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ANALYSIS OF ENDOTHELIAL CELL CONTRACTILITY IN MELANOMA-INDUCED JUNCTION DISRUPTION OF ENDOTHELIAL MONOLAYERS

A Thesis in
Chemical Engineering

by
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ABSTRACT

Tumor metastasis occurs as a multiple step process culminating in tumor cells leaving the bloodstream through the endothelial layer due to the disruption of cell-cell junctions. Soluble signaling from tumor secreted proteins and receptor-ligand binding facilitate interactions between tumor cells and endothelial cells that are known to mediate junction disruption in endothelial cell layers. Previous work has shown that soluble factors alone, such as interleukins which are known to be pro-inflammatory, can promote junction disruption, making a case for endothelial cell contractility as a regulator of junction disruption and extravasation.

In this work we used melanoma as a model of metastatic cancer and sought to elucidate the signaling pathways that are involved in mediating junction disruption in the endothelium, in response to tumor cells. Isolation of endothelial signaling was achieved by incubating endothelial cells with cytoskeletal inhibitors before soluble factor treatment or co-culture experiments. A reduction in gap formation was measured when inhibited monolayers were co-cultured with A2058 cells, as well as with K562 cells which were used as a model to isolate receptor-ligand binding. Inhibitor experiments implicate Rho, Rac and Src pathways in junction disruption mediated by melanoma.

Furthermore, we measured the contractility of groups of endothelial cells in response to an inflammatory cytokine secreted by melanoma, and found that endothelial monolayer contractility decreased more rapidly than control treatments. These data show that soluble factors secreted by melanoma are able to affect contractility in endothelial cells, which is borne both by cell-cell forces as well as cell-substrate coupling.
These results suggest possible therapeutic targets to mitigate the spread of metastatic cancers, through the regulation of endothelial permeability.
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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens junction</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>G-actin</td>
<td>Globular actin</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HPMEC</td>
<td>Human pulmonary microvascular endothelial cell</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cell</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular cell adhesion molecule 1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin 1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin 8</td>
</tr>
<tr>
<td>LFA-1</td>
<td>Leukocyte function-associated antigen 1</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MLC 2</td>
<td>Myosin light chain 2</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MPT</td>
<td>Myosin light chain phosphatase</td>
</tr>
<tr>
<td>PAK</td>
<td>p21 activated kinase</td>
</tr>
<tr>
<td>P</td>
<td>Free phosphate</td>
</tr>
<tr>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
</tr>
<tr>
<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
</tr>
<tr>
<td>ROCK</td>
<td>RhoA kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TC</td>
<td>Tumor cell</td>
</tr>
<tr>
<td>TCM</td>
<td>Tumor conditioned media</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>Vascular endothelial cadherin</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen 4</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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</table>
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Chapter 1: BACKGROUND

1.1 Melanoma

Melanoma is a type of cancer that originates in melanocytes, the melanin pigment producing cells, as well as other pigmented tissues. The most common cause of skin cancer is from exposure to the sun, particularly in the elderly and those with compromised immune systems (NCI 2014). The American Cancer Society estimates that there will be 76,100 new cases of melanoma diagnosed in the United States in 2014, and that 9,710 people are expected to die this year from melanoma. It accounts for 2% of total skin cancer, but causes a majority of deaths (NCI 2014). Melanoma is a skin cancer cell with a particularly invasive phenotype. It has the ability to adhere to the endothelium and transmigrate, as well as degrade extracellular matrix (ECM) proteins and pass through the basement membrane. A highly locomotive cell line, A2058 has been shown to exhibit a chemotactic response to ECM proteins laminin, fibronectin and type IV collagen (Quinones and Garcia-Castro 2004). Additionally, melanoma displays autocrine and paracrine chemotaxis with exposure to its own conditioned media and to conditioned media from other melanoma types (Quinones and Garcia-Castro 2004). A highly metastatic line such as A2058 may also use the chemotactic response to soluble factors to drive migration toward sites of adhesion, via the cytokines released from normal cells. See Figure 1.1 for a schematic of the model system of melanoma interaction with the endothelium. Melanoma metastasizes mainly to the bone, brain, liver, lung, skin and muscle (NCI 2014).
Figure 1.1 Melanoma secretes soluble signaling proteins (interleukins) and expresses surface ligands that are capable of interacting with other cells.

1.2 Metastasis

Metastasis is the process by which cells from a primary tumor detach and form secondary tumors in other locations and microenvironments throughout the body (Figure 1.2). Initially, blood flow increases to the tumor site via angiogenesis, with the generation of new blood vessels formed from already existing vessels. The tumor grows in size and tumor cells detach, and migrate away from the tumor site into the bloodstream. During both intravasation and extravasation, entering and leaving the bloodstream respectively, tumor cells must deform and interact with multiple microenvironments (Wirtz, Konstantopoulos et al. 2011). Tumor cells enter the bloodstream and circulate in very low concentrations throughout the body, from 1-100 cancer tumor cells per billion blood cells (Hou, Warkiani et al. 2013). Circulating cells arrest and attach to the blood vessel wall in organs with a microenvironment suited to the
particular tumor cell (Langley and Fidler 2011); they can also aggregate with platelets or adhere when the size of the vessel becomes small enough so as to restrict movement (Crissman, Hatfield et al. 1988, Jeon, Zervantonakis et al. 2013).

**Figure 1.2** Metastasis occurs in multiple steps, culminating with extravasation and the transmigration through the endothelium to form a secondary tumor.

Melanoma cells have been shown to migrate in a paracellular fashion (through intercellular junctions), in brain endothelial cell monolayers, though this does not preclude transcellular migration (Fazakas, Wilhelm et al. 2011). Expression of cytokine receptors corresponding to soluble factors secreted by endothelial cells was found to be upregulated in invasive tumor cells, and when recombinant cytokines were introduced the invasiveness of tumor cells was amplified further. Furthermore, the presence of an endothelial layer increased tumor invasiveness in transmigration assays into collagen
matrices (Mierke, Zitterbart et al. 2008). It has also been shown that endothelial cells enhance the invasiveness of breast cancer cells, and that the tumor cells are able to decrease endothelial cell stiffness in comparison to endothelial monoculture (Mierke 2011). The mechanistic and temporal signaling specifics of tumor-endothelial cell interactions during metastasis are not well understood, though tumor cell contractility, as well as endothelial junction complex disruption due to receptor-ligand and soluble factor signaling have been implicated in aspects of extravasation and may influence invasion (Alcaide, Newton et al. 2008, Mierke 2008, Gavard 2009, Khanna, Yunkunis et al. 2010, Indra, Undyala et al. 2011, Kraning-Rush, Califano et al. 2012, Kraning-Rush, Carey et al. 2012).

Tumor cells secrete small signaling proteins called cytokines, which are received by receptors on local endothelial cells. Signaling pathways from these binding events and from membrane embedded ligands on tumor cells binding to receptors on the endothelium have been implicated in cytoskeletal rearrangement in endothelial cells and VE-cadherin phosphorylation and subsequent junction breakdown (Schraufstatter, Chung et al. 2001, Alcaide, Newton et al. 2008). These are two potent tools that tumor cells employ to form gaps in the endothelium for extravasation.

It is not well understood how melanoma extravasation is mediated by receptor-ligand binding and soluble signals mechanistically. Both are thought to contribute in extravasation, but it is not known how they work in concert.
1.3 Endothelial cells

1.3.1 Types and microenvironment

Human umbilical vein endothelial cells (HUVECs) are a macrovascular cell type derived from the umbilical vein. These cells are well characterized in the literature and thus provide an excellent model system to study. A drawback in their use is that physiologically, we do not expect many tumor cells to be involved in extravasation events in the umbilical vein.

Human pulmonary microvascular endothelial cells (HPMECs) are a type of cell found in small capillaries in the lung. A cell line derived from HPMECs (HPMEC-st1.6r) was developed and characterized by Krump-Konvalinkova et al. at the Johannes-Gutenberg University. The cell line expresses proteins and receptors similar to the primary cells they are derived from (Krump-Konvalinkova, Bittinger et al. 2001, Unger, Krump-Konvalinkova et al. 2002). This cell line provides a useful model system to study the more physiologically relevant lung microvasculature in melanoma extravasation, complementary to HUVEC.

1.3.2 Endothelial junctions

Endothelial cells are connected to each other in monolayers through a wide variety of junction types and proteins. Principally they are classified as tight junctions, gap junctions and adherens junctions. Tight junctions regulate the passage of small molecules and ions and are comprised primarily of claudins and occludins (Fanning, Mitic et al. 1999). Gap junctions are intercellular channels composed of connexins that enable cell-cell signaling via the passage of small molecules and ions (Giepmans 2004).
Adherens junctions provide mechanical connections between cells and are typically formed from cadherins and catenins, and are of interest in processes that mediate cell-cell junction disruption (Meng and Takeichi 2009).

Cadherins mediate structural connections to the actin cytoskeleton. In the case of endothelial cells, vascular endothelial (VE)-cadherin spans cells as a homodimer. Single connecting cadherins can withstand pulling forces of 35-55 pN in the case of VE-cadherin, and are thought to exhibit greater connective strength through clustering, due to their weak unit binding strength and affinity for transcellular interactions (Baumgartner, Hinterdorfer et al. 2000). It has been shown elsewhere that VE-cadherin bond average adhesion force follows Bell model predictions, dependent on the logarithm of the loading rate, applied to the bond (Panorchan, George et al. 2006). It was found that the VE-cadherin/VE-cadherin bond could withstand forces between 32 and 50 pN depending on loading rate (Panorchan, George et al. 2006). Mechanosensing at cell-cell junctions is thought to be mediated by the connection of VE-cadherin to the actin cytoskeleton, namely through interactions with α-catenin and vinculin, though the mechanism is yet unknown (Huveneers and Rooij 2013).

