junctions over 45 minutes of VCAM-1 stimulation (via FAB cross-linkers) to form gaps (F). High levels of FRET are marked by white arrows and shown by red and blue regions. G-H shows the decrease in colocalization of Rac/PAK signals at endothelial junctions. (A-C, D-E) Scale bar = 10 μm. (F) Scale bar = 2 μm. Results show the mean ± SEM; n = 3.
Furthermore, our studies show that specific binding of VLA-4 expressing k-562 cells result in changes in spatial binding of Rac and PAK molecules (Fig. 5-5).

A. FRET signaling in endothelial cells in response to VLA-4 adhesion

B. FRET signaling in endothelial cell borders in response to VLA-4 adhesion

Figure 5-5: Direct binding of VLA-4 to VCAM-1 results in dissociation of Rac/PAK signaling from VE-cadherin and increase in binding within the cytosol. A. Near endothelial borders, membrane ruffling occurs (right panel). B. FRET analysis shows that Rac/PAK dissociation occurs near endothelial junctions when cells are adhered. To
ensure these events are not activated by the force of the micropipette or wild-type k-562 cells, we repeated these experiments using inert bead (A).

Micropipette was used to hold a transfected or wild-type k-562 cell and position it over endothelial cells co-transfected with Rac-YFP and PBD-CFP (Fig. 5-5A). The adhesion of VLA-4 of VCAM-1 to endothelial cells results in membrane ruffling (fingerlike projections) (5-5A-B) after 3 minutes. To ensure that these events were not triggered by the force of the micropipette or by other surface molecules, wild-type k-562 cells were used to stimulate endothelial monolayers and show little to no change in FRET over time (Fig. 5-5A).

5.3.3 Model simulations reveal that VCAM-1 signals increase Rac and PAK activation levels over time. Our model was constructed using a system of ODEs incorporated into a SBtoolbox module driven by MATLAB software. Our inputs to the model were based on experiments and literature, and simulations were used to compare proteins concentrations over time when perturbing specified signals (Fig. 5-6).

Figure 5-6: Model Schematic. Represents a schematic of data and software used to construct an ODE network model with inputs, a modeling cascades, and simulations.
The signaling map in Fig. 5-7 shows all proteins incorporated into the ODE model based on previous literature (Vera, Sun et al. 2007) and experimental analysis (Hendriks, Hua et al. 2008). In this study, we focused on VCAM-1 mediated Rac/PAK interactions since these proteins are localized to the junctions in neighboring cells where VE-cadherin also resides. Using model simulations we perturbed VCAM-1 to determine the influence of VCAM-1 activation on Rac/PAK signaling. We abolished VCAM-1 activation, but allowed IL-8 and IL-1β stimulation of the network over 45 minutes since gap formation was observed to occur within 45 minutes.

The activation of Rac and PAK eventually reached a steady state at 45 minutes (Fig. 5-8A and 5-9A). Turning on VCAM-1 resulted in a 10% increase in Rac and PAK during the initial activation of these proteins in the first 20 seconds (Fig. 5-8B and 5-9B). Since

**Figure 5-7**: Schematic of network connections and model parameters.
Rac and PAK are upstream of p38 and VE-cadherin, we hypothesized that VCAM-1 may regulate p38 phosphorylation via its Rac and PAK effector proteins.

Figure 5-8: Computational simulations show that shutting off VCAM-1 decreases Rac activation levels. Over 45 minutes, Rac reaches steady state within 10 minutes (A). Peak concentrations of Rac decrease from 2.25 μM to approximately 1.8 μM when turning off VCAM-1 signaling over 20 seconds (B).
5.3.4 Model simulations confirm that VCAM-1 stimulates p38 phosphorylation over short time points. Since VCAM-1 alters upstream Rac/PAK protein interactions, we confirmed that VCAM-1 alone was able to induce p38 phosphorylation (Fig. 5-10A) using

Figure 5-9: Computational simulations show that shutting off VCAM-1 decreases PAK activation levels. Over 45 minutes, PAK reaches steady state within 10 minutes (A). Peak concentrations of active PAK decrease from 0.09 μM to 0.07 μM over 20 seconds when turning off VCAM-1 signaling (B).
western blot analysis. These simulations were compared to computational simulations, which showed similar kinetics as experimental results (Fig.5-10A).

