DEVELOPMENT AND CHARACTERIZATION OF EXTRACELLULAR AND INTRACELLULAR TARGETS IN TUMOR-INDUCED ENDOTHELIAL JUNCTION REGULATIONS

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ABSTRACT

Tumor metastasis is characterized by disassembly of endothelial junctions, which allows for tumor extravasation and migration through the endothelium. The goal of this study was to characterize extracellular stimuli and intracellular targets that induce signaling for VE-cadherin disassembly. A2058 tumor cells, anti VCAM-1, and A2058 tumor conditioned medium, containing cytokines IL-6, IL-8, IL1β, and GRO-α, were all found to induce gap formation in endothelial cells. Each cytokine was added in recombinant and neutralized forms to test for individual effect; all gave results that indicate that they in part mediate junction breakdown. As for investigation of intracellular targets involved in VE-cadherin disassembly, p38 in the MAPK pathway and intracellular Ca\(^{2+}\), PKC, and MLCK in the Ca\(^{2+}\) pathway were investigated. Each was tested to determine its role in mediating gap formation in endothelial cells. All data was collected using Human Umbilical Vein Endothelial Cells (HUVECs) and A2058 melanoma cells. Experimental techniques include cell staining, fluorescence microscopy, and Ca\(^{2+}\) measurement.

Finally, experimental data along with information from literature/databases was incorporated into an integrated signaling map. The graphical map was translated into two kinetic models (Ca\(^{2+}\) individually and combined Ca\(^{2+}\)-p38 MAPK) via ordinary differential equation formation. Global sensitivity analysis was performed to predict influential parameters in VE-cadherin: β-catenin disassembly. In the Ca\(^{2+}\) pathway model, components CaM and Ca\(^{2+}\)store and reactions involving Ca\(^{2+}\)ext, Rac-RhoGDI and MLC are the most influential; in the combined pathway model the most sensitive targets are components MKK6, p38, and Hsp27 and reactions including MK2 and p38P, all of which fall into the p38 MAPK pathway.
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ABBREVIATIONS

$[\text{Ca}^{2+}]_{i}$ Intracellular calcium
CaM Calmodulin
CS Calf serum
DAG Diacylglycerol
DMEM Dulbecco’s modified Eagle’s medium
DPBS Dulbecco’s phosphate-buffered saline
EC Endothelial cell
ELISA Enzyme-linked immunosorbent assay
G protein Trimeric guanine-binding regulatory protein
GPCR G-protein coupled receptor
GRO-α Growth related oncogene-α
GS Goat serum
Hsp Heat shock protein
HUVEC Human umbilical vein endothelial cell
IL-1 Interleukin-1
IL-6 Interleukin-6
IL-8 Interleukin-8
IP$_3$ Inositol trisphosphate
MAPK Mitogen-activated protein kinase
MAPKAPK or MK MAPK-activated protein kinase
MAPKK or MKK Mitogen-activated protein kinase kinase
MAPKKK or MKKK Mitogen-activated protein kinase kinase kinase
MLCK Myosin light chain kinase
NF-κB Nuclear factor-κB
ODE Ordinary differential equation
PAK p21-activated kinase
PIP$_2$ Phosphatidylinositol 4,5-bisphosphate
PKC Protein kinase C
PLC Phospholipase C
<table>
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<tr>
<th>Abbreviation</th>
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<td>RhoGDI</td>
<td>Guanosine-nucleotide dissociation inhibitors</td>
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<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum Ca(^{2+})-ATPase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TAK</td>
<td>TGF-β-activated kinase</td>
</tr>
<tr>
<td>TC</td>
<td>Tumor cell</td>
</tr>
<tr>
<td>TCM</td>
<td>Tumor conditioned medium</td>
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Chapter 1

BACKGROUND

1.1 Cancer

Cancer arises due to uncontrolled cell growth and division. Normal cells undergo cell death, or apoptosis, in old or injured cells so that they can be replaced by healthy cells. This normal cellular process can be altered by genetic (DNA) mutations in a cell. The mutations will cause abnormal growth and division and prevent apoptosis, which result in a mass of cells called a tumor. There are two types of tumors: benign which is not cancerous and malignant which is cancerous (National Cancer Institute 2002).

Cancer is caused by both external and internal triggers. These include tobacco, chemicals, radiation, and infectious organisms, as well as genetic mutations, hormone imbalances, and immune conditions. Some of these factors can be induced by lifestyle choices include tobacco smoking, alcohol use, and tanning. In addition to avoiding the aforementioned list, there are a number of medical screening techniques that aid in cancer prevention (National Cancer Institute 2002).

Behind only heart disease, cancer is the second most deadly disease in the United States today; it is responsible for 25% of all deaths (National Cancer Institute 2002). A 2009 American Cancer Society study indicates that approximately 1,479,350 new cases of cancer would arise
and 562,340 deaths would occur in 2009. The particular type of cancer of focus in this study is melanoma, which represents 5% of all cancers (American Cancer Society 2009).

1.2 Melanoma

Melanoma is a very common type of cancer and the most serious type of skin cancer today (American Cancer Society 2009 and National Cancer Institute 2002). It originates in skin cells called melanocytes which regulate skin pigment. The skin is largest organ of the human body and has a number of significant roles; it shelters the body from external factors including heat, sunlight, injury, and infection. Additionally, the skin regulates the body’s temperature, stores water, stores fat, and makes vitamin D. The skin is composed of an outer layer called the epidermis and an inner layer called the dermis. Melanocytes are located in the lower part of the epidermis and are responsible for producing melanin, the pigment that controls skin color. When a melanocyte becomes malignant, it is considered to be melanoma cancer (National Cancer Institute 2002).

Possible signs of melanoma include formation of a growth on the surface of the skin and size, shape, or color changes in existing growths. These changes take place gradually over a time period of at least one month. Risk factors include a family history of melanoma cancer, prevalence of moles on the body, and skin sensitivity to sunlight. The four commonly noted warning signals of melanoma cancer are a skin growth that is (1) asymmetric with (2) an irregular border, (3) non-uniform in color, and (4) has a diameter greater than 6 millimeters (American Cancer Society 2009).

Based on a study by the American Cancer Society, it is estimated that 68,720 persons were newly diagnosed with melanoma cancer in the United States in 2009 (American Cancer
The incidence rate for this type of cancer has more than doubled over the course of the past 30 years (National Cancer Institute 2002). The most prevalent increases have been found in younger white women and older white men; since 1995, there has been a 3.8% annual increase in white women aged 15 to 34 and since 2003, there has been an 8.8% increase in men over the age of 65. The study also estimated 8,650 deaths to occur due to melanoma in 2009 (American Cancer Society 2009). The severity of melanoma influences a great deal of research today; understanding the mechanisms in which tumor cells operate will help in the development of therapeutic drugs.

1.3 Tumor metastasis

Although benign tumors only grow locally, malignant cancer cells spread from a primary tumor to other locations in the body via a process called metastasis. Tumor metastasis entails escape of the tumor cells (TCs) from the primary site into the circulation, a process called intravasation. The cells travel through the bloodstream and then adhere to the endothelium downstream of the primary site (King 2000). This interaction between tumor and endothelial cells is essential to melanoma metastasis (Dong 2005). Extravasation involves tumor cell migration out of the circulation and into neighboring tissue where the cell can develop into a secondary tumor. This growth and division of the tumor cells is called proliferation (King 2000). Figure 1.1 shows a schematic of the process of metastasis (Alberts 1994).
Figure 1.1 Schematic of tumor metastasis: intravasation and extravasation. Tumor cell overgrowth in the epithelium (primary site) leads to intravasation into the capillary. The cells then travel through the bloodstream and adhere to the capillary wall, where they can extravasate and proliferate in the secondary site (Source: Alberts 1994).

During travel in the circulation, tumor cells interact with numerous types of cells including leukocytes and platelets in the bloodstream and endothelial cells composing the vessel wall. Tumor cells may travel as a single cell, as a homotypic tumor cell aggregate, or as a heterotypic tumor cell, leukocyte, and platelet aggregate. The formation of aggregates may protect the tumor cell from mechanical stresses in the flow as well as aid in attachment to the endothelium (King 2000). Tumor extravasation begins when tumors cell bind to the endothelium; this causes retraction of the endothelial cells and subsequent tumor cell invasion into the surrounding tissue (King 2000, Nicolson 1989). Figure 1.2 shows a diagram of the aforementioned process (King 2000). It is the role of endothelial cells to regulate biomolecules
and cells that pass through the endothelial cell barrier into the bloodstream or tissues. Cell-cell junctions mediate cell permeability and maintain endothelial barrier function. Hence, tumor cells affect endothelial junction proteins, causing them to allow passage through the endothelium into the secondary sight (Bazzoni 2004).

**Figure 1.2** Tumor extravasation. The three steps include TC adhesion to the EC surface, retraction of the ECs due to TC attachment, and TC invasion and migration into surrounding tissue (Source: King 2000).
1.4  Cell junctions

In the body, epithelial cells line the various cavities and surfaces of the body's organs. Intercellular junctions between epithelial cells play an important role in epithelial adhesion, communication, and permeability (Bazzoni 2004, Dejana 1995). Cell-cell junctions between epithelial cells belong to one of the four types: adherens junctions, gap junctions, tight junctions, and desmosomes (Dejana 1995). Adherens junctions inhibit cell growth and control the permeability of cells and solutes passage through the epithelial lining. This movement is via the paracellular pathway, in which cell junctions are opened or rearranged to allow for cell or solute passage across the epithelium. Gap junctions are intercellular mechanisms that mediate cell-cell communication, allowing movement of small solutes between neighboring cells. Tight junctions create the barrier between neighboring cells by controlling cellular permeability and sustaining cell polarity. Lastly, desmosomes are intercellular junctions that are essential for strong adhesion to nearby epithelial cells (Bazzoni 2004).

In order to control these various cellular actions, the junctions are composed of a complex network of transmembrane proteins imbedded in the plasma membrane. In general, junctions exhibit dynamic behavior to allow for structural changes. For example, an architectural shift can allow for passage of solutes and cells between two neighboring cells; this rearrangement is reversible and can occur in the time frame of minutes (Dejana 1995).

1.4.1  Endothelial junctions

Endothelial cells are a specific type of epithelial cell that make up the inner lining of blood vessels; unlike general epithelial cells, endothelial junctions do not have desmosomes. Particular to the endothelium, adherens junctions control the movement of plasma components
and white blood cells across the endothelium; in addition, these junctions direct angiogenesis, or formation of new blood vessels. Gap junctions allow for passage of components within the endothelium and tight junctions form the blood vessel barrier. These three types of junctions are expressed and organized based on the varying functions and capabilities of the different endothelial sites throughout the body (Bazzoni 2004).

The molecular structure of the junction has been suggested to change depending on the particular mechanism required, for example permeability regulation (Bazzoni 2004, Dejana 1995). In the blood vessels of the brain, the junctions have been found to have a complex organization so not to allow passage through the endothelium; on the contrary, in post-capillary venules, the junction structure is extremely simple to allow for crucial cell extravasation and exchange of plasma components (Dejana 1995). Additionally, the combination of junction types also varies depending on requirements. Using the same example, the brain contains a higher percentage of tight junctions than post-capillary venules, to account for the brain’s need for a sealed barrier rather than for permeable exchange (Bazzoni 2004).