Junction complexes are comprised of many different components. VE-cadherin is a transmembrane protein that facilitates homodimeric interactions to connect endothelial cells. On the intercellular side p120, plakoglobin and β-catenin are known to interact directly with VE-cadherin and with α-catenin which is thought to facilitate the connection to the actin cytoskeleton (Lampugnani, Corada et al. 1995). Little is known about many of the indirect protein interactions that occur in the connection between actin and adherens junctions (Dejana, Orsenigo et al. 2008).
Tumor cells have been thought to use a mechanism similar to leukocytes in extravasation. It has been shown recently however, that vascular permeability induced by inflammatory mediators, secreted by tumor cells, is mediated by phosphorylation of a specific tyrosine residue (Wessel, Winderlich et al. 2014). Leukocytes were found to dephosphorylate a different residue in their extravasation (Wessel, Winderlich et al. 2014). It has also been shown that VE-cadherin tyrosine phosphorylation alone is not sufficient to induce increased permeability, however it likely works in conjunction with other signaling events (Adam, Sharenko et al. 2010).

1.3.3 Cytoskeleton

The endothelial cytoskeleton is composed of a diverse range of structural proteins. Of principle importance to this work are actin stress fibers that form a polymeric network throughout the cell. Stress fibers or microfilaments are composed of bundles of filamentous (F)-actin and provide the cell with structure as well as a network for shuttling proteins. The F-actin polymer is formed from polymerization of free globular (G)-actin monomers present in the cytoplasm. Stress fibers also interact with myosin motor proteins to generate contractility in the cell. This actomyosin activity is what allows cells to pull against one another and against their substrate. We are primarily interested in actomyosin contractility generated by this first class of filaments.

Intermediate filaments are a second class of cytoskeletal protein that is also tension bearing, and more stable than microfilaments. They serve a primarily structural purpose by anchoring organelles and are composed of vimentins and keratin.
Finally, microtubules are hollow fibers, and the largest of the cytoskeletal polymers. They serve as a transport system for organelles and are associated with different motor proteins than microfilaments. Microtubules also play an important role in mitosis, separating chromosomes as cells split.

1.4 Mechanics

The actin cytoskeleton is a polymeric network that connects to both cell-cell adhesion molecules as well as cell-matrix complexes that anchor cells to ECM proteins such as fibronectin (FN), collagens, and laminins. Through these connections, cells are able to generate contractile forces by pulling actin stress fibers against one another with myosin activity. Myosin motor proteins move along actin filaments with a “powerstroke” motion that requires adenosine triphosphate (ATP) hydrolysis. Before the stroke begins, the myosin head is bound in an adenosine diphosphate (ADP)-free phosphate (P_i) complex bound weakly to actin. Docking to actin results in the release of P_i, causing the lever arm to swing to the ADP-bound “post-stroke”. The arm swinging motion moves the actin filament approximately 100 Å and culminates in release of ADP. After ADP release, ATP binding occurs, causing the head complex to revert to a weakly bound state for actin and re-cock, for the cycle to be repeated (Vale and Milligan 2000). As previously described, the connection of actin stress fibers to cell-cell junctions and focal adhesions enables cells to generate contractile forces against the ECM and neighboring cells. The actin cytoskeleton can also generate force through a “treadmilling” mechanism which is the result of actin filament polymerization and depolymerization (Bugyi and Carlier 2010).
Cells coupled together can exert significant forces between one another, at greater strength than single cadherin bonds are shown to be capable of. Liu and coworkers have measured endothelial tugging forces of up to 120 pN, normal to the cell-cell contact surface. They found that these tugging forces regulate adherens junction (AJ) assembly, and that junction size is directly correlated to tugging force (Liu, Tan et al. 2010). Both Rho and Rac pathways were found to participate in adherens junction growth, and tugging force was found to be necessary, though the mechanism is yet unknown. Liu et al. suggest models of AJ growth dependent on membrane protrusion activity, which is Rac mediated.

Contractility is known to be regulated by Rho and Rac pathways in endothelial cells (Nobes and Hall 1995, Schraufstatter, Chung et al. 2001, Wojciak-Stothard, Potempa et al. 2001). Rho activity in cells is associated with increased contractility using the actin cytoskeleton and myosin motors. Rho associated protein kinase (ROCK) is able to phosphorylate myosin phosphatase, increasing the amount of phosphorylated myosin light chain 2 (MLC2) and thereby increasing myosin II activity (Sanz-Moreno, Gaggioli et al. 2011, Ito, Medicine et al. 2013). Myosin light chain kinase (MLCK) activation also promotes actomyosin activity by phosphorylating MLC2 (Dudek and Garcia 2001, Wolfson, Lang et al. 2009). Thus, ROCK and MLCK represent two mechanisms that are able to amplify actomyosin activity in cells.

Rac and rho activity is associated with regulating stress fiber formation in endothelial cells, and has also been shown to become activated in response to shear stress (Nobes and Hall 1995, Wojciak-Stothard and Ridley 2003). It has also been shown to be involved in cell protrusions and lamellipodia formation (Nobes and Hall 1995). Rac
also mediates the formation of cell-cell contacts and tends to stabilize junctions (Tzima 2006). The stabilization of cells via cortical actin development, EC barrier enhancement and permeability restoration are regulated by Rac (Wolfson, Lang et al. 2009). Rac and Rho small GTPases have both contrary and complementary roles in junction dynamics (Wojciak-Stothard, Potempa et al. 2001). In their model for endothelial permeability, Wolfson and coworkers suggest that balance of mechanical forces, namely tethering and contraction, regulate barrier function. Tumor cell influence of Rho and Rac activation in such a balance could therefore promote permeability and extravasation.

Previous work has shown that patterned cells on polyacrylamide gels exert an enhanced force response due to thrombin, mediated by Rho activity (Krishnan, Klumpers et al. 2011). Krishnan and coworkers found that gaps form on stiffer substrates local to the measurement of increased forces. Little is known about force enhancement in response to tumor cell cues such as soluble factors like interleukins. We seek to elucidate the connection between tumor cell cues and endothelial cell contractility in junction disruption in this work.

1.5 Receptor-ligand interactions

1.5.1 VLA-4/VCAM-1

Very late antigen (VLA)-4 is a protein ligand (integrin) expressed on the surface by melanoma cells as well as leukocytes (Garofalo, Chirivi et al. 1995, Liang and Dong 2008, Schlesinger, Schmitz et al. 2012, Valcarcel, Carrascal et al. 2014). Integrins are a heterodimeric transmembrane receptor, responsible for mediating contact with the ECM as well as other cells. Integrins can adopt a variety of conformations depending on
antibody and ion binding to the chain subdomains, and exhibit clustering to regulate signaling from ligand binding (Kornberg, Earp et al. 1991). VLA-4 is comprised of an $\alpha 4$ and $\beta 1$ chain, each of which contain many smaller subdomains and binding sites (Byron, Humphries et al. 2009). Integrins have different binding affinities based on the specific conformations they are able to adopt. Integrin structure includes a head region where the two subunits contact, supported by two distinct leg units that are able to bend, and which link to transmembrane domains. Adoption of an unbent conformation is thought to correspond to a high affinity state (Byron, Humphries et al. 2009). The affinity of integrins such as VLA-4 can be regulated by inside out signaling via Rho GTPase or by direct binding to integrin cytoplasmic domains (Masumoto and Hemler 1993, O'Toole, Katagiri et al. 1994, Howe, Aplin et al. 1998). Cells are able to use these mechanisms to regulate the affinity of their integrins for substrate binding and the formation of focal adhesions to mediate force generation. Khanna and coworkers have shown that the stimulation of HUVEC monolayers with anti-VCAM-1 crosslinking antibodies results in a modest increase in gap formation. Crosslinking antibodies approximate the binding of VLA-4 to the endothelium and indicate that this receptor-ligand interaction is important in A2058 melanoma extravasation (Khanna, Yunkunis et al. 2010).

1.6 Soluble factors

1.6.1 Interleukin-8

Interleukin (IL)-8 is a soluble signaling protein secreted by both tumor and normal cells (Khanna, Yunkunis et al. 2010). When endothelial cells are incubated with IL-8 over time, it activates both Rho and Rac pathways over differing timelines through
distinct surface receptors CXCR1 and CXCR2. Schraufstatter and coworkers demonstrate that Rho induced stress fiber formation can occur after 1 minute. On a more intermediate time scale between 5-10 minutes, Rac mediated cell retraction occurs, followed by longer term cytoskeletal activation from 15-30 minutes, measured by stress fiber formation (Schraufstatter, Chung et al. 2001). Lai et al. also measure the activation of Rho/Rac temporally. They find a local maximum of both Rho and Rac activity at 5 minutes following stimulation. Rac activity achieves a maximal activity significantly later, at 4 hours, in contrast to a maximum for Rho between 30-60 minutes which then decreases over the following hours (Lai, Shen et al. 2011). This underscores the importance of temporal signaling in tumor cell extravasation, given the activation of multiple, distinct pathways over time.

1.6.2 Interleukin-6

Interleukin (IL)-6 is another inflammatory cytokine secreted by A2058 cells and is implicated in loss of permeability via the protein kinase C (PKC) pathway (Desai, Leeper et al. 2002). Permeability in HUVEC monolayers was found to be both dose and time dependent in response to IL-6 (Desai, Leeper et al. 2002). Maruo and coworkers also found that IL-6 induced junction disruption in bovine vascular endothelial cells (BVEC) is attributed to contractile action as a result of actin filament rearrangement. They found that after 21 hours of IL-6 incubation, peripheral actin was reduced and perinuclear actin filaments were realigned. Additionally, in some cells IL-6 incubation resulted in irregular filament alignment. Physiological levels of IL-6 have been measured from 20-100 ng/ml in the blood of arthritis patients, 50 ng/ml in the blood of burn
patients and 6 ng/ml in media conditioned with A2058 melanoma over 24 hours (Maruo, Morita et al. 1992, Khanna, Yunkunis et al. 2010).

