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AUTOCRINE STIMULATION OF MELANOMA CAUSES MMP-2 UPREGULATION AND SUBSEQUENT MIGRATION

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ABSTRACT

The goal of this work is to investigate the pathway behind the up-regulation and expression of Matrix Metalloproteinases (MMPs) as a result of autocrine melanoma stimulation. Certain chemokines, such as Monocyte Chemotactic Protein-1 (MCP-1) and Interleukin-8 (IL-8) are characteristic of metastatic melanomas and have previously been implicated to be involved in MMP-2 up-regulation and activation. It is the pathways involving these proteins that are the focus of our research.

In order to study the effect of MMPs on melanoma extravasation through the extracellular matrix, an assay was developed, which involved the extract from murine Engelbreth-Holm-Swarm sarcoma (Matrigel), to simulate the extracellular matrix. An optimal Matrigel volume was determined to allow monitoring the changes in melanoma migration as a result of inhibition of certain steps in the MMP activation pathway. The highly metastatic melanoma cell line (Lu1205) was used in the experiments.

The study focused on the synergistic effect of MCP-1 and IL-8 on melanoma migration through the extracellular matrix. It was shown that blocking MCP-1 and IL-8 reduced the migration and invasion rate of Lu1205 cells. Additionally, it was shown that MMP-2 plays a crucial role in melanoma extracellular matrix digestion. In order to account for incomplete migration of Lu1205 through the Matrigel, a modified flow chamber was designed.

TABLE OF CONTENTS

LIST OF FIGURES……iii

LIST OF TABLES……..iv

ABBREVIATIONS……v

AKNOWLEDGEMENTS……..……vi

Chapter 1. Introduction…….……1
1.1 Cancer and Melanoma……1
1.2 Matrix Metalloproteinases and Melanoma Extravasation……...4

Chapter 2. Experimental Methods……..……...……8
2.1 Cell Culture……..8
2.2 Co-Culture Methods……9
2.3 Enzyme-Linked Immunosorbent Assay……...9
2.4 Matrigel Thickness Assay…….....10
2.5 Matrigel Migration Assay……..……...….....11
2.6 Statistical Analysis.....13

Chapter 3. Results and Discussion……14
3.1 Results ……..13
3.2 Discussion…….…...……..21

Chapter 4. Development of a 2D PDMS migration chamber……..…….24
4.1 Introduction……..……..22
4.2 Flow chamber manufacture.....27
4.3 Results and further development.....27

Chapter 5. Conclusions…….……30
5.1 Further Directions……..30
5.2 Conclusions……...…...……..30

Appendix A. PDMS Flow Chamber design and Implementation.....32

References.....34

LIST OF FIGURES

Figure 1.1 The process of tumor extravasation. Tumor cells travel through the vasculature, attach to the endothelium, and digest the extracellular matrix in order to migrate to the epidermis. ……..…….....3

Figure 1.2 Effect of MCAM and IL-8 on melanoma metasthasis.....5

Figure 1.3 The hypothesis of our paper: Lu1205 uses autocrine signaling to increase MMP-2 levels……..……6

Figure 3.1 IL-8 and MCP-1 concentrations in Lu1205 supernatant (Kwang, 2008). Supernatant from Lu1205 culture is collected and assayed via ELISA A) Concentrations of IL-8 upon co-culture, P=0.11% B) Concentrations of MCP-1 upon co-culture, P=0.03%.....…15

Figure 3.2: Co-culture with neutralizing IL-8 and MCP-1 in Lu1205 and HUVEC/Lu1205 co-culture. Zymography assay is shown with the relative intensity chart.....16

Figure 3.3 Effect of Matrigel volume on Lu1205 migration using 48-well Boyden chamber and 10um pore polycarbonate filters. Migration time is 4 hours……....18