1.4.2 Adherens junctions

Adherens junctions are formed via homophilic calcium-dependent adhesion in the intercellular space (Bazzoni 2004, Leckband 2006). Each cell contains a transmembrane glycoprotein, called a cadherin, connected to an intracellular network of cytoplasmic proteins and the cytoskeleton (Bazzoni 2004). Structurally, a cadherin is a single protein chain that extends through the membrane, with an extracellular domain in the intercellular space and a cytoplasmic region inside the cell. The extracellular part of the cadherin contains a calcium (Ca$^{2+}$) binding domain that allows for cadherin clustering (Dejana 1995). Cadherins on the same
cell can form dimers laterally in the cis direction while cadherins on neighboring cells can adhere in the intercellular space in the trans direction (Leckband 2006).

On the cytoplasmic end, the cadherin is attached to aforementioned cytoplasmic proteins and the cytoskeleton (Yap 1997). Specifically, the cadherin is attached to β-catenin which connects to α-catenin and ultimately connects to actin filaments (Yap 1997). Due to cadherin association with the cytoskeleton, adherens junctions control the structure and shape of the cell. Therefore, adherens junctions are not only responsible for strong adhesion between neighboring cells but also for the morphological changes that allow for paracellular movement of cells through the endothelium (Yap 1997).

Cadherins have four main functions: adhesion, selectivity, clustering, and signaling; each is carried out on a particular structure. Adhesion and selectivity are carried out on the extracellular domain, clustering occurs at the transmembrane portion, and signaling is initiated at the cytoplasmic region and extends into the cell (Leckband 2006). Endothelial cells express both specific and non-specific cadherins in their intercellular junctions. For example, N-cadherin (neural) is not only present in endothelial cells but also in nerve and muscle cells. Conversely, vascular endothelial (VE)-cadherin is specific to the endothelium and maintaining the vascular barrier (Dejana 1995).

1.4.3 VE-cadherin

Vascular endothelial (VE)-cadherin is a type of adherens transmembrane junction that facilitates regulation of the endothelial barrier through disassembly and reassembly (Sandig 1997). The retraction step of tumor extravasation is due to the breakdown of VE-cadherin homodimers which plays an important role in many cellular processes including angiogenesis,

1.5 Extracellular Stimuli

Cells communicate with one another via two different mechanisms: direct contact (juxtacrine signaling) and soluble factor interaction (paracrine signaling). These external stimuli adhere to the endothelial cell membrane and initiate intracellular signaling pathways to direct cell processes (Nelson 2002).

1.5.1 Juxtacrine signaling

In this study, we focused on activation and binding of vascular cell adhesion molecule-1 (VCAM-1), an integrin receptor found on the surface of endothelial cells. This endothelial adhesion molecule has been found to play an important role in the body’s immune system, assisting leukocyte adhesion to the blood vessel wall. It also has been suggested in initiation of signaling pathways during leukocyte- endothelial cell contact. VCAM-1 is a ligand for the integrin dimer very late antigen-4 (VLA-4), which is present on leukocytes and also on certain types of melanoma cells. VLA-4 is only expressed when the leukocytes or melanoma cells are activated by a stimulus to promote adhesion; the integrin then undergoes a conformational
change in order to bind to VCAM-1. A possible mechanism for melanoma adhesion and migration through the endothelium is similar to that which leukocytes use during inflammatory responses (Barreiro 2002).

1.5.2 Paracrine signaling

Other than direct cell-cell interaction via receptor/ligand binding, cells can also respond to soluble proteins which are excreted from neighboring cells. These proteins, known as cytokines, move through the extracellular space carrying signals intended to affect the function of the target cell. Adherence to the appropriate membrane receptor then initiates intracellular signaling cascades in the target cell. In the tumor microenvironment, proinflammatory cytokines are prevalent and have the role of causing inflammation. The cytokines of interest in this study are of the chemokine family, interleukin-8 and growth related oncogene-α, and of the interleukin family, interleukin-1β and interleukin-6 (Dinarello 2000).

1.5.2.1 Interleukin-8

Interleukin-8 (IL-8) is a proinflammatory cytokine that acts through G-protein-coupled receptors (GPCRs) to activate a multitude of intracellular signaling pathways (Hoch 1996, Waugh 2008). The two receptors it regulates are CXCR1 and CXCR2; CXCR1 selectively binds to IL-8 and granulocyte chemotactic protein-2 only while CXCR2 has an affinity for other CXC cytokines. The signaling cascades initiated at these receptors can induce angiogenic responses in endothelial cells, proliferation and survival of endothelial and cancer cells, and migration of endothelial cells, cancer cells, and neutrophils. All of these functions suggest an important regulatory role in the tumor microenvironment. Stimuli in this environment such as
inflammatory signals, chemical/environmental stresses, and steroid hormones are responsible for inducing expression of IL-8 (Waugh 2008). High expression levels of IL-8 and/or CXCR1/2 have been found in endothelial cells, neutrophils, macrophages, and tumor cells. Tumor cells that produce higher amounts of IL-8 have been described as having a higher metastatic potential (Slattery 2005). Constitutive as well as inducible expression of IL-8 in tumor cells makes it a potential therapeutic target in controlling tumor growth and metastasis (Waugh 2008, Xie 2001).

1.5.2.2 Growth related oncogene-α

Growth related oncogene (GRO) is a family of proteins that has been found to be expressed during inflammatory responses in fibroblasts, synovial cells, and endothelial cells (Stoeckle 1991). The GRO proteins have also been discovered in tumors including melanoma, glioblastoma, and carcinoma cells. There are three major isoforms GRO including α, β, and γ, all of which demonstrate similar cellular activity. Structurally, they are 33-40% identical to IL-8 and all share the roles of IL-8 in the cell, inducing chemotaxis, shape change, rise in intracellular free calcium, exocytosis, and respiratory burst in neutrophils (release of oxygen molecules for immune purposes) (Geiser 1993). While both CXCR1 and CXCR2 have a strong affinity for IL-8, only CXCR2 has the same affinity for the GRO family (Geiser 1993, Waugh 2008).

1.5.2.3 Interleukin-1β

The interleukin-1 (IL-1) protein family plays a vital role in the innate immune system, affecting survival and function of the immune cells (Sims 2010). Of the 11 extracellular cytokines in the family, IL-1β is the most commonly studied due to its role in mediating autoinflammatory diseases (Dinarello 2009, Sims 2010). Although IL-1α acts identically to IL-
1β when in solution, their physiological role in the cell differs drastically; while IL-1α acts in an autocrine fashion acting on the plasma membrane of the cell that produced it, IL-1β functions in a paracrine manner traveling in the circulation (Sims 2010). As for its origin, IL-1β can be secreted from normal body cells, monocytes, macrophages, and dendrites, or from cancer cells, melanoma, leukemia, and multiple myeloma cells (Dinarello 2009, Rubartelli 1990). Before secretion from the cell, IL-1β is contained inside intracellular vesicles to guard it from digestion from proteases and is normally expressed at low levels inside the cell (Rubartelli 1990, Sims 2010). After being released from the cell, it can adhere to a neighboring cell membrane via IL-1R1 which is the primary receptor subunit. This complex will then recruit IL-1RAP, receptor accessory protein, to form the receptor heterodimer. The dimer then induces intracellular signaling via the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways, which can lead to a number of cellular actions (Sims 2010). Notably, IL-1β induces inflammation by increasing expression of VCAM-1 on endothelial cells which aids in immune cell migration from the blood to the tissues; likewise, it facilitates tumor metastasis to a secondary site (Dinarello 2009).

1.5.2.4 Interleukin-6

Interleukin-6 (IL-6) is in a family of cytokines that functions through a receptor subunit called gp130 (Fuccini 2009). IL-6 binds to a cell membrane via the IL-6R complex which is comprised of IL-6R and aforementioned gp130. This binding initiates tyrosine phosphorylation which generally leads to activation of the signal transducers and activators of transcription (STAT) pathway. The gp130 stimulation upon binding simultaneously activates Ras which stimulates the p38 MAPK pathway (Taga 1997). IL-6 is produced by various cell types
including endothelial cells, keratinocytes, monocytes, macrophages, T-cells, and a number of tumor cell lines; its most important role is in the circulatory system but it also affects the nervous and endocrine systems (Chun 2009, Taga 1997, Van Snick 1990). In the circulatory system specifically, IL-6 interacts with blood and tumor cells to direct proliferation and differentiation (Taga 1997). Overall, the main functions of IL-6 are regulatory and deal with the immune system, acute-phase response, and bone metabolism (Fulciniti 2009).
Chapter 2

DETERMINATION OF EXTRACELLULAR STIMULI THAT INITIATE TUMOR-INDUCED VE-CADHERIN DISASSEMBLY

2.1 Introduction

The extravasation step of tumor metastasis involves retraction of the endothelium via disruption of the intercellular junctions, namely VE-cadherin. This process includes disassembly of VE-cadherin homodimers which control paracellular passage of molecules and cells through the endothelium (Tremblay 2006). VE-cadherin is commonly known for its role in angiogenesis, tumor metastasis, cell/protein signaling, and endothelial survival (Dejana 1999, Liao 2002). These functions support the importance of studying VE-cadherin in melanoma-mediated extravasation. This chapter will focus on identifying the extracellular triggers, both juxtacrine via receptor/ligand molecules and paracrine via soluble factors, which lead to VE-cadherin breakdown in endothelial cells.

2.2 Methods

2.2.1 Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) were acquired from American Type Culture Collection (ATCC) (Manassas, VA) and cultured in F-12K medium, supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin-streptomycin (pen-strep) (Biofluids, Inc.),
30 μg/ml endothelial cell growth supplement (ECGS), and 50 μg/ml heparin (Mallinckrodt Baker, Inc.). Lu1205 melanoma cells were obtained from Dr. Gavin P. Robertson (Penn State Hershey Medical Center, Hershey, PA) and cultured in Dulbecco’s Modified Eagle Medium/F12 (DMEM/F12) (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 100 units/ml pen-strep. A2058 melanoma cells were acquired from ATCC and cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% FBS and 100 units/ml pen-strep. The cells were cultured at 37°C and 5% CO₂ in a humidified incubator.

2.2.2 Cell preparation

No. 1 cover slips with a 25mm diameter were prepared by a wash with Dulbecco’s phosphate-buffered saline (DPBS) and coated with 1 μg/ml fibronectin to enhance cell adhesion. HUVECs were seeded on these cover slips and placed in a 6-well plate where they were grown to 100% confluency in the conditions described above in Section 2.2.1.

2.2.3 Endothelial-melanoma co-culture

Each cover slip contained approximately 3x10⁵ endothelial cells when grown to 100% confluency. Experimental conditions included pretreatment with F-12K medium supplemented with 2% FBS without additional nutrients for 12 hours prior to experimentation at 37°C and 5% CO₂. A 3:1 ratio of melanoma cells: endothelial cells was used in all co-culture experiments. Suspended in culture medium, melanoma cells were added to the endothelial cells in the 6 well plate. In preparation for co-culture, melanoma cells were lifted using a thin coating of 0.5% trypsin (Gibco, Invitrogen, Carlsbad, CA) incubated for approximately 5 minutes until the cells
detached. The cells were then suspended in fresh medium in a 15ml conical tube and rocked for 1 hour in a 37°C incubator. The recovered melanoma cells were then added to the endothelial monolayer to perform the experiment.

2.2.4 Tumor conditioned medium (TCM) collection

Melanoma cells were first grown to approximately 95% confluency in 150cm² flasks in their appropriate medium and conditions stated in Section 2.2.1 above. Melanoma cell culture medium was replaced with HUVEC medium with 2% FBS, as described above. After 24 hours, the medium was collected from the flask, placed in a 15ml conical tube, and centrifuged for 5 minutes at 1500 revolutions per minute (RPM) to remove any cell components. TCM was then stored at -80°C until use in an experiment.