**A. Experimental validation of p38 phosphorylation kinetics**

![Graph showing p38 phosphorylation kinetics](image)

**B. Experimental comparison of PAK and p38 MAPK kinetics**

![Graphs showing PAK and p38 MAPK kinetics](image)

**Figure 5-10: Comparison of model simulations and experimental data. (A-B).** Fold changes in p38 and PAK phosphorylation in response to VCAM-1 stimulation of endothelial cells was confirmed using western blot analysis and was compared to computational predictions (A). Results are representative of 3 separate experiments. (n = 3). Computational simulations show that VCAM-1 increases phosphorylated p38 levels over 10 minutes and levels decrease over 45 and 90 minutes while PAK levels remained high after 45 minutes and 90 minutes (B).

Our simulations show that p38 phosphorylation increases to a maximum after 10 minutes and then decreases rapidly over 45 and 90 minutes. Since p38 is downstream of Rac/PAK, we hypothesized that Rac or PAK kinetics could effect p38 MAPK phosphorylation. In the next set of western blots we compared levels of
activated/phosphorylated PAK to simulations of active PAK over 90 minutes (Fig. 5-10B). We found some deviation of PAK activation in experimental and computational simulation after 45 minutes. We found that PAK remained active over long periods of time while p38 phosphorylation peaked after 10 minutes and then dramatically decreased over 45 and 90 minutes (Fig. 5-10B). These events were not effected by Rac activation (Fig. 5-11A-B).

Figure 5-11: Rac activation rates do not influence p38 and VE-cadherin phosphorylation. (A-B). Increasing Rac off rates has little effect on p38 and VE-cadherin phosphorylation over time. Changing the off rates by 50 % resulted in no changes in p38 or VE-cadherin phosphorylation.
5.3.5 Model simulations suggest that p38 MAPK and VE-cadherin phosphorylation over time are insensitive to Rac dynamics but sensitive to PAK dynamics. Altering Rac kinetics did not change downstream p38 MAPK and VE-cadherin phosphorylation (Fig. 5-11 A-B). However, increasing PAK off rates had less significant effects of p38 and VE-cadherin phosphorylation rates. Concentration of p38 phosphorylation levels changed from 3 μM to 2 μM when PAK off rates increased from 1 s\(^{-1}\) to 1.5 s\(^{-1}\) (Fig. 5-12).

![Figure 5-12: Increasing the off rate of PAK decreases p38 phosphorylation. Increasing PAK activation off rates from 1 s\(^{-1}\) to 1.5 s\(^{-1}\) resulted in a decrease in p38 phosphorylation levels over 2700 seconds from a peak concentration of 3.2 μM to 2.8 μM concentration.](image)

Interestingly enough, increasing the kinetic off rates by 50% resulted in a decrease in p38 and VE-cadherin phosphorylation levels (Fig. 5-13A-B) within a time frame of 45 min. These results show that altering the upstream kinetics has dramatic effects on downstream p38 MAPK concentrations over time (Fig. 5-12).
Figure 5-13: PAK off rates influence VE-cadherin phosphorylation at early, but not longer timepoints. We found that PAK activation on VE-cadherin phosphorylation only affected the initial VE-cadherin phosphorylation levels where decrease in PAK off rates resulted in faster VE-cadherin phosphorylation states (B-C).
5.4 DISCUSSION

In the above studies we found that melanoma cells induce changes in VE-cadherin disassembly via binding of VCAM-1 receptors on endothelial cells. While other external stimuli are secreted into the tumor microenvironment, we found that IL-8 and VLA-4 have been cited in literature as potential therapeutic targets (Klemke, Weschenfelder et al. 2007). Consequently, it is important to understand how signaling by adhesion molecules is modulated by chemokines to allow development of better therapies targeting these molecules. However, stronger effects on signaling may be modulated by other cytokines other than IL-8, therefore the present model for tumor extravasation needs additional modules and further studies on how these signaling cascades are integrated. In the current model we included the p38 MAPK pathway and calcium pathways that have been deemed important from previous experiments. Inhibiting components of these pathways results in a dramatic decrease in endothelial gap formation (Andrews, Ho et al. 2003; Peng, Hodgson et al. 2005; Peng 2007; Cai, Liu et al. 2008). Furthermore VCAM-1, IL-8 and IL-1β have been shown to regulate the activation of calcium and p38 MAPK pathways.

Our current results show active Rac/PAK signaling near the border of single endothelial cells, but this activity is localized to endothelial junctions when endothelial cells bind VLA-4 ligands and are pulling apart. To study PAK and p38 phosphorylation dynamics in response to changes in VCAM-1 activation. We used model predictions since it is not possible to monitor the changes in protein kinetics in response to VCAM-1 activation rates using experimental methods alone to capture intracellular binding upon the moment of receptor activation. In general, these network models are robust to large variations in model parameters, Measurements of one or a few specific kinetic parameters may not have a large impact on simulations of a complex model (Schmidt
and Jirstrand 2006). So, the question becomes why use the model? In this case we used the model to provide some insights into the changes in the dynamics of signaling pathways during receptor activation, which are not always evident using experimental methods.