Figure 3.4 Effect of MCP-1, IL-8, and MMP-2 on Lu1205 migration. Matrigel migration assay is shown above, human monoclonal antibodies used in concentrations of 1.5ng/mL for MCP-1, 2ng/mL for Il-8, and 20ng/mL for MMP-2……19

Figure 3.5 Comparison of field-of-view of Lu1205 Matrigel migration assay after cell attachment and staining……..20

Figure 3.6 Decrease in migration compared to control as a result of antibody neutralization…….....21

Figure 4.1 Schematic diagram of three-dimensional cell migration device (Chaw *et al, 2007)*……...……..…….....26

Figure 4.2 Flow chamber design, 3D view. More detailed description of device dimensions and implementation is presented in (Appendix A)…..…..…….....28

Figure 4.3 1/5th field-of-view under 10x magnification of Cell-tracker green-stained Lu1205 cells in PDMS migration chamber after 4 hours incubation…….....29

LIST OF TABLES

Table 3-1: Metastatic potentials of melanoma cell lines…….....14

ABBREVIATIONS

EC	Endothelial Cell
TC	Tumor Cell
HUVEC	Human Umbilical Vein Endothelial Cell
MCP-1	Monocyte Chemotactic Protein-1
IL-8	Interleukin-8
VCAM-1	Vascular Adhesion Molecule-1
VLA-4	Very Late Antigen-4
MMP-2	Matrix Metalloproteinase-2
CAM	Cellular Adhesion Molecule
ECM	Extracellular Matrix
PDMS	Polydimethylsiloxane
CIV	Collagen IV

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Chapter 1

INTRODUCTION

1.1 Cancer and Melanoma

Cancer is a disease associated with tissue renewal and uncontrolled growth of affected cells. In the world, about a third of the human population will develop cancer in their lifetime (Dittmar, 2008). The condition is the leading cause of death worldwide, with 7.9 million deaths are predicted in 2007 due to some form of cancer (WHO, 2010). Cancer is a disorder which occurs when the cells in the body become abnormal and start to grow and divide at uncontrollable rates and spread over other parts of the body (NCI, 2011). The cause for such behavior is DNA damage or mutation, which occurs partially as a result of exposure to extrinsic factors, such as tobacco use, unhealthy diet, physical inactivity, and harmful use of alcohol (WHO, 2010). When the cell accumulates enough mutations, it can overcome the body's natural checkpoints, gain an advantage over the neighboring cells, and grow independently of its originating tissue. While a single mutation would not be sufficient, a cell needs at least 3 mutations, accumulated over its lifetime to become cancerous (Alberts, 2004). Once the cell is able to proliferate independently of its host, it passes on its mutations to the daughter cells, which form a growth, known as a tumor. Some tumors are benign, and do not pose a direct threat to their host, while others can become malignant. A malignant tumor, also known as cancer, can quickly spread to other tissues and cause death of the organism (Fidler, 2002). Cancer cells are characterized with an ability to utilize the extracellular signaling mechanisms and the host immune system for their advantage, in order to supply the tumor with

sufficient conditions for growth (Alberts, 2008). These characteristics allowed some cells to detach from their primary location, travel through the vasculature, and extravasate into another location, forming secondary tumors (Liotta, 1991).

Melanoma is a type of skin cancer, characterized by aggressive local growth and metastasis; once metastasized, malignant melanomas are usually fatal (Alberts, 2006). Although not the predominant type of skin cancer, it is responsible for over 75% of all deaths associated with carcinoma (Jerant, 2000). According to World Health Organization, around 65,000 melanoma- related deaths occurred worldwide in the year 2000 (Lucas, 2006). The main cause of melanoma is the exposure to sunlight, specifically ultraviolet radiation, which can cause DNA mutations in the melanocytes, the types of cells responsible for producing skin pigment. Once the primary tumor mass develops, a cell or a group of tumor cells may leave the primary tumor, enter the circulation, attach themselves to a distant vascular bed, and extravasate to form a secondary colony, through a process known as metastasis (Lance, 1991). Figure 1.1 shows the process of cancer cell extravasation through the endothelial wall, basement membrane, the extracellular matrix, and its subsequent formation of the secondary tumor.