2.2.5 Pharmacological antibodies and inhibitors

Cytokine experimentation included both recombinant activation and neutralization of the factors in the HUVECs. Concentrations of recombinant IL-8 at 15 ng/ml, IL-6 at 6 ng/ml, IL-1β at 0.051 ng/ml, and GRO-α at 5.4 ng/ml (Biosource, Inc.) were added to the HUVEC monolayer for 45 minutes to assess endothelial gap formation. Additionally, the four cytokines were neutralized in HUVECs 30 minutes prior to addition of tumor cells in contact co-culture, described in Section 2.2.3.
2.2.6 VE-cadherin cell staining

After HUVECs were stimulated during each experiment, the cells were washed twice with DPBS and fixed with 5% formaldehyde for 20 minutes in a dark environment. Next, the cells were permeabilized with 0.3% Triton-X 100 in DPBS with 5% calf serum (CS) and 2% goat serum (GS) for 15 minutes before being refrigerated overnight at 4°C with 10 μg/ml anti-VE-cadherin primary antibody diluted in DPBS/5%CS/2%GS. The final step was 1 hour incubation at room temperature in solution containing 1 μg/ml Alexa Fluor 488 in DPBS/5%CS/2%GS. Each incubation step was separated by 3 washes with DPBS/5%CS/2%GS.

2.2.7 Fluorescence microscopy analysis

Cell imaging was performed using a Nikon fluorescence microscope. Each cover slip was viewed under a 40x and 100x oil objective and six random fields of view were imaged. These images were analyzed using Image J software (Rasband 1997). The area of gaps present in the images was calculated by Image J; the total percent of gap in the image was then calculated. This was performed by calculating the ratio of total gap area in one image by the total image area of 1920000 (1600x1200) pixels and multiplying by 100 to find a percentage (Serck-Hanssen 2006 and Stevens 2002). The average endothelial gap was calculated from the results of the six separate images and plotted versus the other cases in the experiment. This gap formation is directly related to VE-cadherin disassembly because gaps are formed when VE-cadherin is no longer intact. Intact VE-cadherin is represented by continuous green fluorescent dye at the cell membrane so breakdown of VE-cadherin eliminates the green fluorescence.
2.2.8 Statistical analysis

Each experiment represents three independent trials. Standard deviation was used and significance was found by using a statistical t-test (Sigma Plot 8.0). A p value less than 0.05 is considered significant and is denoted by (*).

2.3 Results

2.3.1 Comparison of melanoma cell lines

In order to determine the means that tumor cells use to initiate VE-cadherin disassembly in endothelial cells, a melanoma cell line study was performed. HUVEC monolayers were grown to confluency before co-culture with WM35, A2058, and Lu1205 melanoma cells or tumor conditioned medium (TCM) for a 45 minute incubation. The metastatic potentials of the three cell lines in question are shown in Table 2.1 and the effect of each cell line on VE-cadherin disassembly is shown in Figure 2.1.

Table 2.1 Metastatic potentials: melanoma cell lines. WM35 has the lowest metastatic potential, followed by A2058 and finally Lu1205 which is a highly metastatic cell line.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Metastatic potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>WM35</td>
<td>_</td>
</tr>
<tr>
<td>A2058</td>
<td>++</td>
</tr>
<tr>
<td>Lu1205</td>
<td>+++</td>
</tr>
</tbody>
</table>

(Khanna 2010)
Figure 2.1 Endothelial gap formation in response to melanoma cell lines. See Table 2.1 for metastatic potential of three representative cell lines. WM35 TCM and TC did not create a significant amount of gap formation compared to the control case. Higher metastatic lines, A2058 and Lu1205, induce significant values for both TCM and TC. Values are means ± SD, * represents p <0.05. WM35 data was kindly provided by Payal Khanna (Ph.D. Candidate of Bioengineering, The Pennsylvania State University).

It was found that low metastatic line WM35 did not induce significant gap formation. However, medium/high metastatic lines A2058 and Lu1205 induced significant increases in gap formation with both TCM and TC. Relative to the control case with no stimulus, A2058 created 6.6-10.1 times more and Lu1205 resulted in 7.9-13.5 times more gap formation.
2.3.2 Stimuli that induce VE-cadherin disassembly and gap formation

Now that it was determined that medium to high metastatic cell lines induce gap formation at a time period of 45 minutes, we have chosen to direct our studies to the medium metastatic line, A2058. To learn more about the manner in which tumor cells induce endothelial gap, the first goal is to investigate the two types of stimuli that tumor cells utilize: receptor/ligand molecules and soluble factors. To maximize the information gathered, a time course experiment was designed to monitor the result of three stimuli: A2058 TCM to simulate soluble means, anti VCAM-1 to induce receptor/ligand-mediated signaling, and A2058 TCs as a positive control. The time course result is plotted in Figure 2.2 and fluorescent images of TCM-stimulated HUVEC monolayers are shown in Figure 2.3.

![Figure 2.2](image_url)

**Figure 2.2** Endothelial gap formation over time. Anti VCAM-1 was added to induce direct contact signaling while A2058 TCM was used to mimic a purely soluble-mediated response; cases of unstimulated HUVEC and A2058 TC addition were used as negative and positive controls, respectively. Anti VCAM-1 data was kindly provided by Payal Khanna (Ph.D. Candidate of Bioengineering, The Pennsylvania State University).
Figure 2.3 Fluorescent VE-cadherin staining in endothelial cells. Panel A represents the control case with no stimulation, panel B represents 10 minute stimulation, panel C represents 45 minute stimulation and panel D represents 90 minute stimulation, by A2058 TCM.

The purpose of testing direct contact and soluble induction separately is to examine the relative importance of each on VE-cadherin disassembly. Over a time course of 90 minutes, A2058 TCs resulted in a value between 24-41% while A2058 TCM and anti VCAM-1 both induced approximately 14-28% gap formation. This suggests that both types of stimuli are necessary for VE-cadherin disassembly and gap formation in endothelial cells. Because only one direct contact mechanism (VCAM-1) was tested in this study, it is possible that there are additional adhesion molecules that play a role in signaling for VE-cadherin disassembly. Despite that, VCAM-1 acted similar to the other two stimuli resulting in a linear increase in %
endothelial gap over time. Although the 90 minute time point gave the result with the largest amount of endothelial gap, the images were dominated by gap; therefore, the remainder of VE-cadherin disassembly experiments will be conducted over 45 minutes which created a good balance between the amount of endothelium and gap.

2.3.3 Identification of cytokines

Now that it has been shown that soluble factors in TCM play an important role in VE-cadherin disassembly, four specific targets will be examined: interleukin-8 (IL-8), growth related oncogene-α (GRO-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Previous data has found that these four cytokines are increased in endothelial-melanoma co-culture. Table 2.2 below shows the concentrations of these cytokines found in the 24-hour TCM of three melanoma cell lines: WM35, A2058, and Lu1205. Continuing with the previous A2058 studies, the highlighted concentrations shown in Table 2.2 will be used.

**Table 2.2 Cytokine concentration in melanoma cell lines.** This table displays the result of an ELISA measuring the concentrations of IL-8, IL-6, IL-1β, and GRO-α in 24-hour TCM. It was kindly provided by Payal Khanna (Ph.D. Candidate of Bioengineering, The Pennsylvania State University).

<table>
<thead>
<tr>
<th>Melanoma Cell Line</th>
<th>Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-8</td>
</tr>
<tr>
<td>WM35</td>
<td>0.084</td>
</tr>
<tr>
<td>A2058</td>
<td>15</td>
</tr>
<tr>
<td>Lu1205</td>
<td>24</td>
</tr>
</tbody>
</table>
To test the individual effect of each of these cytokines, the recombinant forms were added separately to a HUVEC monolayer over a 45 minute time period. The IL-8 concentration used was 15 ng/ml, GRO-α concentration was 5.4 ng/ml, IL-1β concentration was 0.051 ng/ml, and IL-6 concentration was 6 ng/ml. Figure 2.4 below is a representation of the results.

**Figure 2.4** Cytokine induction of VE-cadherin disassembly. Each of IL-1β, IL-6, IL-8, and GRO-α induced a significant amount of gap formation compared to the control case of HUVEC alone. Additionally, TCM still produced a significantly higher amount of endothelial gap than any of the individual cytokines. Values are means ± SD, * represents p <0.05.

Although each cytokine was found to cause a significant amount of gap formation in comparison to the unstimulated control case, each also created significantly less than the TCM case. Because the results of all four cytokines were not significantly different from one another, one particular cytokine does not play the main role in initiating VE-cadherin disassembly. However, physiologically and in TCM, the cytokines do not act individually but in combination;
Therefore, the next step is to examine pairs of cytokines to test resulting gap formation. Through literature evidence that IL-1β regulates the p38 MAPK pathway and that IL-1β has been found to induce IL-6 and IL-8 expression, it was decided that IL-1β paired with IL-6 and IL-8 would be interesting cases to examine (Dinarello 2009, Sims 2010). Again at concentrations of IL-8 at 15 ng/ml, IL-6 at 6 ng/ml, and IL-1β at 0.051 ng/ml, the individual cases IL-1β, IL-6, IL-8 for controls, combinations IL-8/IL-1β and IL-6/IL-1β, and TCM case were tested over a 45 minute time period. The results are plotted below in Figure 2.5.

![Figure 2.5](image_url)

**Figure 2.5** Combination versus individual cytokines. The combination of IL-8/IL-1β induced an additive effect while the combination of IL-6/IL-1β induced a slightly synergistic effect compared to result of the individual cytokines. Both combination cases created significantly less endothelial gap than the TCM case. Values are means ± SD, * represents p <0.05.

In this experiment, all three interleukins and both combinations proved to cause significant endothelial gap compared to the control case; IL-6/IL-1β induced significantly more endothelial gap that the individual cytokines which indicates a slightly synergistic effect while
IL-8/IL-1β resulted in a purely additive effect. Neither combination case however was statistically similar to the TCM value. This indicates that synergy of a cytokine pair is not sufficient stimulation and that other soluble factors are needed to initiate the full response of TCM. These players are not limited to the four being presently discussed (including GRO-α) but can include other cytokines not examined via the ELISA.

2.3.3.2 Neutralization studies

The previous two sections verified the importance of each cytokine individually and suggested that other factors are still necessary to produce the full effect of TCM in endothelial cells. In order to come to a conclusive result regarding the latter implication, a neutralization study was planned to test the opposite outcome. Our hypothesis prior to experimentation was that cytokine neutralization inside endothelial cells would cause a decrease in gap formation relative to the TCM case. As previously stated we know that IL-1β stimulates the p38 MAPK pathway and discovery that IL-8 induces the Ca^{2+} pathway influenced us to focus on these two cytokines. Figure 2.6 shows the result of three cases IL-8, IL-1β, and IL-8/IL-1β after 30 minute neutralization prior to the 45 minute EC co-culture with TC.
Figure 2.6 Neutralization of cytokines. The two controls in this experiment are unstimulated and A2058 TC-stimulated HUVEC. Neutralizing IL-8, IL-1β, and IL-8/IL-1β before A2058 stimulation decreased the endothelial gap formation significantly in comparison to the A2058 TC-induced response. This study was performed over 45 minute stimulation. Values are means ± SD, * represents p <0.05.