Using FRET imaging we confirmed that endothelial junctions contain high levels of active Rac and PAK molecules that dissociate to bind downstream molecules. Using computational simulations, we found that PAK activation off rates dramatically alter downstream p38 phosphorylation concentrations compared to altering the on rates of PAK activation. These results show that decreasing the off rates of PAK_act results in nearly a 2-fold increase in the rate of p38 phosphorylation. Possible reasons could be decreasing the off rate for activated PAK results in an increase in the number of molecules activated when bound to Rac GTPases. Moreover, studies have found that Rac and PAK modulate downstream contractility of endothelial cells that is potentially modulated by the rate at which PAK binds cytosolic molecules, creating large enough gaps for tumor cells to extravasate through the blood vessels. Rac and PAK regulate contractility of the cell by releasing active PAK molecules upon binding of GTP bound Rac molecules (membranes). PAK then dissociates from the endothelial junctions and then binds downstream MLC molecules on their serine residues. These events result in activation of myosin II and assembly of actin and myosin complexes and these events result in a contractile force pulling the junctions apart. In the present model, we did not include mechanical forces; however, several papers have correlated the contractile force with the levels MLC phosphorylation regulated by Rac/PAK molecules. Measurements of these phosphorylation levels can be used to derive the contractile force (Buscemi, Foster et al. 2002; McFawn, Shen et al. 2003; Wirth, Schroeter et al. 2003; Wang, Li et al. 2007).
In this study we find that IL-8 is able to alter PAK activation in response to VCAM-1 signaling. While initially the slight changes in PAK activation rates would not seem significant, we found that slight changes in PAK activation rates has dramatic changes in p38 MAPK signaling. Further studies are needed to determine if completely abolishing IL-8 alters p38 MAPK phosphorylation further downstream of Rac/PAK molecules. Furthermore, our previous studies using sensitivity analysis have found that specifically intracellular calcium levels play a significant role in modulating VE-cadherin15. In the present model, we have not found that PAK affects calcium pathways, which may be attributed to missing proteins or connections in the model. Another possibility may be that intracellular calcium regulates downstream kinases via parallel activation or through other downstream MAPKs such as myosin light chain (MLC) (Buscemi, Foster et al. 2002). In addition proteins not included in the model affect this phenomenon described by model simulations. It is possible signal amplification of downstream signals may be modulated by feedback loops and inhibitory cross-talk between MAPK pathways. Overall, the model is surprisingly robust to large changes in parameter values; thus, the simulations might not be affected by some missing parameters/proteins. On the other hand, the effect of these missing proteins depends largely on their location within the topology of the network. For instance, not including a specific feedback loop may cause discrepancies between theoretical simulations and experimental data. For the most part we found the model predictions are accurately depicting the behavior of specific intracellular proteins. In some proteins the oscillatory behavior due to the multiple phosphorylation sites (MLC, MLCK) are more complex and require advanced partial differential equations to include the compartmentalization of proteins and positive or negative feedback loops (Ferrell, Tsai et al. 2011).
In summary, we present integrated computational and experimental analyses that show that VCAM-1 activation stimulates PAK activation. A decrease in the PAK off rates dramatically affects downstream p38 MAPK phosphorylation. We found that an decrease in PAK off rates result in amplification of p38 phosphorylation, while increasing the off rate results in an decrease of p38 and phosphorylation over longer periods of time. These results show a similar trend to that observed experimentally. In other words, slight there are very small changes in PAK deactivation at 45 minutes, but downstream p38 phosphorylation dramatically decreases to baseline levels.
Chapter 6

CONCLUSIONS AND FURTHER RESEARCH

6.1 Rationale for melanoma metastasis studies. Cancer metastasis, rather than primary tumors themselves, result in the most deaths in melanoma cancer, breast cancer, and liver cancer. As a result, early detection of cancer can be cured by surgery or local irradiation. Cancer metastasis is a complex process governed by several steps (Patel et al., 2001; Woodhouse et al., 1997). While the metastatic process is defined by several events, characterizing the mechanisms involved in this process remains a challenge. A better understanding of this process will lead to the development of improved therapeutics to prevent cancer metastasis. The current work chooses melanoma cancer as the primary cancer of study because it is a model for studying metastasis because of its highly invasive potential (Todaro, Fryling et al. 1980). The studies and methodologies presented in this thesis work could be applied to breast and liver cancers.