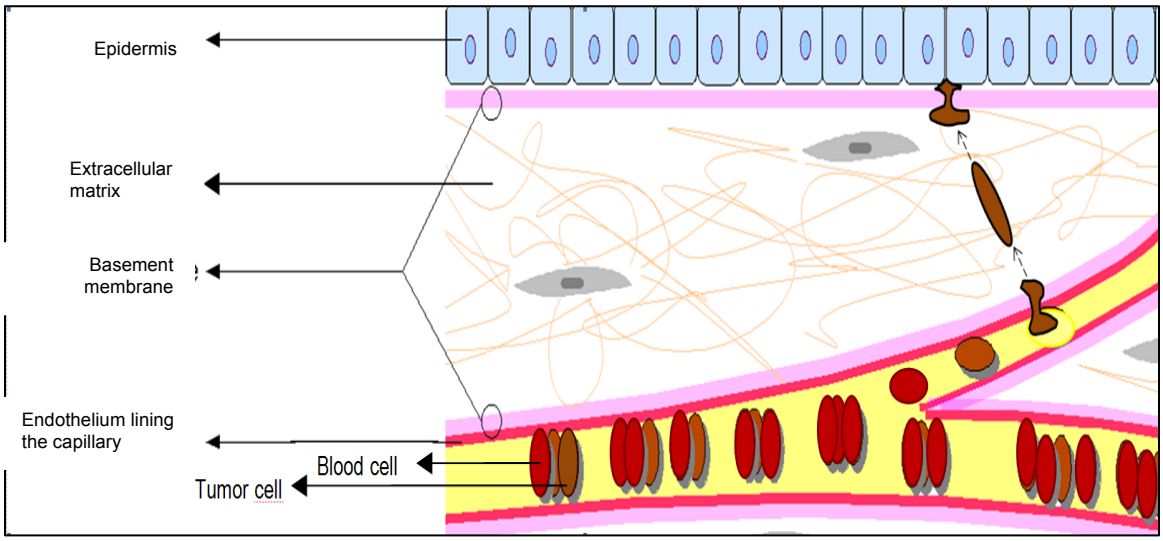

Figure 1.1 The process of tumor extravasation. Tumor cells travel through the vasculature, attach to the endothelium, and digest the extracellular matrix in order to migrate to the epidermis. (Edited from Wikimedia Commons)

Human malignant melanoma is a highly metastatic tumor, with poor prognosis and high resistance to treatment, which is why it is important to understand the mechanisms behind this process and treat it accordingly (Ria, 2010). The process of tumor metastasis consists of a series of interrelated steps. Once healthy cells have transformed into cancerous ones and have grown into a tumor, the select few enter the stage of vascularization by penetrating the lymphatic or vascular channels and being transported by other areas of the body. This process usually occurs when the tumor mass exceeds $1mm^3$ in diameter. Once in the circulation, tumor cells must survive the immune and non-immune defense mechanisms and attach to the capillary bed of distant organs (Fidler, 2002). When tumor cells enter metastatic stage, they express and secrete various cytokines, including VEGF-A, PGF-1, IL-8, and TGF-1, as well as multiple types of

integrins. Cellular adhesion molecules (CAMs) are also known to play an important role in the extravasation process (Hoskins and Dong, 2006). Tumor cells, with conjunction with endothelial cells will also release metalloproteinases to aid in extravasation and angiogenesis (Ria, 2010).