The hypothesis that neutralization of cytokines will cause a decreased amount of gap formation was shown by our data. Additionally, similar to the combination experiment, the neutralization of IL-8/IL-1β showed purely an additive effect on gap formation. A conclusive result thus far is that cytokines of particular interest, IL-8 and IL-1β, are influential extracellular triggers of VE-cadherin disassembly in endothelial cells.

2.4 Discussion

In this chapter, it has been proven that melanoma cells induce VE-cadherin disassembly in order to enable cell migration through the endothelium. We found that this holds true for medium to high metastatic melanoma cell lines but not for cell lines with low metastatic potential, i.e. WM35. After consideration of whether the disassembly was caused by soluble or
contact-mediated processes, it was confirmed that both soluble-mediated and direct contact signaling induce the junction breakdown in endothelial cells. The VCAM-1 contact mechanism and soluble-mediated stimulation via IL-8, IL-6, IL-1β, and GRO-α were proven extracellular stimuli although there may be additional players involved. Concentrating on the soluble factor role, the four cytokines IL-8, IL-6, IL-1β, and GRO-α each played a role in individually causing junction breakdown. Combination studies found that the IL-8/IL-1β pair induces an additive effect; to confirm these results, neutralization of the cytokine pair oppositely and accurately reduced the amount of junction disassembly.

Now that external triggers causing VE-cadherin breakdown have been examined, it is important to look at intracellular players. As previously mentioned, IL-1β is known to regulate the p38 MAPK pathway in endothelial cells (Sims 2010). Additionally, the endothelial Ca^{2+} pathway is stimulated by the CXC receptor family, namely CXCR1 which has a high affinity for IL-8 (Waugh 2008). This makes both the p38 MAPK and Ca^{2+} pathway a possible means for tumor-induced VE-cadherin disassembly. The next chapter will explore various targets in these pathways in order to characterize the signaling cascades that tumor cells utilize to mediate tumor transendothelial migration.
Chapter 3

CHARACTERIZATION OF INTRACELLULAR TARGETS THAT PLAY A ROLE IN TUMOR-INDUCED SIGNALING PATHWAYS

3.1 Introduction

Now that the extracellular stimuli of VE-cadherin disassembly have been examined, the focus will turn to the intracellular signaling pathways that result from these triggers. After a thorough literature search, it was determined that tumor cells may act through the p38 mitogen-activated protein kinase (MAPK) and calcium (Ca$^{2+}$) pathways (Sims 2010, Waugh 2008). The remainder of this chapter will focus on examination of these pathways and characterization of intracellular targets that lead to VE-cadherin disassembly.

3.2 Methods

Cell culture, cell preparation, endothelial-melanoma co-culture, tumor conditioned medium collection, VE-cadherin cell staining, and fluorescence microscopy analysis were performed as described in Sections 2.2.1, 2.2.2, 2.2.3, 2.2.4, 2.2.6, and 2.2.7, respectively.

3.2.1 Pharmacological antibodies and inhibitors

For p38 inhibitor studies, 1 μM of [5-(2-Amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl)imidazole] (SB220025) (Calbiochem; Gibbstown, NJ) was added to pretreat the ECs.
The HUVECs were cultured in F-12K medium supplemented with 2% FBS for 12 hours prior to inhibitor pretreatment. Calcium was chelated using \[\text{N-(2-(((8-(bis(carboxymethyl) amino)-6-methoxy-2-quinoliny1) methoxy)-4-methylphenyl)-N-(carboxymethyl)}\] (quin-2) at a concentration of 40 μM. To block IP3-mediated Ca\textsuperscript{2+} release in ECs, Xestospongion C was administered at a concentration of 1 μM (Calbiochem; Gibbstown, NJ). PKC inhibition in ECs was performed using [2-[1-(3-Dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1H-indol-3-yl) maleimide] (Gö6983) at a concentration of 5 μM (Calbiochem; Gibbstown, NJ). MLCK inhibition in ECs was performed using MLCK inhibitor peptide 18 at a concentration of 50 μM (Calbiochem; Gibbstown, NJ). In all inhibition experiments, HUVEC were incubated with the inhibitor for 30 minutes prior to experimentation. The protein-inhibited HUVECs were then stimulated by TCs as described in Section 2.2.3.

3.2.2 Calcium cell staining

Prior to staining, cells were serum starved in F-12K medium with no nutrients for 30 minutes. The fluorescent dye, Calcium Green-1 AM (Molecular Probes; Carlsbad, CA), was then administered at a concentration of 5 μM and incubated for 30 minutes. The cells were washed three times with DPBS to remove any unattached dye.

3.2.3 Calcium measurements

To determine calcium ion levels inside the cell, the fluorescently labeled cells were viewed under a Nikon fluorescence microscope with a 40x oil objective. A CoolSNAP EZ camera (Photometrics: Tucson, AZ) and NIS-Elements software (Nikon Instruments Inc.; Melville, NY) were used to capture images of the cells and to measure fluorescence intensity.
over time. The program was set to collect a data point every 6 seconds for a period of approximately 6 minutes; the data points from the first minute were used to form a baseline value. When different stimuli were added into the chamber containing the HUVEC monolayer, the system would track any change in fluorescence intensity which was automatically plotted over time.

3.2.4 Statistical analysis

Each experiment represents three independent trials. Standard deviation was used and significance was found by using a statistical t-test (Sigma Plot 8.0). A p value less than 0.05 is considered significant and is denoted by (*). For Ca^{2+} measurement, each case consisted of three independent trials and each trial measured 8-10 individual endothelial cell responses.

3.3 Results

3.3.1 p38 MAPK pathway: p38 inhibition

A generalized version of a MAPK pathway involves three central components, MAPKKK, MAPKK, and MAPK; specifically, the p38 MAPK pathway is composed of TAK1 (MAPKKK), MKK3/6 (MAPKK), and p38 (MAPK). Examining the pathway in its entirety begins with IL-1β stimulation of its membrane receptor which activates TGF-β-activated kinase1 (TAK1). TAK1 then proceeds to activate (mitogen-activated protein kinase kinase) MKK3 and/or MKK6 which are responsible for phosphorylation of p38. It has been found that MKK3 and MKK6 may each be linked to specific isoforms of p38, suggesting differing function in the cell. It also has been found that cytokines and stress signals stimulate particular MKKs to induce a target function (Li 2005). Next, p38 phosphorylates Hsp27 which binds to actin filaments; this
action mediates H$_2$O$_2$ reorganization of the actin cytoskeleton (Nguyen 2004). It has been suggested that the p38 pathway leads to junction breakdown and VE-cadherin disassembly (Nwariaku 2002 and Tremblay 2006). A detailed schematic of the p38 MAPK pathway is displayed in Figure 3.1.

![Figure 3.1 Intracellular p38 MAPK signaling pathway. Extracellular IL-1β is known to initiate the p38 cascade at its receptor on the cell membrane, activating the downstream signaling cascade that ultimately mediates VE-cadherin disassembly.](image)

In summary, we know that tumor cells initiate the p38 MAPK pathway and Hsp27 interacts with the actin cytoskeleton (Nwariaku 2002 and Tremblay 2006). To test if tumor cells
use this p38 MAPK pathway directly to affect VE-cadherin disassembly, an experiment was designed to halt the signaling cascade at p38. To do so, p38 was inhibited in the HUVEC monolayer using the inhibitor, SB220025. Experimental conditions involved HUVEC monolayer incubation in F-12K medium supplemented with 2% FBS and no growth factors for 12 hours; this would assure no outside factors affecting the intracellular signaling within the endothelial cells. Then, 30 minutes prior to the start of the experiment, the HUVECs were incubated with 1 μM SB220025 and finally stimulated with A2058 melanoma for the 45 minute course of the experiment. The results are shown in Figure 3.2.

**Figure 3.2** Effect of p38 inhibition on gap formation. The HUVEC monolayer was pretreated with p38 inhibitor, SB220025, for 30 minutes prior to 45 minute stimulation with A2058 TCs. The inhibitor significantly reduced the amount of gaps formed in comparison to the untreated case. Values are means ± SD, * represents p <0.05.

Physiologically, pretreatment of the endothelial cell monolayer with the p38 inhibitor SB220025 blocks p38-mediated signals to downstream effector proteins in the signaling cascade;
the aim was to verify that VE-cadherin was downstream of p38 in endothelial cells. Figure 3.2 shows that use of the p38 inhibitor prior to tumor cell addition causes a decrease in endothelial gap formation. The positive control with p38 intact produced more than 2 times the amount of gap formation than when p38 was inhibited. This proves that the disassembly of VE-cadherin is a downstream target of the p38 MAPK pathway. Now that we’ve verified the role of the p38 MAPK pathway in VE-cadherin breakdown in endothelial cells, we will turn to examination of a possible connection between VE-cadherin and the Ca$^{2+}$ signaling pathway.

3.3.2 Calcium pathway

The Ca$^{2+}$ pathway begins when extracellular stimulus IL-8 binds to a G-protein coupled receptor (GPCR), which is composed of subunits α, β, and γ. The phosphorylated G$_{α}$ subunit then activates phospholipase C (PLC), specifically PLC$β$. Other subunits of PLC include PLC$δ$, PLC$ε$, PLC$ζ$, and PLC$γ$, which are activated by other types of receptors. PLC then cleaves phosphatidylinositol 4,5-bisphosphate (PIP$_2$) into diacylglycerol (DAG) and inositol triphosphate (IP$_3$). DAG activates protein kinase C (PKC) which leads to activation of Rac and crosstalk with other signaling cascades. IP$_3$ is released into the cytosol and binds to inositol triphosphate receptors (IP$_3$R), which are Ca$^{2+}$ channels in the endoplasmic reticulum (Peng 2005). Ca$^{2+}$ is then released from the endoplasmic reticulum and into the cytosol where it induces intracellular activity (Montcouquiol 2001). External calcium ions (Ca$^{2+}$ext) are taken into the cell from external sources and sarco/endoplasmic reticulum Ca$^{2+}$ ATPase (SERCA) is responsible for the reuptake of Ca$^{2+}$ into the endoplasmic reticulum as calcium storage (Ca$^{2+}$store). Ca$^{2+}$ also mediates the Rac-RhoGDI complex which regulates Rac and crosstalk to other pathways. Additionally, Ca$^{2+}$ binds to calmodulin (CaM) which then activates myosin light-chain kinase.
(MLCK) causing contraction of the extracellular matrix by initiating myosin-mediated binding to actin filaments. This is thought to affect VE-cadherin interaction with β-catenin and ultimately VE-cadherin disassembly (Peng 2007). A graphical representation of the pathway is shown in Figure 3.3.

Figure 3.3 Intracellular Ca\(^{2+}\) signaling pathway. Extracellular IL-8 is known to initiate the cascade by adhering to a G-protein coupled receptor, which activates the downstream signaling cascade.
Our goal was to validate this pathway via laboratory experimentation and mathematical modeling so our first step was testing tumor cells’ ability to initiate intracellular Ca\(^{2+}\) release in endothelial cells.