6.2 Gene expression in early and late stage melanomas. In late stage cancers, inhibiting both mutant (V600E) and wild/type B-Raf genes inhibit the invasiveness capabilities of these melanomas(Liang, Sharma et al. 2007); however, this is not the case in early stage melanoma cells. Our current hypothesis is that in highly invasive cell lines such as UACC 903M, A375M, and A2058 cells, there is no CD82 expression so knocking down B-Raf upstream of CD82 tetraspanins results in a decrease in IL-8 secretion and possibly VLA-4 expression. However, in early stages of melanoma cancer we find that knocking down B-Raf in early stage or primary melanoma cells including WM35 and SbCl2 cells result in inhibition of CD82. Current results show that knocking
down CD82 expression in early stage melanoma cells increases its invasive capabilities as shown by \textit{in vitro} flow chambers and collagen IV assays.

\textbf{6.3 CD82 expression regulates IL-8 secretion and VLA-4 binding.} While previous studies have found that CD82 regulates VLA-4 adhesion affinities, few studies have shown the role of CD82 in IL-8 secretion. Studies by Jee et-al. show that expression of CD82 attenuates the maturation of the \(\beta_1\) subunit, which regulates the migratory function of melanoma cells towards collagen IV (Hodgson, Henderson et al. 2003). Furthermore, the \(\beta_1\) subunit of the VLA-4 integrins functions in allowing adhesion of VLA-4 to its ligand, VCAM-1. Studies by (Chigaev, Zwartz et al. 2004) et-al. show that IL-8 secretion and binding to melanoma cells, activates cGMP pathways that increase the affinity states of VLA-4, mediated by the \(\beta_1\) subunit. Here, studies by Chigaev et-al. used fluorescent ligand binding to evaluate the integrin activation state on live cells in real-time (Chigaev, Blenc et al. 2001; Chigaev, Zwartz et al. 2003; Chigaev, Zwartz et al. 2004; Chigaev, Waller et al. 2007; Chigaev, Smagley et al. 2011). These studies showed that cGMP signaling pathway (activated by IL-8) a can rapidly down-modulate binding of a VLA-4 specific ligand on cells pre-activated through three G\(\alpha_i\)-coupled receptors: wild type CXCR4, CXCR2 (IL-8 receptor type B). Our current results show that CD82 expression suppresses IL-8 secretion. These events could prevent adhesion of melanoma cells by suppressing maturation of the \(\beta_1\) subunit of VLA-4 integrins and decrease the overall affinity states of the VLA-4 integrin through suppression of IL-8 signaling (Chigaev, Smagley et al. 2011). Consistent with previous studies, we have found that transfecting CD82 cDNAs into late stage melanomas prevents activation of VLA-4 integrins (Durpes, Hardy-Dessources et al. 2011)

Furthermore, our results show that CD82 binding to DARC receptors prevent IL-8 binding to these receptors. While DARC has been shown to be a “chemokine sink” to
modulate the binding of cytokines, in endothelial cells DARC translocates IL-8 to endothelial cell surface. IL-8 has long been shown to be a chemoattractant for other melanoma cells and PMNs (Dawson, Lentsch et al. 2000; Zarbock, Bishop et al. 2010). Our current work shows that 5AzaC (a reagent that restores CD82 expression) reduces recruitment of PMNs to inflammatory sites on endothelial cells and subsequent melanoma cell extravasation in late stage melanoma cells. However, further studies are needed to show whether DARC and IL-8 binding influence the recruitment of PMNs to inflammatory sites that facilitate melanoma cell extravasation (Zarbock, Bishop et al. 2010).

6.4 IL-8 secretion and VLA-4 binding regulates endothelial gap formation. The above work shows the role of CD82 in regulating extracellular signals from the tumor microenvironment. The question remains as to how these extracellular signals (including IL-8 and VLA-4) regulate endothelial signaling during gap formation. Previous studies have shown that calcium signaling plays a key role in endothelial gap formation when endothelial cells are in contact with melanoma cells (Peng et-al., 2007). Consistent with these results, neutralizing IL-8, an activator of calcium and cGMP signaling, reduces gap formation in endothelial cells. Furthermore, our current studies using siRNA against IL-8 nucleofected into melanoma cells reduces endothelial gap formation.