1.2 Matrix Metalloproteinases and Melanoma Extravasation

Melanoma and endothelial cells interact via two main mechanisms: receptor-ligand interactions via direct cell contact and exchange of soluble factors, known as cytokines, secreted by both types of cells (Hsu, 2002). Vascular adhesion molecule (VCAM-1) is expressed by cytokine-activated endothelium and is an endothelial ligand for very late antigen-4 (VLA-4). Their interaction has been implicated in melanoma binding to the endothelial wall and its subsequent extravasation.

Matrix components are degraded by extracellular proteolytic enzymes called proteases. A special class of proteases called matrix metalloproteinases rely on Ca^{2+} and Zn^{2+} metal ions and degrade matrix proteins such as laminin, fibronectin, and collagen (Alberts, 2008). Melanoma and tumor cells express several MMPs, as well as tissue inhibitors TIMPs. The most extensively studied MMPs are MMP-2 and MMP-9 (Ria, 2010). MMP-2 is a gelatinase and degrades fibronectin and is localized on the surface of the cells to allow increased invasive characteristics. MMP-2 expression specifically was highly correlated with metastatic spread and low survival rates (Hofmann, 2000). Thus, the focus of this research is to investigate different autocrine melanoma cell interactions through which MMP-2 is expressed and up-regulated in order to develop a method for its inhibition.

Prior to and during melanoma invasion, there is a wide variety of cytokine and direct contact interactions between the various host cells and melanoma. Overexpression of interleukin -1 and -8 (IL-1, IL-8) and monocyte chemoattractive protein protein-1 (MCP-1) were found to be characteristic of invasive melanoma and related to MMP-2 activation. (Hsu, 2002). The effects of IL-8 on melanoma extravasation are demonstrated in (Figure 1.2)

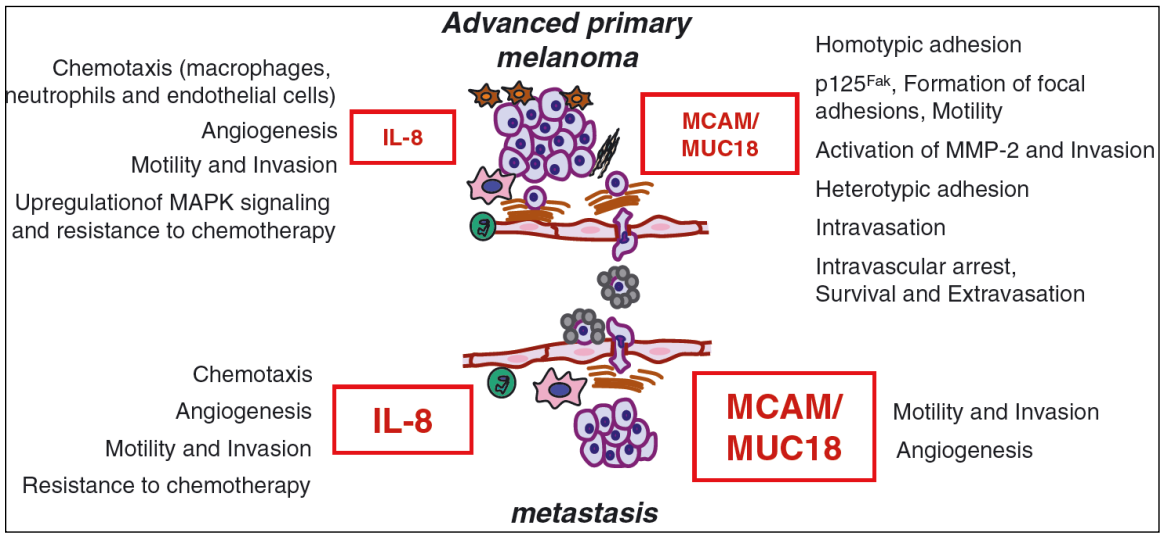

Figure 1.2 Effect of MCAM and IL-8 on melanoma metasthasis (Melnikova, 2006).