1.6.3 Interleukin-1β

Interleukin (IL)-1β is involved in the sustained, delayed inflammatory response (4-6 hours) to bodily injury. IL-1β promotes expression of adhesion molecules such as VCAM-1, for recruiting leukocytes in the case of an injury (Zhang 2008). IL-1β also induced increased contractility in endothelial aorta cells from hypertensive rats with a significant increase over baseline force generation, and an increase was measured at 20 minutes, with a plateau after 1 hour (Dorrance 2007). Furthermore, IL-1β stimulated human pulmonary artery endothelial cell (HPAEC) migration in a simulated wound closure assay on collagen matrices, and that IL-1β treated HPAEC were able to further contract collagen gels in comparison to control (Kanaji, Sato et al. 2011). Tumor cells may use high local concentrations of inflammatory cytokines such as IL-8, IL-6 and IL-1β to influence endothelial cell behavior and aid in extravasation.

1.7 Objectives

In this work, we seek to understand how soluble factors secreted by melanoma and receptor-ligand binding effects endothelial cell contractility in the context of gap formation. Our hypothesis is that increased cellular contractility is required for the formation of gaps between cells, and to cause cell-cell junction disruption. It is not well known if forces exerted by endothelial cells will be enhanced or attenuated in response to cues from tumor cells to mediate trans-endothelial migration. To elucidate this
connection we use traction force microscopy to measure endothelial contractility and the temporal aspect of physical forces in extravasation.

The work will be accomplished in several main thrusts: first by measuring melanoma mediated junction disruption in endothelial monolayers, second measuring junction disruption after blocking pathways known to be involved in contractility, and third by measuring the contractile response in endothelial cells following stimulation.

We demonstrate that melanoma is able to facilitate junctional disruption of endothelial monolayers via receptor-ligand interactions and soluble factors, and that these effects are mediated by signaling pathways that lead to endothelial contractility.
Chapter 2: INDUCTION OF ENDOTHELIAL JUNCTION DISRUPTION BY MELANOMA TUMOR CELL CO-CULTURE

2.1 Introduction

The capability of melanoma cells to pass through the endothelium has been well characterized. Fazakas and coworkers have investigated the ability of melanoma to navigate the blood brain barrier. They find that melanoma attach to junctional sites in order to mediate paracellular migration, and release large amounts of serine proteases to cleave protein peptide bonds (Fazakas, Wilhelm et al. 2011). Both soluble factors secreted by melanoma and surface expressed ligands have been implicated in melanoma extravasation (Khanna, Yunkunis et al. 2010). Another in vitro study by Ghislin et al. shows that tumor conditioned media from tumor cells increases expression of intercellular cell adhesion molecule (ICAM)-1, and that blocking ICAM-1 mitigated trans-endothelial migration of melanoma. They also propose that passage may be cooperative, with leukocyte function-associated antigen (LFA)-1 and ICAM-1 interaction mediating clumping of melanoma cells (Ghislin, Obino et al. 2012).

Increased F-actin levels have been observed in endothelial cells local to sites of melanoma adhesion, in conjunction with changes in melanoma morphology and F-actin distribution (Voura, Sandig et al. 1998). Sandig and coworkers find that during migration, endothelial cells become more flat, local to tumor adhesion sites and VE-cadherin localization was reduced prior to melanoma-endothelial cell intercalation. They conclude that endothelial cells actively participate in transmigration through junction remodeling (Sandig, Voura et al. 1997).
Of interest are the mechanisms melanoma employs to mediate junction disruption—particularly soluble factors such as interleukins and surface adhesion molecules—and the specific signaling that occurs in endothelial cells as a result of interactions with melanoma.

2.2 Methods

2.2.1 Cell culture

Human umbilical vein endothelial cells (HUVEC; HUV-EC-C, CRL-1730) were purchased from the American Type Culture Collection (ATCC). Human pulmonary microvascular endothelial cells (HPMEC), A2058 melanoma cells and “K562-α4” mutant myelogenous leukemia (K562) cells were a gift from Cheng Dong (The Pennsylvania State University). Mutant K562-α4 were originally from Martin Humphries at the University of Manchester, UK (Byron, Humphries et al. 2012) and were transfected to express the α4 integrin. HPMEC were originally a gift from Charles Kirkpatrick at the University of Mainz, Germany (Krump-Konvalinkova, Bittinger et al. 2001, Unger, Krump-Konvalinkova et al. 2002). Wild type (WT) K562 cells were a gift from Ross Hardison (The Pennsylvania State University). HUVEC were maintained in F-12K media (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals), 50 μg/ml heparin (Alfa Aesar), 15 μg/ml endothelial cell growth supplement (ECGS; BD Biosciences), and 50 U/ml-50 μg/ml Penicillin-Streptomycin (Pen-Strep; Life Technologies). HPMEC were cultured in media 199 (M199; Life Technologies) supplemented with 10% FBS, 15 μg/ml ECGS, 2 mM L-glutamine (Life Technologies) and 50 U/ml-50 μg/ml Pen-Strep. A2058 melanoma were maintained in
Dulbecco’s Modified Eagle medium (DMEM; Corning) supplemented with 10% FBS and 50 μg/ml gentamicin (Life Technologies). K562 cells were cultured in RPMI 1640 media (Life Technologies) supplemented with 10% FBS and 50 U/ml-50 μg/ml Pen-Strep; mutant cells were additionally cultured with 1 mg/ml geneticin (Santa Cruz Biotechnology). All cell lines were grown in a controlled atmosphere at 37°C and 5% CO₂.

2.2.2 Monolayer formation

HPMEC: 12 mm glass coverslips were sterilized with ethanol and incubated with 10 μg/ml human fibronectin (FN; BD Biosciences) in 1× phosphate buffered saline solution (PBS) for 2 hours at room temperature. Slips were placed in 24-well plates and washed with 1× PBS before seeding 1×10⁵ HPMECs in 500 µl of complete media 199. Monolayers were monitored for approximately 40 hours until confluent and media was changed to M199 supplemented with 2% FBS, 24 hours prior to experiments. HUVEC: 25 mm glass coverslips were sterilized with ethanol and incubated with 1 μg/ml FN in F-12K media in 6-well plates for 24 hours. After 24 hours, media was removed and 3×10⁵ HUVECs in 2 ml of complete F-12K media. Monolayers were monitored over 72 hours until confluent and media was changed to F-12K media supplemented with 2% FBS, 12 hours prior to experiments.

2.2.3 Cytokines

Human recombinant interleukin 8 (IL-8; Sigma) was reconstituted in a solution of 0.1% BSA in autoclaved deionized water (dH₂O).
2.2.4 Endothelial-tumor cell co-culture

Confluent endothelial monolayers were washed with media supplemented with 2% FBS. A2058 melanoma cells at about 80% confluence in 10 cm culture dishes were washed with 1× PBS and incubated with 0.05% trypsin-EDTA (Life Technologies) to lift cells from the surface. Melanoma were centrifuged for 5 minutes at 800 rpm and re-suspended in 1 ml of endothelial cell media supplemented with 2% FBS. HPMEC: 1.8×10^5 A2058 cells were added to monolayers on 12 mm coverslips and incubated at 37°C and 5% CO₂ for 10 or 45 minutes. HUVEC: 9.0×10^5 A2058 cells were added to monolayers on 25 mm coverslips and incubated at 37°C and 5% CO₂ for 45 minutes. After co-culture, monolayers were washed with 1× PBS and fixed with 4% paraformaldehyde for 15 minutes. After fixing, monolayers were washed with 1× PBS.

2.2.5 Immunofluorescence staining

After fixing, endothelial monolayers were incubated for an hour with a blocking solution comprised of 1× PBS, 5% goat serum (GS; Sigma) and 0.3% Triton™ X-100. Monolayers were incubated with a primary monoclonal antibody (mAb) for VE-cadherin (#2500; Cell Signaling Technology) at a 1:100 dilution in antibody dilution buffer for 16 hours. The antibody dilution buffer was composed of 1× PBS, 1% bovine serum albumin (BSA; EMD Millipore), and 0.3% Triton™ X-100. After incubation, monolayers were washed with 1× PBS. Monolayers were then incubated for 1 hour, protected from light, with Alexa Fluor® 488 Goat Anti-Mouse IgG secondary antibody (Life Technologies) at
a 1:500 dilution in antibody dilution buffer. After secondary antibody incubation, monolayers were washed with 1× PBS and covered to protect from light. Nuclei were stained with Hoechst 33342 (1:10,000) in 1× PBS for 10 minutes, protecting from light. Monolayers were washed again with 1× PBS, mounted to microscope slides, and covered with drops of Fluoromount G (Southern Biotech) and a 22×40 mm coverslip. Slides were stored at 4°C, protected from light.

2.2.6 Junction disruption analysis

Images of monolayer samples were taken using a Nikon Plan Fluor 100×/1.30 oil objective and Nikon Ti-E inverted fluorescence microscope equipped with a Photometrics CoolSNAP HQ² camera. The percentage of each image corresponding to gap area between cells was calculated using ImageJ software (Schneider, Rasband et al. 2012). Gap regions were outlined and “% endothelial gap” was defined as the ratio of pixels in gap regions to total pixels in each image (Khanna, Yunkunis et al. 2010). Each replicate data point was reported as the average of 24 images.