In addition, we found that stimulating VCAM-1 receptors on endothelial cells, a ligand to VLA-4, increasing gap formation over 45 and 90 minutes. Previous studies show integrin signaling via formations of the Src/FAK complex is involved in many signaling pathways and play an important role in adhesion and transendothelial migration ((Na, Collin et al. 2008). The localization of Src and FAK play an important role in regulating the actin cytoskeleton; however, Rac is directly activated by adhesion and these events are not depend on lipid rafts in the plasma membrane, but cytoskeletal
integrity ((Poh, Na et al. 2009)). We wondered whether direct binding of VLA-4 with VCAM-1 increases gap formation via Rac GTPases and downstream p38 MAPK(Nwariaku, Chang et al. 2002; Liu, Guevara et al. 2009). We show that inhibitors targeting p38 MAPK proteins reduce tumor induced gap formation by more than 50% when compared to the control case. Since IL-8 and VCAM-1 regulate upstream Rac GTPases, we hypothesized that perhaps IL-8 signaling was able to modulate VCAM-1 signaling in endothelial cells. However, the complexity of signaling networks and numerous upstream effectors of p38 MAPK make it difficult to pinpoint proteins that specifically regulate p38 phosphorylation during stimulation of the networks with multiple stimuli. As a result of the complexities of intracellular networks, we used network modeling to study p38 phosphorylation kinetics in response to IL-8 and VCAM-1.

6.5 VCAM-1 activation increases PAK kinetics to amplify downstream p38 phosphorylation activation. Since our previous work shows the importance of the calcium and p38 MAPK signaling pathways to play a vital role in gap formation, we used literature to assemble a network model of proteins in each of these signaling networks(Schoeberl, Eichler-Jonsson et al. 2002; Hendriks, Hua et al. 2008). Since we were interested in how different stimuli influence the p38 MAPK kinetics, an ODE model was used to simulate changes in protein concentrations over time. Using steady state sensitivity analysis, we perturbed signaling networks with three inputs: IL-8, IL-1β, and VCAM-1. Using K-S statistics, proteins most sensitive to VE-cadherin phosphorylation were calculated. Our results show that PAK and VCAM-1 activation functions as important mediators of gap formation along with p38 MAPK and Src. Model predictions were supported by experimental FRET imaging showing that VLA-4/VCAM-1 interactions induce dissociation of Rac and PAK proteins within endothelial cells. These results are consistent with previous studies showing rapid Rac activation near sites of
integrin mediated adhesion (Poh, Na et al. 2009). Furthermore, inhibiting Rac (a direct activator of PAK) decreases gap formation in endothelial cells. Currently, we hypothesized that the cytoskeleton could play a role in transducing Rac signaling to other parts of the cell. While our experimental data shows that our theoretical predictions are correct, further studies are needed to validate model findings.
### APPENDIX

**MODEL PARAMETERS AND INITIAL CONDITIONS**

**Table 5-1: Reactions and parameters used in network**

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<th>Description</th>
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<th>Description</th>
<th>Value</th>
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<td>MKK6P/PhosphataseMKK6\textsuperscript{4}</td>
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<td>K29</td>
<td>Ca\textsubscript{2+} $\rightarrow$ Ca\textsubscript{2+} ext\textsuperscript{1}</td>
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Table 5-2: Initial conditions of network

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<tr>
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<td>Gaβγ²</td>
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<td>PIP2¹</td>
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Table 5-2: Initial conditions of network continued...

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<th>Parameter</th>
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Table 5-2: Initial conditions of network continued...

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<th>Parameter</th>
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<td>p38P (nucleus)</td>
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<td>p38P:MK2</td>
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<td>MK2 (nucleus)</td>
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<td>p38P:MK2P (nucleus)</td>
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<tr>
<td>p38P:MK2P</td>
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<td>MK2P</td>
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*The above parameters were pulled from several sources including:

1*(Peng 2007)*
2*Not found in literature (estimated as 1 or 0) or estimated using software*
3*(Nishikawa, Sellers et al. 1984)*
4*(Hendriks, Hua et al. 2008)*
REFERENCES


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 Thesis: HMG-CoA Reductase Inhibitors Act Synergistically With UCN-01 through Ras inhibition.
Advisors: Prof. Steven Grant and Prof. Gary Bowlin

Virginia Commonwealth University May 2002
B.S., Biomedical Engineering

JOURNAL PUBLICATIONS


7. Khanna, P, Chung CY, Neves R, Robertson, GP. CD82/KAI expression Prevents IL-8 mediated endothelial gap formation in late stage melanomas via p21/Waf1 signaling. Submitted; December 2011

8. Chung CY, Khanna P, Targeted Drugs Promote Epigenetic Changes leading to more Aggressive Cancer. Submitted; December 2011