IL-8 (CXCL8) has been initially identified as a leukocyte chemoattractant. It serves many roles, most related to the body's immune response; it induces lysosomal enzyme release from neutrophils, and possesses a chemotactic activity for T lymphocytes and basophils (Mukaida et al., 1998). It has been shown to be involved in increasing melanoma metastatic potential, as well as to be involved in MMP-2 expression (Luca et al., 1997).

MCP-1 (CCL-2) is a monocyte chemotactic factor and triggers the release of lysosomal enzymes in monocytes. It acts as a chemoattractant for T lymphocytes and increases the multi-bond strength of VLA-4 on T-lymphocytes. MCP-1 is also implicated in vascular remodeling, which generally involves extracellular matrix degradation (Mukaida et al., 1998). It is the lysosomal enzyme release and vascular remodeling that made these two chemokines a target of this paper's investigation. The general hypothesis of this thesis is presented in (Figure 1.3)

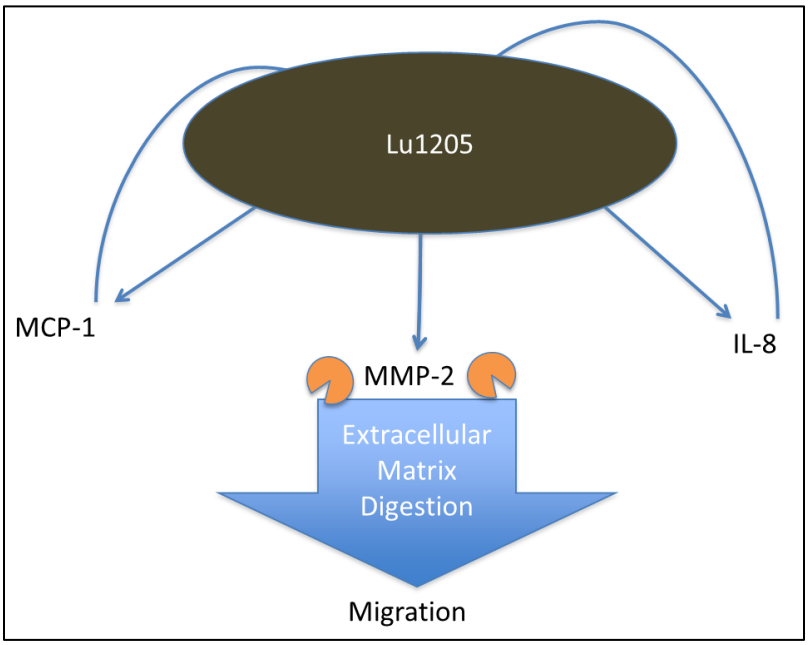

Figure 1.3 The hypothesis of this paper: Lu1205 uses autocrine signaling to increase MMP-2 levels

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The main objective of our research is to investigate the hypothesis that melanoma cells with metastatic potential use autocrine signaling to migrate through the extracellular matrix. The specific aims of our research include:

1. Developing an assay to assess the effect of chemokines and matrix metalloproteinases on the rate of digestion of extracellular matrix (ECM) by melanoma.
2. Using the developed assay to assess the autocrine effect of IL-8 and MCP-1 on melanoma ECM digestion and subsequent migration.
3. Determining whether the effect of IL-8 and MCP-1 on ECM digestion is synergistic in nature.
4. Building a chamber that would allow partial melanoma migration through the Matrigel to be monitored.

Chapter 2

EXPERIMENTAL METHODS

2.1 Cell Culture

Human melanoma cell line Lu1205 (green fluorescent protein [GFP]) was provided by Dr. Garvin P. Robertson (Penn State Hershey Medical Center, Hershey, PA). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS; Biosource, Inc.), 100 units/ml penicillin-streptomycin (Biosource, Inc.). Cells were cultured in passages 1-100 on polystyrene Petri dishes in a 37°C, 5% CO_2 environment.