2.2.7 Statistical analysis

Statistics were performed using two way ANOVA in Microsoft Excel for inhibitor studies, combined with Tukey’s HSD multiple comparison test using KaleidaGraph 4.1 software (Synergy). A minimum of three independent experimental replicates were performed for all studies. Differences were considered significant for P < 0.05. All reported values are mean ± standard error of the mean.
2.3 Results

2.3.1 Endothelial-tumor cell co-culture with time

To determine the effect that melanoma has on endothelial junction disruption as a function of time, experiments were designed for the observation of melanoma-endothelial cell co-culture. Using an antibody for VE-cadherin, junction integrity could be observed and quantified by fluorescence imaging. It was found that junction disruption increased with time from 10 to 45 minutes for endothelial cells co-cultured with A2058 melanoma (Figure 2.1). At the 45 minute time point, the average percentage of monolayer disruption increased significantly compared to control as well as at 10 minutes. The control was not different compared to 10 minutes of A2058 co-culture at a statistically significant level. This model of the endothelium illustrates that melanoma is able to induce the formation of space between endothelial cells that would be required for extravasation. In agreement is previous work done showing junction disruption in HUVEC mediated by A2058 that increases in time over 90 minutes(Khanna, Yunkunis et al. 2010).
Figure 2.1 HPMEC monolayers co-cultured with A2058 melanoma cells have increased junction disruption over time compared to intact monolayers. There is an increase in junction disruption at 45 minutes compared to 10 minutes of co-culture. A. Brightfield images of HPMEC monolayers with corresponding images of immunofluorescence staining of VE-cadherin junctions. Dotted line outlines gap region between cells. An arrow shows the location of an A2058 melanoma cell. B. Plot of the ratio of pixels in gap regions compared to total pixels in each image. Each replicate is comprised of 24
Co-culture of endothelial monolayers with melanoma induced loss of cell-cell junctions, and gap formation local to A2058 tumor cell adhesion. In a physiological environment, junction disruption would be followed by passage of the melanoma cell into the stroma. The mechanism that melanoma uses to facilitate junction disruption is not well known however, with respect to signaling in endothelial monolayers, and the downstream induction of contractility. Co-culture gap size measurements are not sufficient to determine the effect that soluble factors and integrin signaling contribute individually to junction disruption. Further experiments were needed to elucidate single contributions in junction disruption, and how in comparison they might work together to facilitate disruption. We seek to measure effects from soluble signaling and receptor-ligand binding separately; to compare with the disruption that melanoma facilitates using a combination of both.

2.3.2 Junction disruption from interleukin signaling

To isolate the effect of soluble signaling due to inflammatory cytokines secreted by melanoma, HPMEC monolayers were treated with recombinant IL-8 over a range of concentrations. Over 24 hours, confluent layers of A2058 are known to secrete IL-8 at 15 ng/ml in the bulk supernatant(Khanna, Yunkunis et al. 2010). The concentration that endothelial cells experience local to tumor cell adhesion may be much higher however. Experiments were performed over a range of IL-8 concentrations, finding a significant
increase in junction disruption over control for all concentrations used. Figure 2.2 shows that junction disruption increases with concentration for monolayers incubated with IL-8 for 45 minutes. This experiment shows that signaling from interleukin 8 alone is sufficient to induce junction disruption in endothelial monolayers. This has been shown previously in HUVEC (Khanna, Yunkunis et al. 2010); we demonstrate it in the HPMEC line as well.

Figure 2.2 HPMEC monolayers incubated with increasing concentrations of interleukin (IL)-8 for 45 minutes showed an increase in junction disruption. A. HPMEC are stained with VE-cadherin antibody to visualize cell-cell junctions and their integrity. B. Plot of the ratio of pixels in gap regions compared to total pixels in each image. Each replicate is comprised of 24 images. *P<0.05, **P<0.01, n=3, Tukey post hoc test; 100× objective. Error reported as mean ± SEM, scale bar: 10 µm.
This result is interesting, because it demonstrates that the soluble factors melanoma secretes are able to induce changes in endothelial cells capable of facilitating gap formation, which is advantageous to melanoma.

2.3.3 VLA-4/VCAM-1 binding induces endothelial junction disruption, modeled by K562 co-culture

Melanoma cells express VLA-4 on their surface that is capable of activating signaling in endothelial cells after binding VCAM-1. It has been suggested previously that signaling induced from VLA-4/VCAM-1 binding plays a role in endothelial junction disruption, through experiments treating HUVEC monolayers with a cross-linked antibody for VCAM-1 (Khanna, Yunkunis et al. 2010). To further explore this effect, K562 cells transfected to express VLA-4 were used as a model system; the cells secrete interleukins at levels significantly less than A2058 cells and therefore isolate signaling from VLA-4/VCAM-1 (Dedoussis, Mouzaki et al. 1999). The wild type K562 cells were used as a control to account for any effects that the cells have on their own in co-culture with HUVEC, such as forces from contact, from landing after falling through solution or intracellular contractility while the cells are in contact.
Figure 2.3 HUVEC monolayers co-cultured with K562 mutant expressing VLA-4 resulted in an increase over those co-cultured with WT K562 and untreated monolayers. Plot of the ratio of pixels in gap regions compared to total pixels in each image. Each replicate is comprised of 24 images. *P<0.05, **P<0.01, #P<0.05 compared to 45 and 90 minute mutant K562, n=4, Tukey post hoc test; 100× objective. Error reported as mean ± SEM.

Figure 2.3 shows that upon co-culturing HUVEC and K562 cells, the K562 mutants expressing VLA-4 were found to increase junction disruption over time, though there was no significant difference between the later timepoints at 45 and 90 minutes. Based on this data, the extent of junction disruption has a local maximum beginning at 45 minutes, and does not increase between 45 and 90 minutes, but plateaus. At both 45 and 90 minutes, junction disruption from mutant K562 co-culture was found to be significant in
comparison to HUVEC monolayers alone, as well as against WT control. This experiment confirms the suggestion that VLA-4/VCAM-1 signaling induces junction disruption, using a more physiologically relevant model system, in comparison to cross-linked antibodies.

2.4 Discussion

In the previous sections, the role of melanoma-to-endothelial signaling in endothelial junction disruption was examined. It is clear that melanoma is able to disrupt endothelial cell-cell junctions in vitro. Assays in the literature, with endothelial monolayers on transwell inserts, have shown invasion capabilities for several melanoma cell lines (Fazakas, Wilhelm et al. 2011, Ghislin, Obino et al. 2012). Three dimensional modeling of invasion through a layer of dermal fibroblasts and into a fibrin based model with highly metastatic melanoma lines has shown a recapitulation of in vivo invasive phenotypes (Ghajar, Suresh et al. 2007). Using different models of invasion in combination allow the measurement of multiple properties, unavailable to any one model alone. Our model provides a readout on endothelial junction integrity and gap size, an important readout to measure the potential for extravasation.

Interestingly, we show that both soluble factors and receptor-ligand binding each induced junction disruption as an individual stimulating factor. Since melanoma has both of these at its disposal it is possible that there are additive or even synergistic effects, and that melanoma is able to mediate the separate interactions to drive endothelial cytoskeletal dynamics temporally to more effectively induce junction disruption.
Work by Yu and coworkers demonstrates both concentration and time dependent induction of endothelial permeability with IL-8 (Yu, Huang et al. 2013), which is in agreement with our findings for monolayers incubated with IL-8. Furthermore, tumor cell adhesion to the endothelium has been shown to be enabled by polymorphonuclear leukocytes (PMNs), which are activated by and produce more IL-8 in the presence of melanoma (Dong, Slattery et al. 2005). Thus, IL-8 is not just important for facilitating permeability of the endothelium, but also for melanoma arrest and adhesion mediated by PMNs.

We demonstrate induction of junction disruption via VLA-4(α4β1)/VCAM-1 binding using K562 cells expressing surface VLA-4 ligands. Leukocyte-endothelium interactions have been well studied with respect to VLA-4/VCAM-1 interactions, which are required for leukocyte transmigration and recruitment to sites of injury (Wetering, Berk et al. 2003, Wessel, Winderlich et al. 2014). VLA-4/VCAM-1 binding and signal transduction stimulates Rac1 activation in endothelial cells as well as p38 MAPK and cytoskeletal changes (Cook-Mills, Marchese et al. 2011). Additionally, crosslinking VCAM-1 with antibodies induces stress fiber formation and endothelial junction disruption in HUVEC monolayers (Wetering, Berk et al. 2003). Van Wetering and coworkers found that Rac activation occurs with VCAM-1 stimulation, and is responsible for the induction of endothelial gaps mediated by the loss of cell-cell adhesion. They find that this is regulated by the formation of reactive oxygen species (ROS) and that the induction of stress fiber formation alone is not sufficient to induce intercellular gaps.

Our results isolating VLA-4/VCAM-1 binding suggest that melanoma may employ a similar mechanism during extravasation, to leukocytes based on the opening of
endothelial junctions and loss of cell-cell contacts. Based on findings in the literature, and the results of this work, melanoma mediated endothelial cell-cell junction disruption requires both soluble signaling and receptor-ligand binding for successful opening of cell-cell junctions, to facilitate passage out of the bloodstream and into the stroma.
Chapter 3: ENDOTHELIAL CELL CONTRACTILITY SIGNALING INDUCED BY RECEPTOR-LIGAND INTERACTIONS AND SOLUBLE SIGNALS

3.1 Introduction

Signaling cascades in endothelial cells are complex and control many distinct processes such as apoptosis, growth, survival, and migration, for example. Endothelial cells release and secrete soluble factors that contribute to both paracrine and autocrine signaling (Cozzolino, Torcia et al. 1990, Marin, Montero-Julian et al. 2001, Li, Varney et al. 2014). Because of observed endothelial junction disruption in response to soluble factors that are secreted by melanoma and VLA-4/VCAM-1 binding, we are interested in pathways in endothelial cells that lead to downstream contractility. Pathways including the Rho family GTPases Rho and Rac are known to mediate downstream contractility and junction integrity in endothelial cells and have been shown to act cooperatively as well as antagonistically (Wojiack-Stothard, Potempa et al. 2001, Burridge and Wennerberg 2004, Sailem, Bousgouni et al. 2014). Rho and Rac are essentially molecular switches that can be turned off and on through interaction with guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) and stimulate downstream pathways(Schwartz 2004). As we are interested in the induction of contractility in endothelial cells mediated by melanoma, intermediate proteins in the Rho and Rac cascades provide valuable targets to aid in our understanding of the process. The Src pathway is also of interest in junction disruption due to Src phosphorylation of VE-cadherin that can lead to junction protein complex dissolution(Orsenigo, Giampietro et al. 2012). Inhibition of proteins that mediate downstream contractility or phosphorylation and measurement of the degree to which junction disruption is mitigated can provide
clues to the importance of different pathways and offer potential therapeutic targets to block metastasis.