3.3.2.1 Ca\(^{2+}\) measurements

Our focus turned to the Ca\(^{2+}\) pathway because IL-8 was found to be an important extracellular regulator of the pathway (Waugh 2008). From our previous ELISA data (Table 2.2), we know that tumor cells secrete IL-8 so our hypothesis is that, like IL-8, tumor cells will stimulate the Ca\(^{2+}\) pathway. To test our hypothesis, we planned experiments using cell staining and fluorescence microscopy to measure intracellular calcium ion ([Ca\(^{2+}\)]\(_i\)) concentration. Prior to experimentation, the HUVEC monolayer was treated with 5 \(\mu\)M Calcium Green for 30 minutes. Calcium staining was done on unstimulated cells and live cell measurements were taken as the stimulus is added over the course of the experiment. The first stimulus to be tested was A2058 TC, see results below in Figure 3.4.
Figure 3.4 Ca$^{2+}$ response due to A2058 TC stimulation. A. A2058 TC stimulation induced a significant amount of $[\text{Ca}^{2+}]_{i}$ in HUVECs ($n=9$). Over three independent trials, $90 \pm 5.77\%$ of HUVECs responded to the stimulus. Values are means $\pm$ SD, * represents $p < 0.05$. B. A2058 TCs were added into the chamber at the point of the arrow at approximately 1 minute. The fluorescence intensity and consequently Ca$^{2+}$ concentration increased immediately upon A2058 TC addition. Over the course of the triplicate experiment, $90 \pm 5.77\%$ of the endothelial cells responded to the TC addition. On average it took about 90 seconds for the endothelial cells to return to the baseline value, signifying the amount of time it took the endothelial cells to reuptake the $[\text{Ca}^{2+}]_{i}$ into the Ca$^{2+}$ stores. This result proves our first hypothesis to be true; TCs stimulate the Ca$^{2+}$ pathway and induce calcium ion
release from intracellular stores. Again, we move on to examine whether soluble factors play a role in the tumor-induced response, in this case Ca^{2+} release inside endothelial cells. The next experiment uses A2058 TCM as the stimulus to test for Ca^{2+} response; see Figure 3.5.

**Figure 3.5** Ca^{2+} response due to A2058 TCM stimulation. A. A2058 TCM stimulation induced a significant amount of [Ca^{2+}]_i in HUVECs (n=9). Over three independent trials, 90 ± 5.77% of HUVECs responded to the stimulus. Values are means ± SD, * represents p <0.05. B. A2058 TCM was added into the chamber at the point of the arrow at approximately 1 minute. The fluorescence intensity and consequently Ca^{2+} concentration increased immediately upon addition of the A2058 TCM.

In this case, A2058 TCM induced a significant 2.1 fold increase in [Ca^{2+}]_i response compared to the baseline. Similar to TC stimulation, over three trials 90 ± 5.77 % of the
endothelial cells responded to TCM addition and on average the cells took about 90 seconds to return to the baseline value. The next logical step was to examine whether individual cytokines would induce a Ca$^{2+}$ response in EC. Since IL-8 has been linked to the Ca$^{2+}$ pathway, we wanted to verify that IL-8 will elicit a Ca$^{2+}$ response in our experiment (Waugh 2008). The result can be seen in Figure 3.6.

![Figure 3.6 Ca$^{2+}$ response due to IL-8 stimulation.](image)

A. IL-8 stimulation induced a significant amount of [Ca$^{2+}$]$_i$ in HUVECs (n=7). Over three independent trials, 80 ± 5.77% of HUVECs responded to the stimulus. Values are means ± SD, * represents p <0.05.

B. Recombinant IL-8 at a concentration of 15 ng/ml was added into the chamber at the point of the arrow at approximately 1 minute 45 seconds. The fluorescence intensity and consequently Ca$^{2+}$ concentration increased immediately upon addition of IL-8.
Addition of recombinant IL-8 produced a significant result of nearly 1.9 times the baseline value. In this case over three trials 80 ± 5.77 % of the endothelial cells responded to the IL-8 stimulus and returned to the baseline value after approximately 90 seconds, on average. This result establishes that IL-8 may be an extracellular trigger that tumor cells use to regulate the Ca^{2+} pathway and furthermore VE-cadherin disassembly.

3.3.2.2 Ca^{2+} chelation

Now that we have proved that tumor cells have the ability to initiate Ca^{2+} signaling and ion release through soluble factors, i.e. IL-8, we next want to move to the second half of the pathway: the downstream section between Ca^{2+} and VE-cadherin disassembly. To check this connection, we depleted intracellular calcium in the endothelial cells before stimulation with tumor cells in order to test the stability of VE-cadherin junctions. The experimental results are shown in Figure 3.7.
Figure 3.7 Chelation of intracellular calcium. \([\text{Ca}^{2+}]_{i}\), chelator, quin-2, was administered at a concentration of 40 μM for 30 minutes prior to 45 minute stimulation by A2058 TCs. A. After calcium chelation, \(\text{Ca}^{2+}\) release due to TC stimulation was significantly lower than the untreated A2058 TC stimulated case. B. Endothelial gap formation was significantly reduced in the calcium chelation case in comparison to the untreated A2058 TC stimulated case. Values are means ± SD, * represents p <0.05.

It is apparent from Figure 3.7 that \(\text{Ca}^{2+}\) plays a role in inducing VE-cadherin disassembly and endothelial gap formation. When intracellular \(\text{Ca}^{2+}\) was depleted from the cell, it was
confirmed that TCs were unable to induce Ca\(^{2+}\) release and that TCs created significantly less endothelial gap. The last remaining question regarding the validity of the Ca\(^{2+}\) pathway in VE-cadherin disassembly is whether the intracellular Ca\(^{2+}\) release is controlled by the members of this particular pathway.

3.3.2.3 IP\(_3\)-mediated Ca\(^{2+}\) inhibition

As previously stated, IP\(_3\) functions to bind to IP\(_3\)R which causes release of intracellular Ca\(^{2+}\) from the endoplasmic reticulum into the cytosol. In order to test whether this is the Ca\(^{2+}\) responsible for the signaling of VE-cadherin breakdown, we blocked IP\(_3\)-mediated Ca\(^{2+}\) in the endothelial monolayer using 1 μM Xestospongin C. The effect on endothelial gap formation is shown in Figure 3.8.
Figure 3.8 Inhibition of IP$_3$-mediated Ca$^{2+}$ release. Xestospongin C was added at a concentration of 1 μM for 30 minutes prior to 45 minute stimulation by A2058 TCs. A. After inhibition of IP$_3$-mediated Ca$^{2+}$ release, Ca$^{2+}$ due to TC stimulation was significantly lower than the untreated A2058 stimulated case. B. Endothelial gap formation decreased significantly when IP$_3$-mediated Ca$^{2+}$ was inhibited in comparison to the untreated A2058 TC stimulated case. Values are means ± SD, * represents p <0.05.
This last experiment confirms that the intracellular Ca\textsuperscript{2+} largely responsible for VE-cadherin disassembly is mediated by IP\textsubscript{3} within the Ca\textsuperscript{2+} pathway. Furthermore, within these past three sections, we have verified that extracellular IL-8 induces intracellular Ca\textsuperscript{2+} release, that intracellular Ca\textsuperscript{2+} is necessary for VE-cadherin breakdown, and that it is intracellular Ca\textsuperscript{2+} induced by IP\textsubscript{3} that is specifically needed for the VE-cadherin disassembly. After reaching that conclusion, the study switched gears to examine the other intracellular pathway components.

3.3.2.4 PKC inhibition

The protein kinase C (PKC) family of kinases has 11 isotypes organized into three classes based on the manner in which they are activated. nPKCs are activated solely by DAG, cPKCs are activated by both DAG and Ca\textsuperscript{2+}, and aPKCs are the remaining isotypes that are not activated by DAG, Ca\textsuperscript{2+}, or phorbol esters, which are DAG derivatives (Montcouquiol 2001). A common mode of operation for an activated PKC is to translocate to the membrane and initiate catalytic activity (Nishizuka 1995). PKC functions to phosphorylate Rho guanosine-nucleotide dissociation inhibitor (RhoGDI) which dissociates itself from Rac allowing Rac to translocate to the membrane and interact with membrane proteins (George 2010).

Similar to the p38 inhibition experiment described in Section 3.3.1, we now aimed to block the function of PKC to test whether VE-cadherin disassembly will be affected. The inhibition of several PKC isoforms was performed using Gö6983 at a concentration of 5 μM for 30 minutes prior to addition of A2058 TCs. The result of PKC inhibition is displayed in Figure 3.9.
Figure 3.9 Inhibition of PKC. At a concentration of 5 μM, Gö6983 was used to pretreat the HUVEC monolayer for 30 minutes before 45 minute stimulation by A2058 TCs. Although there was a slight decrease in endothelial gap formation, it was not statistically significant compared to the untreated case. Values are means ± SD, * represents p <0.05.

Although numerically the PKC inhibition case produced less gap formation than the untreated case, the result was not statistically significant. This indicates that PKC may not be directly involved in the part of the Ca^{2+} signaling pathway that leads to gap formation.

3.3.2.5 MLCK inhibition

The family of myosin light chain kinases (MLCKs) are soluble factors that phosphorylate the MLC causing it to change its structure. This then induces actin-myosin interaction, Mg^{2+} activity in myosin, and ultimately actin-myosin contraction (Gutjahr 2005, Shen 2010). Due to MLCK’s association with calmodulin (CaM), it becomes activated when Ca^{2+} binds to CaM. MLCK then phosphorylates MLC in order to direct such cell processes as cell migration, muscle contraction, endo/exocytosis, and endothelial cell-cell adhesion signaling. It has been proven
that MLCK is a principal factor in various signaling pathways that lead to inflammatory responses. Specifically, MLCK’s involvement has been noted in inducing endothelial permeability at trauma sites (Shen 2010); likewise, it has been implicated in the regulation of neutrophil transendothelial migration (Saito 1998).

Since MLCK is thought to play a role in the processes of cell migration and endothelial permeability, it is an extremely attractive target for our signaling pathway. It is thought to be directly upstream of VE-cadherin and our experiment will aim to validate that fact. To do so, we inhibited MLCK in EC using MLCK inhibitor peptide 18 at a concentration of 50 μM. Figure 3.10 shows the endothelial gap formation after MLCK inhibition and TC stimulation.

**Figure 3.10** Inhibition of MLCK. MLCK was inhibited using 50 μM MLCK Inhibitor Peptide 18 for 30 minutes prior to 45 minute stimulation by A2058 TCs. The result shows a significant decrease in endothelial gap compared to the untreated case. Values are means ± SD, * represents p <0.05.
Referring back to Figure 3.3 it is shown that MLCK mediates the most downstream portion of the Ca\(^{2+}\) pathway. The goal of this entire chapter has been to connect intracellular components of the Ca\(^{2+}\) pathway to gap formation, in vitro. With the results of Figure 3.10, it is apparent that there is a connection between MLCK and VE-cadherin disassembly at the cell membrane.

### 3.4 Discussion

We knew from literature that through association with receptor IL-1R, IL-1β regulates the p38 MAPK pathway and we found from experimentation that inhibition of p38 decreases tumor-induced endothelial gap formation (Sims 2010). Therefore, we were able to connect extracellular trigger IL-1β to intracellular signaling pathway p38 MAPK with the end product being VE-cadherin disassembly. Similarly, we learned from literature that through activation of receptors CXCR1 and CXCR2, IL-8 mediates the Ca\(^{2+}\) pathway (Waugh 2008). After experimental verification that TCs, TCM, and IL-8 induce a release of intracellular Ca\(^{2+}\), we were able to establish the relation between extracellular target IL-8 (Chapter 2) to the intracellular Ca\(^{2+}\) pathway. Furthermore, chelation of general intracellular Ca\(^{2+}\) and inhibition of specific IP\(_3\)-mediated intracellular Ca\(^{2+}\) confirmed the role of Ca\(^{2+}\) in VE-cadherin disassembly. Similar to the p38 pathway, the Ca\(^{2+}\) pathway is now proven to extend from extracellular stimulus IL-8 through intracellular signaling components to the final outcome of VE-cadherin disassembly.