Human umbilical vein endothelial cells (American Type Culture Collection, Manassas VA) were cultured in Ham's F12-K media (Biofluids, Inc., Gaithersburg, MD). The media was supplemented with 10% FBS, 10μg/ml endothelial cell growth supplement (ECGS, Sigma Chemical Co.),100μg/ml heparin (Sigma Chemical Co., St. Louis, MO), and 100 units/ml penicillin-streptomycin (Biosource, Inc.). Cells were cultured in passages 1-10 on polystyrene Petri dishes in a 37°C, 5% CO_2 environment.

Cells were passed by first washing with DPBS, adding a thin layer of 0.05% trypsin (Gibco, Invitrogen, Carlsbad, CA) for 5 minutes until the cells have shown detachment. The cells were then suspended in fresh medium and re-plated on new Petri dishes.

2.2 Co-Culture Methods

For contact co-cultures, HUVEC was added to a 6-well plate and grown to confluence. One of the wells was detached and the number of endothelial cells was determined to be around $1x10^6$ cells/ml. The wells were washed with DPBS and 3ml Lu1205 cells were added, suspended in assay media (Ham's F12-K media [Biofluids, Inc., Gaithersburg, MD]) supplemented with 1% fetal bovine serum (FBS; Biosource, Inc.), at a concentration of $1x10^6$ cells/ml. For antibody blocking studies, anti-Vascular Cell Adhesion Molecule-1 (VCAM-1) antibody (R&D Systems, Inc., Minneapolis, MN) was added to the media at a concentration of 3ug/ml. The co-culture was incubated for 12 hours in a 37°C, 5% CO_2 environment. The media was then collected for further experiments.

2.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Co-culture supernatant was collected and centrifuged at 1500rpm for 5minutes. The upper layer of the centrifuged supernatant was removed and stored at -20°C until ELISA was to be performed at the Penn State General Clinical Research Center. ELISA detection of supernatant protein concentrations was performed by diluting mouse anti-human capture antibody for MCP-1 (MAB679, R&D Systems, Minneapolis, MN) was diluted to 2μg/ml in coating buffer (0.1M $NaHCO_3$) and 50μl was added to each well of a 96-well ELISA plate and incubated at 4°C for 24 hours. After washing the plate 4 times with 20% Tween 20 in phosphate-buffered saline solution (PBST) [pH 7.0], 200 μl/well blocking solution (PBS containing 1% bovine serum albumin (BSA)) was added and

incubated at room temperature for 2 hours. Following 3 washes with PBST, 100µl/well of samples or target cytokine standards were added and incubated at 4°C for 24 hours. The plate was then washed 4 times with PBST and 100µl of 0.2µg/ml biotinylated affinity purified goat anti-human MCP-1 polyclonal antibody (BAF279, R&D Systems, Minneapolis, MN) at 0.2µg/ml was added to each well and the plate was sealed and incubated for 2 hours at room temperature. After 6 washes with PBST, the 10µl of streptavidin peroxidase (1µg/ml in PBS, Sigma Chemical Co.) was added to each well, sealed, and incubated for 30 minutes at room temperature. 100µl of 100 µl of 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Sigma Chemical Co.)/peroxide substrate solution was then added. It was then incubated for 1 hour at 4°C in the dark. The plate was read using a microtiter plate reader (Packard, Downers Grove, IL) at a wavelength of 405nm.

2.4 Matrigel thickness assay

Polycarbonate 10µm pore filters (Corning Inc., Corning, NY) were placed in Petri dishes, and soaked in 70% ethanol/water solution for 5 minutes. The ethanol/water solution was aspirated, and the filters were dried under ultraviolet light for 30 minutes in a laminar flow hood. Growth factor reduced Matrigel (BD Biosciences, Franklin Lakes, NJ) was diluted to a concentration of 0.33mg/mL with sterile distilled, deionized water, kept on ice in 0^{o} C, using pre-cooled pipettes and eppendorf tubes to avoid Matrigel polymerization, which occurs at room temperature. The diluted Matrigel was deposited on the rough surface of the filter, covering an area of 4cm X 1.25cm in volumes ranging from 200ul to 550ul. The filters were incubated for 40 minutes at 37^{o} C. Confluent