3.2 Methods

3.2.1 Pharmacological inhibitors

Prior to A2058 co-culture (previously described in 2.2.6), endothelial monolayers were incubated separately with pharmacological inhibitors for 30 minutes. Inhibitors were reconstituted in dimethyl sulfoxide (DMSO; Sigma). HPMEC monolayers were incubated with ML-7 (5 µM; Sigma), Blebbistatin (25 µM; Sigma), Cytochalasin D (0.01 µg/ml; Tocris Bioscience), Y27632 (5 µM; Tocris Bioscience), NSC23766 (200 µM; Tocris Bioscience), and PP1 (0.17 µM; Sigma). HUVEC monolayers were incubated with 5 µM ML-7, 25 µM Blebbistatin, 0.01 µg/ml Cytochalasin D, 10 µM Y27632, 100 µM NSC23766 and 10 µM PP1. After 30 minutes incubation, monolayers were washed with 2% FBS supplemented endothelial cell media and co-cultured with A2058 melanoma cells (previously described in 2.2.6).

ML-7 is a selective inhibitor of myosin light chain kinase (MLCK) by competing with ATP binding(Saitoh, Ishikawa et al. 1987). Blebbistatin inhibition occurs by locking the myosin head in a complex with low affinity for actin(Kovács, Tóth et al. 2004). Cytochalasin D is an inhibitor of actin polymerization. Y27632 inhibits the Rho-associated protein kinase (ROCK) by competing with ATP for binding(Narumiya, Ishizaki et al. 2000). NSC23766 is an inhibitor of Rac1 by preventing interaction with its guanine exchange factors (GEFs). PP1 is a Src family tyrosine kinase inhibitor.
3.3 Rho pathway signaling

Signaling in the Rho pathway is responsible for regulation of the actin cytoskeleton with respect to stress fiber formation, adherens junction clustering, focal adhesion formation and cell contractility (Wojciak-Stothard, Potempa et al. 2001, Popoff and Geny 2009). Extracellular stimuli such as lysophosphatidic acid (LPA) have been shown to induce stress fiber formation in fibroblasts; the response was completely inhibited by C3 transferase, a coenzyme that inactivates Rho proteins(Aelst and D’Souza-Schorey 1997). The mechanism of cytoskeletal organization functions through many different proteins downstream of Rho including Rho associated kinase (ROCK), myosin light chain (MLC), and myosin light chain kinase (MLCK) with Rho activation occurring on the order of seconds to tens of seconds(Spiering and Hodgson 2011). Figure 3.1 illustrates pathway dependencies downstream of Rho activation.
**Figure 3.1** Rho pathway with specific inhibitors shown. Pharmacological inhibitors are indicated in red, portrayed to act on specific molecules in the signaling cascade.

Immediately downstream of Rho is ROCK, a kinase with multiple substrates and functions in contractility. ROCK is known to phosphorylate MLC which leads to activation of myosin ATPase and the myosin powerstroke enabling cell contraction (Amano, Ito et al. 1996, Amano, Nakayama et al. 2010) and stress fiber formation (Aelst and D’Souza-Schorey 1997). Another avenue ROCK uses to promote cell contractility is by phosphorylation of another substrate, myosin light chain phosphatase (MPT). Upon phosphorylation, MPT activity decreases, resulting in higher levels of phosphorylated MLC and therefore greater contractility (Sanz-Moreno, Gaggioli et al. 2011, Ito, Medicine et al. 2013). ROCK then principally affects cell contraction through the regulation of MLC activity. Experimentally, we inhibit the function of ROCK with the small molecule Y27632 which binds to the catalytic site, competing with ATP (Ishizaki, Uehata et al. 2000).

A second activator of MLC is MLCK which also phosphorylates MLC (Dudek and Garcia 2001, Wolfson, Lang et al. 2009), at the same site as ROCK (Amano, Ito et al. 1996). Interestingly, there is evidence that MLCK phosphorylates MLC in a distinct spatial location in the cell periphery compared to ROCK, at the cell center (Totsukawa, Yamakita et al. 2000). Selective inhibition of MLCK in our *in vitro* studies was accomplished using ML-7, a small molecule that acts through ATP binding competition (Saitoh, Ishikawa et al. 1987).
Myosin light chain mediates contractility through its activation of myosin II ATPase, facilitating the myosin powerstroke via ATP hydrolysis. MLC is preferentially phosphorylated at Ser19 and when simultaneously phosphorylated at the second site, Thr18, further increases myosin II ATPase activity (Ikebe and Hartshorne 1985, Ikebe, Hartshorne et al. 1986). Blebbistatin inhibits the action of myosin II ATPase and was used experimentally in this work (Kovács, Tóth et al. 2004).

Finally, actomyosin activity is dependent on the formation and organization of actin stress fibers. Rho stimulation of fibroblasts resulted in rapid stress fiber formation which was inhibited by blocking endogenous Rho (Ridley and Hall 1992). Induction of Rho mediated stress fiber formation has been shown to occur with IL-8 stimulation in endothelial cells (Schraufstatter, Chung et al. 2001). Cytochalasin D, an inhibitor of actin filament formation was used experimentally to isolate the effect of stress fibers on actomyosin contractility in junction disruption.

3.4 Rac pathway signaling

Rac signaling is associated with the formation of lamellipodia and membrane ruffles; it also drives actin polymerization and membrane protrusion (Aelst and D’Souza-Schorey 1997, Burridge and Wennerberg 2004).
**Figure 3.2** Rac pathway with specific inhibitors shown. Pharmacological inhibitors are indicated in red, portrayed to act on specific molecules in the signaling cascade.

Downstream, Rac activates p21 activated kinase (PAK) which has an adverse effect on myosin activity and the formation of stress fibers through inhibition of myosin heavy chain and MLCK (Burridge and Wennerberg 2004). In most cases, Rac is found to antagonize Rho mediated formation of stress fibers, but some results that counter this have also been observed via the downstream activation of Rho by Rac. This activation did not stimulate fiber formation as quickly, or to the extent that direct activation through LPA did, and occurred only in serum starved cells (Ridley and Hall 1992, Ridley, Paterson et al. 1992). The role of Rac in junction disruption may be multifaceted based on the possibility of crosstalk and Rho pathway activation. In experiments, the inhibitor
NSC23766 was used to block Rac from association with GEFs Trio and Tiam1 (Gao, Dickerson et al. 2004).

3.5 Src pathway signaling

The Src signaling pathway can be activated by integrin binding and clustering in normal cells (Playford and Schaller 2004). Integrin adhesion mediates the autophosphorylation and Src phosphorylation of FAK which stimulates Rac1 and later the Rho pathway (Huveneers and Danen 2009). Other studies have also found Src to be mechanoresponsive, becoming activated following integrin activation (Felsenfeld, Schwartzberg et al. 1999, Matsui, Harada et al. 2012). Src signaling is also implicated in phosphorylation of VE-cadherin (Wallez, Cand et al. 2006). Phosphorylation can occur on different residues of VE-cadherin with differing outcomes. Src phosphorylates residue Tyr685 on VE-cadherin (Wallez, Cand et al. 2006) which has been shown to be the residue responsible for regulating vascular permeability in vivo (Wessel, Winderlich et al. 2014). Interestingly, Wessel and coworkers find phosphorylation at Tyr685 to have the distinct consequence of hyper-permeability in contrast to the leukocyte phosphorylation of Tyr731 in extravasation.
Figure 3.3 Src pathway with specific inhibitors shown. Pharmacological inhibitors are indicated in red, portrayed to act on specific molecules in the signaling cascade.

Additionally, phosphorylation of VE-cadherin by vascular endothelial growth factor (VEGF) may induce endothelial junction disruption and permeability by regulating stability (Dejana, Spagnuolo et al. 2001). Src activity is implicated both in cytoskeletal dynamics as well as VE-cadherin phosphorylation related to permeability, both of which can contribute directly to the extravasation of melanoma in response to integrin binding on endothelial cells. This pathway (Figure 3.3) increases the potential for crosstalk between soluble and receptor-ligand signaling that occurs with melanoma co-culture and adhesion as it can affect Rho and Rac pathways downstream. The inhibitor PP1 was used experimentally to investigate the effect of blocking Src on junction disruption.
3.6 Results

3.6.1 Rho, Rac and Src pathways in micro- and macro-vascular endothelial monolayers influence melanoma induced junction disruption

Signaling in endothelial cells resulting from melanoma co-culture was measured using junction disruption of HPMEC monolayers as an output after incubating with pharmacological inhibitors. Endothelial monolayers were grown to confluence on glass slides covered with fibronectin as a model of the endothelium. To target and isolate specific signaling molecules in endothelial cell signaling we incubated monolayers with pharmacological inhibitors and washed them away completely before introducing A2058 melanoma cells. In this way, the inhibitors are only able to influence signaling in endothelial cells.

![Figure 3.4](image.png)

**Figure 3.4** Pharmacological inhibitors mitigated junction disruption in microvascular HPMEC monolayers co-cultured with A2058 melanoma cells for 45 minutes. Plot of the ratio of pixels in gap regions compared to total pixels in each image. Each replicate is
comprised of 24 images. **P<0.01, ***P<0.001, n=3 for inhibitors, n=4 for untreated and DMSO, Tukey post hoc test; 100× objective. Error reported as mean ± SEM.

Control monolayers that remained untreated or were incubated with carrier solution (DMSO) had a significant degree of gap formation after A2058 co-culture for 45 minutes. This data agrees with earlier experiments that measured junction disruption in HPMEC monolayers as a function of time (Figure 2.1). Inhibitor incubation can have an effect by itself on monolayer integrity, so monolayers were treated with inhibitors without co-culture to establish baselines for each inhibitor. This was also important to consider with different cell lines, since a concentration that was appropriate for HPMEC culture could kill a significant amount of HUVEC and abolish monolayer integrity.

Comparing the effect of monolayers inhibited with different inhibitors shows that most inhibitors did not completely abolish junction disruption that resulted from A2058 co-culture, but drastically reduced the effect. Each of the inhibitors resulted in a decrease of junction disruption in comparison to monolayers treated with DMSO (Figure 3.4).