Now that laboratory experimentation has validated VE-cadherin disassembly via signaling through the p38 MAPK and Ca\(^{2+}\) signaling pathways, we turned to identification of the intracellular components in the Ca\(^{2+}\) pathway. We found that PKC inhibition did not prove to
significantly reduce the endothelial gap, suggesting that IL-8 signaling does not necessarily activate downstream targets via PKC. To understand why this may be, let’s look upstream at the PIP₂:PLCβ:Gα-P complex where PLCβ cleaves PIP₂ into IP₃ and DAG (Peng 2009). IP₃ moves on to mediate Ca²⁺ release from intracellular stores which we proved to be significant in VE-cadherin disassembly; DAG activates PKC which was not a statistically significant regulator of gap formation. Therefore, it is possible that when the signal reaches the PIP₂:PLCβ:Gα-P complex, it moves downstream through IP₃ rather than DAG, explaining why the role of PKC may not be as important in Ca²⁺-mediated signaling of VE-cadherin disassembly. MLCK inhibition on the other hand, resulted in a significant decrease in endothelial gap compared to the untreated case. Since we know that MLCK activation induces phosphorylation of MLC, we can now postulate that there is a direct connection between MLCP and VE-cadherin. According to literature, ROCK plays the same role as MLCK, phosphorylating MLC, and inhibition of ROCK has been found to stabilize VE-cadherin in endothelial cells (Shen 2010). This work supports our conclusion that VE-cadherin is a downstream target of MLCK and MLCP in the Ca²⁺ signaling pathway.

After investigation of VE-cadherin disassembly due to signaling from the p38 MAPK and Ca²⁺ pathways, the goal of the next chapter is to elucidate how these pathways interact to induce VE-cadherin disassembly whether it be via crosstalk or activation in a parallel manner. We will examine the Ca²⁺ pathway individually to form a comparison to the combined pathway. Both signaling maps will be translated into kinetic models via ordinary differential equation (ODE) formation in order to perform global sensitivity analysis (GSA). GSA will drive further laboratory experimentation via predicting the most influential parameters involved in VE-cadherin disassembly from β-catenin.
Chapter 4

INTRACELLULAR MAP CREATION AND MATHEMATICAL ANALYSIS

4.1 Introduction

In the previous two chapters we have examined the extracellular triggers and intracellular p38 MAPK and Ca\(^{2+}\) pathways. In this chapter we will model our findings using an ordinary differential equation (ODE) mathematical model. As previously stated, IL-1\(\beta\) has been found to initiate the p38 MAPK pathway and IL-8 has been found to activate the Ca\(^{2+}\) pathway (Sims 2010, Waugh 2008). The final step is to establish a connection between the pathways at the intracellular level. In literature, it has been found that there is crosstalk between the p38 MAPK and Ca\(^{2+}\) pathways through PKC and Ca\(^{2+}\) mediation of the Rac-RhoGDI complex (Montcouquiol 2001, Vouret-Craviari 1998). A graphical representation of this combined signaling map will be displayed in Section 4.2.1 below.

4.2 Methods

4.2.1 Development of signaling map

Data regarding extracellular stimuli, Ca\(^{2+}\) pathway, p38 MAPK pathway, crosstalk between pathways, (including Rac and \(\beta\)-catenin signaling), and finally VE-cadherin was collected from laboratory experimentation, literature, and databases. A graphical map of signal transduction pathways was created starting from membrane receptor stimulation and ending with
VE-cadherin disassembly. The final signaling cascade is displayed in Figure 4.1, with (1) in being the Ca$^{2+}$ pathway, (3) in being the p38 MAPK pathway, and (2) in and (4) in representing newly created pathways Rac and β-catenin, respectively.

Of the new additions to the overall pathway, let’s first examine the Rac pathway, which is initiated by stimulation of VCAM-1 at the cell membrane and denoted as (2) in Figure 4.1. VCAM-1 is an integrin receptor located on the surface of endothelial cells that can initiate a signaling cascade when activated by its respective integrin, VLA-4. VLA-4 can be found on leukocytes and certain types of melanoma cells during activated states. This interaction leads to tumor adhesion and migration through the endothelium; the signaling for this mechanism is largely due to VCAM-1 activation of Rac in endothelial cells (Barreiro 2002, Van Wetering 2003). Rac is normally found as an inactive form in a complex with RhoGDI. RhoGDI is responsible for holding Rac in the cytosol so it is upon dissociation from RhoGDI that Rac translocates to the membrane where it performs its function of mediating cytoskeletal rearrangement and consequently cell migration (Price 2003). The family of p21-activated kinases (PAKs) are activated by binding with active Rac and Cdc42 GTPases. PAKs function to regulate cytoskeletal dynamics and cell migration as well as to mediate other intracellular signaling processes (Bokoch 2003). Next, we will examine the addition of the β-catenin pathway, (4) in Figure 4.1. VE-cadherin is an adherens junction specific to the endothelium that regulates vascular permeability and cell migration (Vestweber 2008). The VE-cadherin: β-catenin complex is anchored to the actin cytoskeleton via α-catenin; this allows for its control over cell-cell adhesion and maintaining the endothelial barrier (Guo 2008). The four signaling pathways are shown with appropriate kinetic constants (per description in Section 4.2.2) in Figure 4.1.
Figure 4.1 Signaling cascades that lead to VE-cadherin breakdown in EC. The portion on the left (1) represents the Ca^{2+} pathway as developed in (Peng 2009); the part on the right (3) is based on the p38 MAPK model developed in (Hendriks 2008); through experimentation and literature review, the section in the center (2) was our addition of the Rac pathway, and the section in the center (4) was our addition of the β-catenin pathway (Montcouquiol 2001, Van Wetering 2003, Vestweber 2008, Vouret-Craviari 1998). Payal Khanna (Ph.D. Candidate of Bioengineering, The Pennsylvania State University) and Francisco Vital-Lopez (Ph.D. Candidate of Chemical Engineering, The Pennsylvania State University) aided in map creation and kinetic constant formation.
4.2.2 Translation into kinetic model

Conversion from a graphical representation into a mathematical model is essential for studying the dynamics of the pathway. To do so, a system of ordinary differential equations (ODEs) was developed using Michaelis-Menten kinetics. Each interaction between components was represented by a kinetic expression to describe its respective rate. Kinetic constants denoted by $k$ are shown on the map in Figure 4.1.

As an example of ODE formation, let's focus on one particular step in the Ca$^{2+}$ pathway, the cleavage of PIP$_2$ by the PLC-G complex. The association rate of PLC-G-PIP$_2$ (1), dissociation rate of PLC-G-PIP$_2$ (2), and PIP$_2$ cleavage rate (3) are shown in Table 4.1, where $r_i$ indicates the rate of reaction $i$, $k_i$ is the corresponding rate constant, and $C_i$ represents the concentration of species $i$. The conservation equation for the PLC-G-PIP$_2$ complex, or the rate at which its concentration changes over time, is a combination of the three aforementioned reaction rates and is displayed as equation (4). After all reactions in the pathway have been translated into this form (concentration rate), the final model equations can be written as equation (5), where $C$ is the vector of concentrations of all of the factors (over time), $S$ is a matrix defining the reaction stoichiometry, $r$ is the vector of reaction rates, and $k$ is the vector of kinetic constants. As in equation (4), the final equations were written as rate of change of concentration per time, which required initial conditions to be solved. These initial concentrations and kinetic constant ranges were determined based on experimental results, literature, and databases/model repositories including BioModels Database, DOQCS database, and CellML Model Repository (Le Novere 2006, Lloyd 2008, Sivakumaran 2003).
Table 4.1 List of equations for translation into Michaelis-Menten kinetic model.

<table>
<thead>
<tr>
<th>Equation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( r_1 = k_1 C_{PLC-G} C_{PIP_2} )</td>
<td>PLC-G-PIP(_2) association rate</td>
</tr>
<tr>
<td>( r_{-1} = k_{-1} C_{PLC-G-PIP_2} )</td>
<td>PLC-G-PIP(_2) dissociation rate</td>
</tr>
<tr>
<td>( r_2 = k_2 C_{PLC-G-PIP_2} )</td>
<td>PIP(_2) cleavage rate</td>
</tr>
<tr>
<td>( \frac{dC_{PLC-G-PIP_2}}{dt} = r_1 - r_{-1} - r_2 )</td>
<td>PLC-G-PIP(_2) conservation</td>
</tr>
<tr>
<td>( \frac{dC}{dt} = S \cdot r(C,k) )</td>
<td>Mathematical model</td>
</tr>
</tbody>
</table>

4.2.3 Global sensitivity analysis (GSA)

Global sensitivity analysis was used to predict the most influential components and reactions in the mathematical model. Multi-parametric sensitivity analysis (MPSA) examines relative importance of each parameter in the model to the final output of the model (Cho 2003). Briefly, the method consists of simulating the model for an ensemble of different parameter sets and computing a metric of the model output to the parameters based on the deviation of the predicted output from a reference value. The parameters are generated randomly (within a predefined range) via a method called Latin hypercube sampling. Then, a scoring function (6) is created which calculates the difference between the predicted model output and a reference value.

In equation 6, \( f(k_p) = \sum_{i=1}^{n} (C(i,k_p) - C(i,k_0))^2 \) created which calculates the difference between the predicted model output and a reference value.

In equation 6, \( f \) represents the scoring function, \( C(i) \) is the concentration at time \( i \), \( k_0 \) is the vector of nominal parameter values, \( k_p \) is the vector of parameter values sampled from the parameter space, and \( n \) is the number of time points. The scoring function is examined over a collection of different parameter sets \( k_p \). The average value over the collection is then compared to each individual parameter set; if the individual parameter set has a scoring function value less than the
collection’s value, the set is considered acceptable, or unacceptable otherwise. As each parameter is examined over increasing values, the cumulative frequency distributions of acceptable and unacceptable cases are calculated and compared with the other parameters using Kolmogorov-Smirnov (K-S) statistics. K-S statistics is defined as the maximum distance between the cumulative frequency distribution of the acceptable and unacceptable cases. This method calculates a statistical value that denotes relative sensitivity of the output of the model to each model parameter; parameters with higher values have more influence on the model output (Zi 2008).

4.3 Results

The model of the Ca\textsuperscript{2+} pathway was composed of 45 total parameters: 33 kinetic constants (representing reaction rates) and 12 initial conditions (non-zero concentrations at time equal to zero for each component). All GSA simulations were performed using IL-8 as the extracellular stimulus. The model output predicted which parameters in the Ca\textsuperscript{2+} pathway were most influential on VE-cadherin: β-catenin disassembly.

4.3.1 Ca\textsuperscript{2+} pathway simulation: IL-8 stimulus

Figure 3.3 above shows all of the components included in our Ca\textsuperscript{2+} model, which includes connections to members of the Rac and β-catenin pathways. IL-8 was used as the extracellular stimulus for activation of the pathway. The simulations displayed in Figure 4.2 represent three independent trials with varying parameter bounds, extending from magnitudes of 10\textsuperscript{6} to 10\textsuperscript{2}.
Figure 4.2 Kolmogorov-Smirnov statistics of IL-8 stimulation of Ca$^{2+}$ pathway. Moving from panel A to C, the parameter bounds shift from the most conservative to least conservative. The bounds in A span a range of 10$^6$, in B span a range of 10$^4$, and in C span a range of 10$^2$. 