This result gives positive evidence for our hypothesis that endothelial cytoskeletal contractility plays an important role in melanoma mediated endothelial cell-cell junction disruption, because inhibiting important proteins in signaling cascades that lead to contractility is able to mitigate junction disruption.

There is always a question of how unique a measured effect may be or if observations made in one model system are true generally, so we also measured the effect of melanoma induced junction disruption in the macrovascular endothelial cell line, HUVEC. The umbilical vein is not necessarily a location of physiological relevance in
the study of melanoma metastasis and extravasation, however HUVEC is a well-studied cell line that represents another microenvironment, cells from a larger vessel, in contrast to those found in lung capillaries such as HPMEC.

We find that control HUVEC monolayers (untreated, DMSO) exhibit a large increase in junction disruption and gap formation in response to A2058 co-culture, more than twice as large as in HPMEC (Figure 3.5). After incubation with pharmacological inhibitors, HUVEC displayed an attenuated response in junction disruption similar in trend to HPMEC.

**Figure 3.5** Pharmacological inhibitors mitigated junction disruption in macrovascular HUVEC monolayers co-cultured with A2058 melanoma cells for 45 minutes. Plot of the ratio of pixels in gap regions compared to total pixels in each image. Each replicate is comprised of 24 images. ***P<0.001, n=3 for inhibitors, n=6 for untreated and DMSO, Tukey post hoc test; 100× objective. Error reported as mean ± SEM.
The result in Figure 3.5 shows that inhibitors cause mild increases in junction disruption alone, and that each inhibitor studied significantly reduced gap formation in HUVEC monolayers in comparison to inhibitor control, DMSO. While the magnitude of response in HUVEC and HPMEC lines was different, the trend after inhibitor incubation was conserved. In both cases this implies that melanoma is able to induce signaling in endothelial cells through Rho, Rac and Src pathways.

Activation of these pathways by melanoma or leukocytes may mediate junction disruption through changes in endothelial cell contractility or through the dissolution of junction complexes that hold endothelial cells together. Upstream activation of Rho, for example, has the potential to increase the activity of actomyosin interactions.

3.6.2 Junction disruption mediated by VLA-4/VCAM-1 binding in K562 co-culture is mitigated by Rho, Rac and Src pathway inhibitors

To determine if signaling from VLA-4/VCAM-1 binding influences endothelial contractility through the pathways that melanoma does, we used a mutant K562 cell line that had already been generated, to express VLA-4 in order to isolate effects from integrin signaling. There was a significant increase in junction disruption after mutant K562 cell co-culture, indicating that signaling from VLA-4/VCAM-1 binding is able to induce gap formation (Figure 2.3). Signaling from VLA-4/VCAM-1 binding was found to be mitigated by some of the same pharmacological inhibitors that were used with A2058 co-culture to target myosin II ATPase, Rac, ROCK and Src signaling (see Figure 3.6).
Figure 3.6 Pharmacological inhibitors mitigated junction disruption in macrovascular HUVEC monolayers co-cultured with K562 cells for 45 minutes. Plot of the ratio of pixels in gap regions compared to total pixels in each image. Each replicate is comprised of 24 images. ***P<0.001, **P<0.01, *P<0.05 for VLA-4 expr. K562 co-culture with inhibitors against VLA-4 expr. K562 co-culture with DMSO, n=7 untreated control and WT, n=6 for untreated VLA-4 expr., n=3 for all others, Tukey post hoc test; 100× objective. Error reported as mean ± SEM.

A wild type K562 cell line was not found to increase junction disruption, which was expected because it does not express the α4β1 integrin in its membrane to facilitate interaction with VCAM-1. VLA-4/VCAM-1 binding did not induce gap formation to the same degree that melanoma co-culture did in HUVEC implying that other factors, such as soluble signaling, contribute in conjunction with receptor-ligand binding to facilitate extravasation in melanoma. Based on our results in Figure 3.6, the Rho, Rac and Src
pathways are all involved in mediating junction disruption from VLA-4/VCAM-1 binding interactions.

It is not unexpected that several pathways are implicated in VLA-4/VCAM-1 binding induced junction disruption, due to the potential for signaling between cascades discussed previously.

3.7 Discussion

In examining the potential for endothelial cell contractility induced by melanoma, several pathways were identified that may play an important role. The Rho and Rac pathways have been well characterized for their involvement in promoting the formation of contractile machinery such as stress fibers and for regulating cytoskeletal organization (Nobes and Hall 1995, Schraufstatter, Chung et al. 2001, Wojciak-Stothard and Ridley 2003). In support of our hypothesis that the mediation of endothelial cell contractility is responsible for junction disruption and extravasation of melanoma, we found that inhibiting key signaling proteins in cascades leading to contractility attenuated gap formation between endothelial cells.

Untreated endothelial monolayers exhibited significant junction loss local to sites of melanoma binding and adhesion, however upon treatment with pharmacological inhibitors of contractility and Src this response was reduced even when tumor cells bound and adhered.

In support of a mechanism similar to leukocyte trans-endothelial migration, work by Stroka and Aranda-Espinoza shows that incubating endothelial monolayers with Blebbistatin and ML-7 reduces the fraction of transmigrating neutrophils compared to
control; they found in addition that fewer holes formed on substrates treated with ML-7 (Stroka and Aranda-Espinoza 2011). This data agrees with our observations of decreased junction disruption in endothelial monolayers incubated with ML-7 and Blebbistatin prior to melanoma co-culture. Stroka et al. also correlate the effect of endothelial contractility and neutrophil transmigration with subendothelial stiffness.

Furthermore, work by Huynh and coworkers finds that increased matrix stiffness is able to promote leukocyte transmigration which was reduced after treating endothelial layers with Y27632, a ROCK inhibitor (Huynh, Nishimura et al. 2011). The context of the work by Huynh et al. was in atherosclerosis—understanding monolayer function is advantageous both for understanding cardiovascular disease as well as its implications in cancer models. Due to the similarity of findings in melanoma and leukocyte transmigration, it may be interesting to probe a connection between the incidence and progression of metastasis in individuals with cardiovascular disease that results in vessel stiffening.

Eosinophil transmigration has also been shown to be mediated by endothelial cell contractility, with inhibition of ROCK in HUVEC decreasing transmigration (Cuvelier, Paul et al. 2005). Eosinophils do not exhibit Src family kinase activation downstream of integrin activation, as PP1 had no effect on transmigration. The finding with eosinophils is in contrast to previous data from Cuvelier and coworkers which demonstrated Src activation downstream of integrin binding in neutrophils (Cuvelier, Paul et al. 2005).

Interestingly, the invasion potential of several types of white blood cells has been shown to decrease when endothelial cell contractile signaling pathways are inhibited (Cuvelier, Paul et al. 2005, Huynh, Nishimura et al. 2011). This agrees well
with our findings that melanoma employ endothelial signaling in transmigration, induced by VLA-4/VCAM-1 and soluble signal activation which are shared by neutrophils.

Support of involvement by the endothelium in metastasis has been observed in the case of breast cancer metastasis. Mierke found that MDA-MB-231 cells were three fold more invasive with endothelial co-culture compared to collagen matrices. Additionally, in agreement with our data for melanoma, Mierke finds that the percent invasion of cells derived from MDA-MB-231 expressing high levels of α5β1 integrin, was decreased after incubating HPMECs with ML-7 (MLCK), Y27632 (ROCK) and Rac Inh (Rac1) inhibitor (Mierke 2011).

Together, findings in the literature for white blood cells and for metastatic breast cancer support our data indicating that small GTPase signaling through endothelial contractility pathways mediates melanoma induced junction disruption. This provides a role for the endothelium in tumor cell extravasation and offers potential therapeutic targets.
4.1 Introduction

Cells are able to signal to other cells, and receive cues from their environment through mechanotransduction. It has been shown that factors such as substrate stiffness and fibronectin concentration are able to influence cell traction stress, spread area and downstream MLC phosphorylation (Tan, Tien et al. 2003, Polte, Eichler et al. 2004, Krishnan, Klumpers et al. 2011). Having identified signaling molecules important in contractility that mediate junction disruption in endothelial-melanoma co-culture we seek to measure the response of groups of endothelial cells to factors secreted by melanoma. The contractility of groups of endothelial cells grown on polyacrylamide substrates can be measured in response to stimuli, and used as a model to study contractility of the endothelium. In particular, we are interested in determining if groups of endothelial cells contract following stimulation, leading to junction disruption. This will provide insight to the conditions necessary for melanoma mediated junction disruption.

4.2 Traction force microscopy

We perform traction force microscopy using groups of HPMECs patterned onto squares of fibronectin protein stamped onto polyacrylamide (PA) substrata. This method provides a system of cells in contact with a constant area, in order to measure endothelial cell contractile response to tumor cell cues (Figure 4.1A). Figure 4.1B shows VE-cadherin junctions in a group of patterned HPMECs on a PA substrate 48 hours after seeding. PA substrata were synthesized with an embedded layer of fluorescent beads that
are displaced based on endothelial cell contraction at the surface. Because the PA gel is a compliant viscoelastic substrate cells are able to deform the surface of the gels and cause movement in embedded fluorescent beads. The substrate and beads return to their initial unstressed positions after cells are removed from the surface, allowing calculation of the bead displacement caused by cells stressing the substrate. This enables the subsequent calculation of traction stress due to physical properties of the substrate such as stiffness and Poisson ratio.

Traction stresses are calculated using the LIBTRC analysis library (version 2.4) developed by Dr. Micah Dembo of Boston University. Phase images of tissues and fluorescent images of bead distributions in the gel below each tissue are taken before treatment with soluble factors. Cells are incubated in the LiveCell chamber for 30 minutes, followed by a 60 minute baseline with images taken every 5 minutes. Reagents are added at time $t = 0$ minutes, and images are taken every 2 minutes for 90 minutes. Finally the tissues are removed from the surface and fluorescent images are taken of the bead distributions with no applied tissue force (null force). The locations of beads at the null force position are compared to positions under stress from the cells using LIBTRC to calculate bead displacements. From the bead displacements and substrate properties relating to deformation and pliability such as Poisson’s ratio and the Young’s modulus, traction stresses can be calculated for the tissues using Green’s function (Jacobs, Huang et al. 2012). Single point forces and displacements can be modeled if assumed to act in the same direction, using equation 1.

$$u = \frac{(1+\nu)}{\pi Ed} F$$

(1)
In this equation, \( u \) is the displacement, \( \nu \) and \( E \) are Poisson’s ratio and Young’s modulus of the substrate respectively, and \( d \) is the distance separating the force, \( F \), from the displacement. To calculate tissue tractions, a similar relation is solved by numerical integration for all bead displacements, including both \( x \) and \( y \) contributions and non-collinear terms (Jacobs, Huang et al. 2012). Baseline comparisons (\( t = -60 \) to \(-5 \) minutes) and treatment comparisons (\( t = 0 \) to \( 90 \) minutes) are made to determine the effect of soluble signaling.

TFM measures the cell- or tissue-ECM traction stresses that occur endogenously or in response to agonists. It has been shown, that cell-cell force, our metric of interest, is directly proportional to the total cell-ECM traction force (Maruthamuthu, Sabass et al. 2011). Since cell-cell force correlates well with cell-ECM force, measured force increases by traction force microscopy can be thought of as an approximate way to measure cytoskeletal mediated contractility applied through adherens junctions between cells, though the measured quantity is due to cell-ECM interaction only.

**Figure 4.1** A: Brightfield image of patterned HPMEC monolayers on a polyacrylamide substrate. B: Fluorescence staining of VE-cadherin (green) and nuclei (blue) for an HPMEC patterned monolayer. Scale bar: 25 µm.
It is important that mature junctions form in the patterned groups, because of the mechanosensing that occurs at cell-cell contacts by cadherins (Leckband, le Duc et al. 2011). Interestingly, Liu and coworkers found that junction size increased with tugging force for geometrically constrained cells; they determine that Rac activity is cooperative, but that tugging force is necessary and sufficient for adherens junction assembly (Liu, Tan et al. 2010). These force dependent studies underscore the importance of cell mechanics in regulating cell-cell junctions and interaction.

4.2.1 Methods
4.2.1.1 Substrate synthesis
Polyacrylamide (PA) substrata with two layers were synthesized following an adapted protocol (Pelham and Wang 1997, Bridgman, Dave et al. 2001, Tse and Engler 2001). Glass coverslips were cleaned using 0.1 M sodium hydroxide for 15 minutes. Coverslips were incubated with 2% aminopropyl trimethoxysilane (APTMS; Sigma) in acetone, washed, and later incubated with 0.5% glutaraldehyde (Sigma) in PBS. A set of circular glass coverslips was treated with Rain-X™ to make the glass hydrophobic, and easier to peel from the gels. For a gel stiffness of approximately 1.80 kPa (Tse and Engler 2001), a solution of 5% acrylamide and 0.06% bis-acrylamide (Bio-Rad) was used. This stiffness was used so that the gel was compliant enough for bead movements to be measured in response to cell contractility. Additionally, this is close to the approximate stiffness of the extracellular matrix, which is between 1-4 kPa (Sinkus, Lorenzen et al. 2000, Stroka and Aranda-Espinoza 2011). Solutions were degassed and polymerization
initiated by adding 1:200 (v/v) 10% ammonium persulfate (Bio-Rad) solution with dH$_2$O and 1:2000 (v/v) N,N,N',N'-tetramethylethlenediamine (TEMED; Bio-Rad).

Fluorescent beads (0.5 µm; Life Technologies) were included in the top layer solution. Polymerized substrata were activated with 1 mg/ml sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-SANPAH; Pierce) in 50 mM HEPES buffer at pH 8.5 and exposed to UV light for 10 minutes to crosslink the sulfo-SANPAH to the PA substrate.

### 4.2.1.2 Micropatterning PDMS stamps

Initially, silicon wafer master templates were made using standard photolithography techniques (Gomez and Nelson 2011) to prepare arrays featuring square posts, 10,000 µm$^2$ in area. Polydimethylsiloxane (PDMS; Dow Corning) was mixed 10:1 with silicone elastomer curing agent (Dow Corning), degassed and poured onto wafers to form a master stamp with square indentations. To cure, stamps were baked at 60°C for 4 hours, peeled from wafers and treated with UV/ozone for 10 minutes. PDMS stamps were then silanated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (Sigma) and used to create stamps with arrays of square posts as described above. Prior to stamping protein, activated PA substrata were washed with sterile HEPES buffer and dried on a hotplate at 60°C for 20 minutes (Choi, Vincent et al. 2012). Before use, PDMS stamps were sterilized with ethanol, dried and incubated with 100 µg/ml FN for 20 minutes. Stamps were rinsed with PBS and dried mildly with a stream of nitrogen and pressed gently on activated substrata for 10 minutes. After 10 minutes, stamps were removed and PA substrates were incubated in HEPES buffer overnight at 37°C (Figure
4.2. The next day, 1.0×10⁵ HPMECs were seeded to each micropatterned substrate. To control cell adhesion, substrates were rinsed after 25 minutes to remove non-adherent cells. Substrates with adhered cells were incubated with 2% FBS supplemented media for 48 hours prior to experiments.

**Figure 4.2** Synthesis and activation of polyacrylamide gels with embedded fluorescent micro-beads. Bottom gel layer contains no beads (turquoise) and upper layer contains fluorescent beads (pink).
4.2.1.3 Traction force images

Images were taken of patterned substrates with a phase filter for outlines of patterned cells and Texas Red filter for fluorescent bead distributions and a baseline set of images was taken for 1 hour (imaged every 5 minutes). Patterned cells were then incubated with cytokines or carrier solution (cytokine control) for 90 minutes (imaged every 2 minutes). Bead distribution images were taken before treatments, at different time points during experiments, and after removing cells from the substrate surface using 0.05% Triton™ X-100 for a null force image to compare with substrata under stress from cells. A neutral density filter setting of 8 was used to allow 12.5% of light from the lamp to be transmitted to the sample.

4.2.1.4 TFM Analysis

Phase and fluorescent bead images were aligned using the TurboReg plugin (Thevenaz, Ruttimann et al. 1998) in ImageJ. Bead displacement and traction stresses were calculated using the LIBTRC analysis library (version 2.4) developed by Professor Micah Dembo of the Boston University. Calculations were done at endogenous levels before co-culture or cytokine incubation to provide a baseline traction stress level for comparison. Traction stress and net contractile moment were calculated using LIBTRC. Input parameters are listed in Appendix A.

Net contractile moment is a scalar quantity that describes the total contractile input to the substrate in the x and y directions, weighted by the x and y coordinates of the applied tractions (Butler, Tolic-Norrelykke et al. 2002). It can be thought of as the
strength of two equal and opposite force generating points that bear the entirety of the force being generated by the cells. The condensed force couple “pinches” the substrate equivalently to the extent that the cells do together (Wang, Tolic-Norrelykke et al. 2002).

Net contractile moment is useful here because we are observing multiple cells, and it provides a reliable way to condense the contractility of the group of cells into a single scalar quantity.

4.3 Contractility with soluble factor incubation

4.3.1 Results

Patterned HPMEC squares were monitored for 90 minutes following 100 ng/ml IL-8 treatment after a 60 minute baseline period. In most of the cases, cells pulling apart to form gaps were not observed. A typical time series of traction maps is shown for control and IL-8 treatment in Figures 4.3 and 4.4 respectively, with tissues that did not show gap formation or cell-cell retraction. There are small changes in the localization of stress in the tissue over time, but no large changes were observed.
Figure 4.3 Traction maps showing the contractility over time in a group of cells treated with control vehicle. Minor changes in the pattern of traction stress over 90 minutes were observed.

In the time series of traction maps showing an example of IL-8 incubation (Figure 4.4), there is little change over the tissue in the magnitude of traction stresses, similar to control data (Figure 4.3). However, it is noticeable that in an area of higher stress in the
upper left corner of the group of cells, some minor retraction occurs over time (Figure 4.4).

**Figure 4.4** Time series traction maps for a group of cells incubated with IL-8 shows minor changes in the pattern of traction stress over 90 minutes and minor cell retraction in the upper left corner beginning at 30 minutes.

Measuring contractility over time in groups of cells with control and IL-8 treatment shows a trend of decreasing net contractile moment with time (Figure 4.5). Comparing magnitudes of the contractile moment data does not show a significant
difference between control and IL-8 treatment, as both series are well within the standard error calculated for each. Interestingly however, the decrease of net contractile moment over time with IL-8 treatment (i.e. slope of net contractile moment plotted against time), was significantly greater than the decrease in the control time series. This was confirmed by regression analysis of the data from 0 to 90 minutes. The analysis was performed by calculating the slope of each series from 0 to 90 minutes via a weighted least squares linear model and calculating the standard error of the slope. Using the respective slopes and standard errors, a T-test was performed between the two independent sample slopes, with null hypothesis that the slopes are equal (Figure 4.5). This comparison resulted in P<0.00005 indicating that it is highly unlikely that the slopes have the same value, and therefore that the IL-8 time series decreases more rapidly than the control series (see Appendix B).

The control data may decrease over time due to exposure to fluorescent light used to take the bead images, or because the chamber temperature, humidity and CO₂ concentrations are briefly disrupted when the cover is removed and replaced, to add either IL-8 or carrier solution to the cells.
Figure 4.5 HPMEC generate decreased average net contractile moment with 100 ng/ml interleukin 8 incubation over time. Control data points have sample size n=13 and IL-8 have n=15. Error reported as mean ± SEM.