54
In all three cases, kinetic constants $29 (k_{29})$, 30 ($k_{30}$), and 32 ($k_{32}$) and initial concentrations 39 (CaM) and 42 ($Ca^{2+}_{\text{store}}$) were found to be the most influential (tallest bars in histograms). Analysis of these parameters will be examined in Section 4.4.1 below. First, we’ll examine the simulation results of the combined pathway with stimuli IL-8, VCAM-1, and IL-1β.

4.3.2 Combined pathway simulation

The final mathematical model included 128 total parameters: 101 kinetic constants (reaction rates) and 27 initial conditions (non-zero concentrations at time equal to zero for each component). See Table 4.2 for specific parameters in each of the four pathways. GSA was performed for four different cases: IL-8 stimulation, VCAM-1 stimulation, IL-1β stimulation, and simultaneous IL-8/VCAM-1/IL-1β stimulation. The model output predicted the influential parameters on VE-cadherin: β-catenin disassembly. All results were gathered in collaboration with Francisco-Vital Lopez (Ph.D. Candidate of Chemical Engineering, The Pennsylvania State University).

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reaction rates</td>
</tr>
<tr>
<td></td>
<td>(kinetic constants)</td>
</tr>
<tr>
<td>p38</td>
<td>1-54</td>
</tr>
<tr>
<td>Rac</td>
<td>55-66</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>67-98</td>
</tr>
<tr>
<td>β-catenin</td>
<td>99-101</td>
</tr>
<tr>
<td></td>
<td>Components</td>
</tr>
<tr>
<td></td>
<td>(initial concentrations)</td>
</tr>
<tr>
<td>p38</td>
<td>102-113</td>
</tr>
<tr>
<td>Rac</td>
<td>114-115</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>116-127</td>
</tr>
<tr>
<td>β-catenin</td>
<td>128</td>
</tr>
</tbody>
</table>
4.3.2.1 IL-8 stimulus

This first case examined stimulation from extracellular IL-8 alone; the initial concentration values of both IL-1β and VCAM-1 were set to zero in the model. Physiologically, the model is predicting which intracellular components are the most important in mediating VE-cadherin: β-catenin disassembly due to activation by IL-8. The simulation results are shown in Figure 4.3.

**Figure 4.3** Kolmogorov-Smirnov statistics of IL-8 stimulation of combined pathway. The histogram is coded to show which pathway each parameter belongs to: p38, Rac, Ca^{2+}, or β-catenin. Parameters 1-101 represent the kinetic rates and 102-128 characterize the physiological components. Larger K-S values represent the more influential parameters in the model. Simulation kindly provided by Francisco Vital-Lopez (Ph.D. Candidate of Chemical Engineering, The Pennsylvania State University).

Throughout the course of this study, it was believed that IL-8 acted through the Ca^{2+} pathway to mediate VE-cadherin disassembly. Figure 4.3 however, shows that the most influential targets (tallest bars in histogram) are from the β-catenin and p38 MAPK pathway. Parameters 1-101 are the kinetic rates for each pathway; specifically parameters 99-101 represent
the reactions of the β-catenin pathway. The direct interaction between β-catenin and VE-cadherin explains why the simulation predicted the reactions in the β-catenin pathway to be so influential. Looking beyond these obvious predictions, the four tallest bars all fall between parameters 1-54, which represent reactions in the p38 pathway. As for the actual components in the map, the three tallest bars again fall into the p38 range, between parameters 102-113. These influential parameters will be analyzed and discussed in Section 4.4.2. Now rather than examining only the result of IL-8 activation, we will view the predicted parameters from additional of solely VCAM-1.

4.3.2.2 VCAM-1 stimulus

In the previous case, we found that the predicted influential parameters due to IL-8 stimulation were most significantly found to be in the p38 MAPK pathway. Next, we examined the kinetic model results after stimulation by VCAM-1 only; in this case, initial concentrations for IL-8 and IL-1β were set to zero. This simulation predicted the most significant parameters in VE-cadherin: β-catenin disassembly due to VCAM-1 activation of the Rac signaling pathway. The model predictions are displayed in Figure 4.4.
Figure 4.4 Kolmogorov-Smirnov statistics of VCAM-1 stimulation of combined pathway. The histogram is coded to show which pathway each parameter belongs to: p38, Rac, Ca$^{2+}$, or β-catenin. Parameters 1-101 represent the kinetic rates and 102-128 characterize the physiological components. Larger K-S values represent the more influential parameters in the model. Simulation kindly provided by Francisco Vital-Lopez (Ph.D. Candidate of Chemical Engineering, The Pennsylvania State University).

Referring back to Figure 4.1, it can be seen that the VCAM-1 → Rac interaction is connected to both the Ca$^{2+}$ or p38 MAPK signaling pathways, but may only move downstream in the p38 MAPK cascade. Rac is connected to the Ca$^{2+}$ pathway through signals from PKC or Ca$^{2+}$ (via Rac-RhoGDI) and to the p38 pathway through downstream signaling through PAK to MKK3 or MKK6. The results shown in Figure 4.4 indicate that Rac initiates signaling for VE-cadherin disassembly through the p38 MAPK pathway; like the simulation for IL-8 activation, the four most influential reaction rates and three most significant components are p38 MAPK parameters. For a second time, β-catenin reactions were predicted to be influential parameters, again agreeing with the evident physiological interaction between β-catenin and VE-cadherin. The last case examined individual stimulation of IL-1β and its influence on VE-cadherin: β-catenin disassembly.
4.3.2.3 IL-1β stimulus

IL-1β stimulation is the final extracellular stimulus to investigate via our kinetic model simulations. Following the same methodology of the prior two cases, IL-8 and VCAM-1 were now set to have initial concentrations of zero. Although IL-1β is the extracellular stimulus largely responsible for activation of the p38 MAPK pathway, it is intriguing to examine if IL-1β uses this pathway for the purpose of mediating VE-cadherin: β-catenin disassembly. The outcome of the model prediction is shown in Figure 4.5.

![Kolmogorov-Smirnov statistics of IL-1β stimulation of combined pathway](image)

**Figure 4.5** Kolmogorov-Smirnov statistics of IL-1β stimulation of combined pathway. The histogram is coded to show which pathway each parameter belongs to: p38, Rac, Ca^{2+}, or β-catenin. Parameters 1-101 represent the kinetic rates and 102-128 characterize the physiological components. Larger K-S values represent the more influential parameters in the model. Simulation kindly provided by Francisco Vital-Lopez (Ph.D. Candidate of Chemical Engineering, The Pennsylvania State University).

It is now apparent that the same results hold true for each of the three extracellular stimuli: IL-8, VCAM-1 and IL-1β. Firstly, β-catenin is very significant in regulating VE-cadherin due to its direct physiological association at the cell membrane. Additionally, the
predicted conclusion of the past three sections is that when only one of the extracellular stimuli is present, it uses the p38 MAPK pathway as a means for regulating VE-cadherin: β-catenin disassembly.

4.3.2.4 IL-8/ IL-1β/ VCAM-1 stimuli

Now the focus turns to testing the pathway due to simultaneous IL-8/ IL-1β/ VCAM-1 stimulation where all three stimuli have a nonzero initial concentration value. The model is examining the relative significance of the intracellular components on VE-cadherin: β-catenin disassembly due to activation by three extracellular factors; this is a more accurate depiction since physiologically there are a large number of stimuli that can initiate signaling cascades. The simulation results are shown in Figure 4.6.

![Figure 4.6](image)

**Figure 4.6** Kolmogorov-Smirnov statistics of IL-8/ IL-1β/ VCAM-1 stimulation of combined pathway. The histogram is coded to show which pathway each parameter belongs to: p38, Rac, Ca²⁺, or β-catenin. Parameters 1-101 represent the kinetic rates and 102-128 characterize the physiological components. Larger K-S values represent the more influential parameters in the model. Simulation kindly provided by Francisco Vital-Lopez (Ph.D. Candidate of Chemical Engineering, The Pennsylvania State University).
Even now that intracellular signaling is being initiated from three external sources, we see that again the same results hold true. Other than the reactions of β-catenin, the four most influential kinetic parameters and three most significant components are in the p38 MAPK pathway. This provides interesting insight into the relationship between the Ca$^{2+}$, Rac, and p38 MAPK pathways. This indicates that the crosstalk is apparently a crucial part of the functioning of the network as a whole. Although IL-8 is known to regulate Ca$^{2+}$ and VCAM-1 to activate Rac, it seems that in order to specifically mediate VE-cadherin: β-catenin disassembly, both extracellular stimuli send their signal through the p38 MAPK pathway. Possible mechanisms for this signaling can be viewed in previous Figure 4.1 and are listed in Table 4.3.

### Table 4.3 Possible crosstalk between pathways: simplified form.

<table>
<thead>
<tr>
<th>Pathway 1</th>
<th>Pathway 2</th>
<th>Pathway 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8 → PLC → PIP$_2$</td>
<td>IP$_3$ → Ca$^{2+}$ → Rac-RhoGDI → Rac → PAK → MAPK</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>DAG → PKC → Rac → PAK → MAPK</td>
<td>(2)</td>
</tr>
<tr>
<td>VCAM-1 → Rac → PAK → MAPK</td>
<td></td>
<td>(3)</td>
</tr>
</tbody>
</table>

#### 4.4 Discussion

The next two sections will focus on the identification and characterization of the specific components and reaction rates found to be most sensitive in the Ca$^{2+}$ and combined pathway models.
4.4.1 Analysis of Ca\textsuperscript{2+} pathway model

In section 4.3.1, we briefly covered the results of the Ca\textsuperscript{2+} pathway simulations. Kinetic constants 29 (k\textsubscript{29}), 30 (k\textsubscript{30}), and 32 (k\textsubscript{32}) and initial concentrations 39 (CaM) and 42 (Ca\textsuperscript{2+} store) were found to be the most sensitive targets in the model. The results are displayed in a qualitative manner with influential kinetic parameters shown in bold in Figure 4.7.

**Figure 4.7** Graphical result of GSA for Ca\textsuperscript{2+} pathway. The two components shown in bold print, Ca\textsuperscript{2+} store and CaM, and three kinetic rates displayed by bold arrows k\textsubscript{29}, k\textsubscript{30}, and k\textsubscript{32}, were found to be the most influential in the Ca\textsuperscript{2+} pathway model. The three reactions are described below in Table 4.4.
Given that Ca\(^{2+}\) store and calmodulin (CaM) were predicted to be the most influential components, let’s examine their roles in the Ca\(^{2+}\) pathway. Ca\(^{2+}\) store controls the release of Ca\(^{2+}\) into the cytosol in response to signaling from upstream target IP\(_3\). This intracellular Ca\(^{2+}\) is ultimately returned to the Ca\(^{2+}\) store through reuptake by SERCA. When Ca\(^{2+}\) is released however, it is responsible for initiating a number of intracellular processes, one of which results from binding to CaM. CaM is considered to be an effector protein for Ca\(^{2+}\), due to its high affinity, specificity, and ability to simultaneously bind four calcium ions. Upon binding with Ca\(^{2+}\), it can then form a complex with MLCK to initiate cytoskeletal rearrangement (Peng 2007). This effect on contractility may be linked to VE-cadherin disassembly and gap formation. The next Ca\(^{2+}\) model predictions to examine are the reactions described in Table 4.4.