Interestingly, the net contractile moment was found to decrease steeply for a group of HPMECs incubated with IL-8 that showed considerable junction disruption (see Figure 4.6), which was not typical behavior with IL-8 treatment, but illustrates a connection between junction disruption and decreased net contractile moment.
Figure 4.6 Gap formation in a group of endothelial cells induced by 100 ng/ml IL-8 results in decreased net contractile moment as a function of time. A: Brightfield images patterned HPMECs show junction disruption with IL-8 stimulus. B: Plot of net contractile moment over time with measurements for baseline (every 5 minutes) and IL-8 treatment (every 2 minutes).
Figure 4.7 Contractility in patterned HPMECs slightly decreases with modest junction disruption for 50 µM LPA treatment. A: Phase image series showing gap formation in a group of patterned HPMECs. B: Junction disruption corresponds to a slight decrease in net contractile moment and lower baseline moment.
IL-8 incubation induced junction disruption in endothelial monolayers has been observed in experiments in Chapter 2 on glass slides (Figure 2.2). In general we do not observe the induction of junction disruption from IL-8 incubation during traction force experiments on much less rigid substrates (1.8 kPa), however treatment is accompanied by a steady decrease in net contractile moment over time.

Interestingly, preliminary data for HPMEC monolayers seeded to polyacrylamide substrata of varying stiffness shows an attenuated response to IL-8, compared to that measured on glass substrates (Figure 2.2). This indicates that substrate stiffness may also mediate junction disruption, as far as it regulates the contractility that endothelial cells are able to exert. This result agrees well with the observation by Krishnan and coworkers that differences exists in studies of endothelial permeability on glass and more compliant substrates, with a larger response observed on glass. This observation motivates further study of junction disruption and monolayer permeability using substrates on more physiologically relevant scales.

![Figure 4.8](image)

**Figure 4.8** Net contractile moment in patterned HPMECs slightly decreases with LPA carrier solution (deionized water) treatment over time.
A small effect on junction disruption has been observed, after treating a group of HPMECs with 50 µM lysophosphatidic acid (LPA), a Rho activator. There was a decrease from baseline contractile moment that corresponded to an increase in gap size, and stabilized at a lower contractile moment after the gap size remained constant (Figure 4.7). This suggests a connection between increased Rho activity, contractility and junction disruption. Net contractile moment in response to control solution slowly decreases over time with no sudden decrease like that observed with junction disruption (Figure 4.8).

4.4 Discussion

Our data suggests that treatment of endothelial cells with interleukin 8, a soluble factor secreted by melanoma, corresponds to a decrease in net contractile moment. With IL-8 addition, the decrease was slight, and control data decreased as well, though at a slower rate.

It has been shown in the literature that endothelial junction disruption mediated by treatment with a VE-cadherin blocking antibody resulted in a decrease in contractile moment over 8 minutes, with increased effects at higher substrate stiffness (Krishnan, Klumpers et al. 2011). For the decrease over time of contractile moment that we observe with gels of 1.8 kPa stiffness in response to IL-8, the contractile moment magnitude measured agrees with published data from Krishnan and coworkers at a stiffness of 1.2 kPa (Figure 4.9). They measure a larger decrease over a shorter period of time with the use of VE-cadherin antibodies that mediate junction disruption, but the data has a similar decreasing trend.
In the literature there has been no significant difference in traction force after LPA incubation in comparison to control (Polio, Parameswaran et al. 2014). Additionally, McGrail and coworkers show a decrease in traction stress on “soft” substrates (2.83 kPa) with LPA treatment and an increase in traction stress on “hard” substrates (34.88 kPa) with LPA treatment (McGrail, Kieu et al. 2014). This finding is in agreement with data we present showing a decrease in net contractile moment with LPA treatment on a gel close in stiffness to the “soft” substrate (Young’s modulus, 1.8 kPa).

Figure 4.9 “Substrate stiffening enhances vascular endothelial (VE)-cadherin-borne forces. A: treatment with a VE-cadherin monoclonal blocking antibody disrupted cell-cell junctions (left, before treatment; right, 500 s after treatment). B: correspondingly, overall EC monolayer forces (represented by the net contractile moment, CM) decreased. C: the VE-cadherin-borne forces were enhanced significantly with substrate stiffening. D: the
fraction of overall monolayer forces supported by VE-cadherin was similar across all stiffness substrates. Values are means ± SE; n = 10, 9, and 9 for the 1.2-, 4-, and 11-kPa groups, respectively. *P < 0.05, 1.2 vs. 4 kPa. **P < 0.05, 1.2 vs. 11 kPa. Scale bar = 20 μm.” (figure and caption reprinted with permission, from Krishnan, Klumpers et al. 2011).

We have shown in previous chapters that contractile inhibition of endothelial cells reduces junction disruption in monolayers co-cultured with melanoma cells and incubated with soluble factors and here we show a correlation between soluble factor incubation (IL-8) and decreasing contractile moment. This suggests that contractile forces may be borne by VE-cadherin junctions, as well as between focal adhesions and the matrix. Krishnan and coworkers proposed the concept of intercellular forces borne by the monolayer, which may explain why a treatment which affects junctions can lead to a loss of contractility in groups of cells with corresponding junction disruption. They find that intact monolayers bear roughly one half the contractile forces through VE-cadherin junctions, which is lost during junction dissolution (Krishnan, Klumpers et al. 2011). This would agree with the slightly sharper decrease in contractility over time observed with IL-8 treatment, though gap formation was not observed.

Preliminary data observing VE-cadherin junction disruption on compliant polyacrylamide gels suggests that the response to soluble factors such as IL-8 will be attenuated at more physiologically relevant rigidities, compared to glass. In one replicate, very little junction disruption was observed over the range of 1.8-200 kPa when cells were incubated with 100 ng/ml interleukin 8, in contrast to experiments performed on glass. Because junction disruption and traction force microscopy experiments are
performed on very differently compliant substrates, a more meaningful comparison of
data relating contractility and junction disruption will require replication of gap
experiments on substrates of the same compliance used for TFM.
Chapter 5: CONCLUSIONS AND FUTURE DIRECTIONS

Melanoma is able to mediate junction disruption in endothelial monolayers due to contributions from both soluble factors and receptor-ligand binding. Furthermore, by inhibiting pathways implicated in contractility, the formation of gaps between endothelial cells that form in response to melanoma co-culture is attenuated.

Isolating soluble factors and receptor-ligand binding resulted in individual contributions to junction disruption which were both of a lesser magnitude than directly culturing endothelial cells with melanoma. Inhibiting endothelial contractility and co-culturing with K562 cells to model receptor-ligand binding resulted in a reduction of the amount of junction disruption in comparison to control.

Interestingly, it was found that IL-8 treatment of HPMECS adhered to patterned matrix protein on polyacrylamide gels corresponds to a steeper decrease in contractile moment in comparison to control. This is interesting because data in the literature has shown a decrease in net contractile moment with junction disruption due to VE-cadherin antibodies (Krishnan, Klumpers et al. 2011). A small observed difference in the rate of change of contractility indicates that the effect of soluble factors like IL-8 on endothelial cells may be more subtle for matrices with physiologically relevant stiffness compared to glass.

Taken together, the results of this work implicate signaling from Rho, Rac and Src pathways in mediating junction disruption from melanoma and receptor-ligand interactions, and confirm the effect that soluble factors have on vascular gap formation.

Mechanistically, it is possible that Rho and Rac activity are required to pull apart cell-cell junctions initially, and that upon separation, monolayer contractility decreases in
order to allow melanoma to pass through, though contact between cell and matrix remains intact.

Since an increase in contractile moment was not observed with IL-8 incubation, it is possible that soluble factors and receptor-ligand binding influence contractility and junction disruption differently to facilitate junction disruption over several steps.

Further experiments using traction force measurements with patterned groups of HPMEC and tumor cell co-culture will be useful to determine if an increased contractile response is demonstrated in comparison to the response from soluble factors alone. This would provide insight into the relative impacts of soluble signaling, receptor-ligand binding and the complete melanoma system.

In particular it will be important to study contractility in response to soluble cues such as interleukin 8 and tumor conditioned media, VLA-4/VCAM-1 binding and A2058 co-culture on substrates of varying stiffness for both gap experiments and measurements of contractility over time. The replication of junction disruption experiments performed on glass in this work can be done using more compliant substrata on a more physiological relevant scale—this will also enable the comparison of results measuring contractility using traction force microscopy to junction disruption experiments performed on substrates of the same compliance. The relationship between microenvironmental stiffness and melanoma mediated junction disruption is an interesting area for further research.

It will also be interesting to develop a stronger tie to Rho activation than with just inhibitor experiments. Rho activity in endothelial cells can be quantified in response to soluble factors and tumor cell binding using a FRET biosensor, such as the one
developed by the Hahn lab (Pertz, Hodgson et al. 2006). Using a FRET sensor can provide directly observable spatial and temporal data for Rho activation which may provide further clues into the mechanism that melanoma uses to activate endothelial cells and endothelial contractility.
## APPENDIX A: LIBTRC input parameters

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APPENDIX B: WEIGHTED LEAST SQUARES REGRESSION ANALYSIS

Mathematica code using the “LinearModelFit” function to perform a weighted linear least squares model fit and calculate the standard error for the slopes.

```
lmctrl = LinearModelFit[datactrl, x, x, Weights -> 1/ctrlSEM^2]
FittedModel[-17.541 + 0.0168955 x]

lmctrl["ParameterErrors"]
{0.0724475, 0.00150358}

lmIL8 = LinearModelFit[dataIL8, x, x, Weights -> 1/IL8SEM^2]
FittedModel[-19.4211 + 0.0207881 x]

lmIL8["ParameterErrors"]
{0.0938286, 0.00168146}

rootsos = Sqrt[0.00150058^2 + 0.00168146^2]
0.00225367

Tvalue = Abs[(0.0168965 - 0.0267881)]/rootsos
4.3891
```

Calculation of regression analysis, comparing the slopes of net contractile moment data for IL-8 and control treated groups of cells in a weighted linear least squares regression analysis.

<table>
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<tr>
<th>T-value</th>
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<tbody>
<tr>
<td>Deg. Of F.</td>
<td>88</td>
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<tr>
<td>Alpha</td>
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<tr>
<td>P</td>
<td>3.15E-05</td>
</tr>
<tr>
<td>Critical T</td>
<td>1.987</td>
</tr>
<tr>
<td>Signif.?</td>
<td>yes</td>
</tr>
</tbody>
</table>

= yes if p<alpha