**Table 4.4 Predicted influential reactions in Ca\(^{2+}\) pathway.**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{29}) (i) (\text{Ca}^{2+} \rightarrow \text{Ca}^{2+}\text{ext})</td>
<td>Transport of Ca(^{2+})</td>
</tr>
<tr>
<td>(k_{30}) (ii) (\text{Rac-RhoGDI}^* \rightarrow \text{Rac-RhoGDI})</td>
<td>Deactivation of Rac-RhoGDI</td>
</tr>
<tr>
<td>(k_{32}) (iii) (\text{MLCP} \rightarrow \text{MLC})</td>
<td>Phosphatase(_{\text{MLC}}) catalytic rate</td>
</tr>
</tbody>
</table>

Reaction (i) \(\text{Ca}^{2+} \rightarrow \text{Ca}^{2+}\text{ext}\) represents the transport of calcium ions from the cytosol to the extracellular space. In order for this transport to occur, Ca\(^{2+}\) store (which was already discussed as an important parameter) must first release calcium ions into the cytosol. This transport reaction may then take place (Peng 2007). This is a new and interesting concept that we have not yet reviewed; examination of the role of extracellular Ca\(^{2+}\) would be an interesting approach to address in our studies. Reaction (ii) \(\text{Rac-RhoGDI}^* \rightarrow \text{Rac-RhoGDI}\) corresponds to the deactivation of the Rac-RhoGDI complex. The activation/deactivation reactions are
responsible for Rac translocation to the cell membrane. Deactivation of this complex induces Rac and RhoGDI to remain in a complex as to disallow Rac to translocate to the cell membrane (George 2010) while activation of Rac-RhoGDI induces protein-activated Rac mediation of cytoskeletal rearrangement and cell migration at the membrane (Price 2003). It will be a goal of our future work to understand the role of this activation/deactivation in VE-cadherin disassembly. Lastly, reaction (iii) $\text{MLCP} \rightarrow \text{MLC}$ signifies the catalytic rate of phosphatase$_{\text{MLC}}$. This reaction removes a phosphate group from MLC which is one half of a two-part cyclic reaction that is involved in VE-cadherin disassociation from $\beta$-catenin. This process of phosphorylation/dephosphorylation may be the controlling mechanism for VE-cadherin disassembly in the $\text{Ca}^{2+}$ pathway. Next, we will investigate the predicted results of the GSA simulation of the combined pathway.

4.4.2 Analysis of combined pathway model

Although the histograms in Figures 4.3, 4.4, 4.5, and 4.6 have provided a quantitative prediction, we must now return to the signaling map to identify the physiologically significant parts. The predicted influential components and reactions are highlighted in a bold font in Figure 4.8.
Figure 4.8 Graphical result of GSA for combined pathway. The three components shown in bold print were found to be the most sensitive: MKK6, p38, and Hsp27. The four reactions displayed by bold arrows are described in Table 4.5: $k_{p25}$, $k_{p29}$, $k_{p33}$, and $k_{p34}$.

Let’s first discuss the three most influential components predicted by the kinetic model: MKK6, p38, and Hsp27. Of these three, MKK6 is the most upstream target and it has been found that activation of MKK6 as well as MKK3 is necessary for phosphorylation of p38. It is interesting to consider why the model predicted only one form of MKK, MKK6, to be influential over the other. Although the model does not take into consideration the various isoforms of p38, it is interesting to see that MKK3 and MKK6 have been known to vary in the specific isoforms of p38 that they activate. MKK6 activates p38α, p38β, and p38γ while MKK3 only activates p38α and p38γ (Li 2005). Therefore, if the model’s prediction is accurate, it could be explained physiologically by the possibility that the p38β isoform is largely responsible for p38 function, which would make MKK6 selectively more influential than MKK3. Additionally, since MKK6 shares only 82% of its identity with MKK3, there is a possibility that structural differences play a role in their differing functions (Stein 1996). The next influential parameter predicted by the kinetic model is p38, which has proved to be closely associated with previously discussed MKK6. The family of p38 MAPKs, which includes isoforms α, β, γ, and δ, can be activated by both inflammatory cytokines and environmental stresses. They function to regulate a large variety of cellular processes including cell proliferation, differentiation, survival, and migration (Wagner 2009). Numerous studies have indicated that p38 MAPK mediates phosphorylation of Hsp27 and that phospho-Hsp27 is linked to regulation of the actin cytoskeleton (Okamoto 1999). Phospho-Hsp27 has been found to bind to actin, which promotes actin-myosin interaction (Antonov 2008). This association with the cytoskeleton supports the prediction of Hsp27 as the third and final significant component regulating VE-cadherin disassembly from β-catenin. Next, we will investigate the model’s predicted significant reactions, shown previously in Figure 4.8 as bold arrows and described below in Table 4.5.
Table 4.5 Predicted influential kinetic rates in combined pathway. The four reaction rates listed below correspond to the bold arrows in Figure 4.8.

<table>
<thead>
<tr>
<th>Kinetic rate</th>
<th>Description</th>
<th>Overall reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{c_MK2}$ (i)</td>
<td>$k_{c_MK2}$ irreversible</td>
<td>phospho-MK2 catalytic rate</td>
</tr>
<tr>
<td>$k_{i_p38P}$ (ii)</td>
<td>$k_{i_p38P}$ reversible</td>
<td>phospho-p38 nuclear import rate</td>
</tr>
<tr>
<td>$k_{o_p38P_MK2P}$ (iii)</td>
<td>$k_{o_p38P_MK2P}$ reversible</td>
<td>phospho-MK2 nuclear co-export rate</td>
</tr>
<tr>
<td>$k_{r_p38P_MK2P}$ (iv)</td>
<td>$k_{r_p38P_MK2P}$ irreversible</td>
<td>phospho-p38: phospho-MK2 dissociation rate</td>
</tr>
</tbody>
</table>

Rate (i) $k_{c\_MK2}$ represents the irreversible catalytic reaction of inactive kinase, MAPK-activated protein kinase-2 (MK2), in the process of Hsp27 phosphorylation/ dephosphorylation. This again confirms the importance of Hsp27 in regulation of cytoskeletal dynamics. Next, rate (ii) $k_{i\_p38P}$ is the rate that defines the reversible transport of activated cytosolic kinase, phospho-p38, into and out of the nucleus. The significance of this reaction may be due to location-dependent function of phospho-p38 both in the cytosol and inside the nucleus; further study will need to be performed to validate this predicted reaction. Lastly, rates (iii) $k_{o\_p38P\_MK2P}$ and (iv) $k_{r\_p38P\_MK2P}$ regulate interactions of the phospho-p38: phospho-MK2 complex, reversible transport in and out of the nucleus and irreversible dissociation, respectively. Rate (iv) is involved in the reaction of MK2 activation/ deactivation. MK2 is a protein located directly downstream of a MAPK, in our case p38 MAPK. Results in literature have shown that the p38→MK2→Hsp27 signaling cascade leads to rearrangement of the actin cytoskeleton, clearly demonstrating the importance of MK2-mediated reactions (Kayyali 2002). Although some of
the predicted influential reactions reaffirmed the importance of p38 and Hsp27, new implications that resulted were the significance of MK2 and phospho-p38 translocation.

Although we came to some interesting conclusions regarding the Ca\textsuperscript{2+} pathway in Section 4.4.1, it seems that our latest GSA results for the combined pathway indicate that the role of the p38 MAPK pathway may be more influential overall. Specifically, when we simulate the Ca\textsuperscript{2+} pathway individually, components CaM and Ca\textsuperscript{2+}store and reactions involving Ca\textsuperscript{2+}ext, Rac-RhoGDI and MLC are the most influential. Conversely, when the combined pathway is simulated the most sensitive targets are components MKK6, p38, and Hsp27 and reactions including MK2 and p38P, all of which fall into the p38 MAPK pathway. These results indicate that when examining the signaling cascade in its entirety, the signal for VE-cadherin: β-catenin disassembly most likely travels downstream in the p38 MAPK pathway, rather than the Ca\textsuperscript{2+} pathway. Overall, the GSA was helpful in pinpointing components and reactions in both pathway simulations that may play an important role in VE-cadherin disassembly from β-catenin. We have to keep in mind that this is only the initial simulation of our combined kinetic model and validation is a necessary next step. Experiments proving the significance of the predicted seven influential targets would demonstrate that the model simulations were valid and accurate.
Chapter 5

CONCLUSIONS

5.1 Summary conclusions

Throughout the last three chapters, we have addressed both experimental and computational means of examining signaling pathways leading to VE-cadherin disassembly in endothelial cells. Chapters 2 and 3 focused on the laboratory experimentation used to identify and characterize the extracellular stimuli and intracellular targets of the signaling pathways. It was concluded in Chapter 2 that medium to high metastatic lines induce a significant amount of endothelial gap through both TC and TCM addition. Next, we found that TC, TCM, and anti-VCAM-1 cause a linear increase in endothelial gap formation over a time course of 90 minutes. Cytokines IL-6, IL-1β, IL-8, and GRO-α caused gap formation individually and in the pairs IL-1β/IL-6 and IL-1β/IL-8. Neutralization of the cytokines in the endothelial cells confirmed our results. Next in Chapter 3, p38 inhibition proved p38 MAPK to be an intermediate between TC stimulation (mediated by IL-1β) and VE-cadherin disassembly. Similarly, Ca$^{2+}$ induction via TCs, TCM, and IL-8 and Ca$^{2+}$ chelation/inhibition of IP$_3$-mediated Ca$^{2+}$ established the Ca$^{2+}$ pathway as a means for tumor cells to break down VE-cadherin. Lastly, PKC was found not to be a means for signaling leading to endothelial gap formation while MLCK played an important role in VE-cadherin disassembly. Figures 5.1 and 5.2 are graphical representations of these experimental results.
Figure 5.1 Summary of experimental work: extracellular stimuli.

Figure 5.2 Summary of experimental work: intracellular targets.
After performing laboratory investigations of various targets, we chose to model both the Ca$^{2+}$ pathway individually and combined via crosstalk to the p38 MAPK pathway. From experimental data and literature confirmation of the IL-8 $\rightarrow$ Ca$^{2+}$ $\rightarrow$ VE-cadherin pathway, we developed the final Ca$^{2+}$ signaling map. Adding the IL-1β $\rightarrow$ p38 MAPK $\rightarrow$ VE-cadherin pathway via crosstalk linkages was achieved through a thorough literature review. Both pathways were then translated into a mathematical model by writing ordinary differential equations based on Michaelis-Menten kinetics. Global sensitivity analysis using Latin Hypercube sampling was then performed and Kolmogorov-Smirnov statistics were used to plot the simulations as histograms showing the most influential parameters. This is summarized graphically in Figure 5.3.

Figure 5.3 Summary of computational work.
5.2 Future directions

As seen from the diagram shown in Figure 4.1, there are an immense number of targets in our combined signaling pathway. It would be overwhelmingly time consuming and impractical for a laboratory to test each one experimentally. This was the backbone of our thinking when we decided to create a combined kinetic model to analyze the pathway. The model’s simulations were able to direct us to influential targets which can then be validated experimentally. The three predicted components MKK6, p38, and Hsp27 can be studied and their significance verified. We have already completed in depth studies of p38 to confirm its importance in VE-cadherin disassembly so the next step will be to examine MKK6 and Hsp27. Additionally, investigation into altering reaction rates would be an interesting approach in validating the predicted kinetic parameters. Likewise, any map connection assumptions that were made will need to be tested experimentally to assure that the signaling map is accurate. The results from all of these experiments can then be added back into the model to refine it. Being that this was the first trial of our model, the set parameter bounds were conservative, or spanned a large value range. After validation via laboratory experimentation, adjustment of these parameter bounds will be essential to improve the accuracy of the model. Future simulations will become increasingly more accurate and insightful regarding the dynamics of intracellular signaling for VE-cadherin disassembly in endothelial cells.
BIBLIOGRAPHY


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