Lu1205 culture was serum starved overnight in growth media (DMEM media containing 1% FBS). The cells were detached with 1mL 0.05% trypsin (Gibco, Invitrogen, Carlsbad, Ca). The cells were separated by centrifugation at 1500g for 5 minutes, and resuspended in media (1% FBS DMEM) to a concentration of 500,000 cells/mL. The Lu1205 cells were deposited in the top well of Boyden chamber at 40ul/well and incubated for 4 hours at 37^{o} C, 5% CO_2. The media in the bottom well (DMEM media, 1% FBS) contained 100ug/ml of collagen IV (BD Biosciences, Franklin Lakes, NJ). After migration, the top of the filter was wiped twice with wetted wipes, and the cells attached to the bottom of the filter were stained using Giemsa stain (Harleco Hemacolor, EMD Chemicals, Gibbstown, NJ).

2.5 Matrigel Migration Assay

Polycarbonate 10μm pore filters (Corning Inc., Corning, NY) were placed in Petri dishes, covered with blocker, and soaked in 70% ethanol/water solution for 5 minutes. The ethanol/water solution was aspirated, and the filters were dried under ultraviolet light for 30 minutes in a laminar flow hood. Growth factor reduced Matrigel (BD Biosciences, Franklin Lakes, NJ) was diluted to a concentration of 0.33mg/mL with sterile distilled, deionized water and coated at 200μl /per 25mm x 80mm 10μm pore filter. The filters were incubated for 40 minutes at 37^{o} C. Confluent Lu1205 culture was serum starved overnight in growth media (DMEM media containing 1% FBS). The cells were detached with 1mL 0.05% trypsin (Gibco, Invitrogen, Carlsbad, Ca). The cells were separated by centrifugation at 1500g for 5 minutes, and resuspended in media (1% FBS DMEM) to a concentrations ranging from 100,000 to 700,000 cells/ml.

In the neutralizing cases, 1.5μg/ml of anti-MCP-1 human monoclonal antibody (R&D Systems, Minneapolis, MN) and/or 2μg/ml of anti-Il-8 human monoclonal antibody ((R&D Systems, Minneapolis, MN) was added to the cell suspension and shaken at 37^{o} C for 1 hour.

In the case of MMP-2 neutralization, 20ng/mL anti-MMP-2 monoclonal antibody (R&D Systems, Minneapolis, MN) was added to cell suspension and shaken at 37^{o} C for 1 hour.

The Lu1205 cells were deposited in the top well of Boyden chamber at 40ul/well and incubated for 4 hours at 37^{o} C, 5% CO_2. The media in the bottom well (DMEM media, 1% FBS) contained 100ug/ml of collagen IV (BD Biosciences, Franklin Lakes, NJ). After migration, the top of the filter was wiped twice with wetted wipes, and the cells attached to the bottom of the filter were stained using Giemsa stain (Harleco Hemacolor, EMD Chemicals, Gibbstown, NJ). One frame per well was taken and cells counted for analysis using bright field microscopy under 10x magnification. Cells were distinguished from debris and filter pores by finding the nucleus, which has a round shape and darker color, surrounded by round or elongated lighter-colored cell cytoplasm. Cells attached to each other were distinguished by their nuclei. An average cell size is 15um when it is round, and 25um when it is elongated. The cells were counted using NIS elements software (Nikon Instruments Inc., Melville, NY). The cells in the first frame taken was counted by hand, and the automated cell counting settings, such as separation, circularity, diameter dimensions, and threshold were set until the automated cell count matched the hand cell count. Subsequent frames of view were counted using the software, with visual confirmation of every cell in the frame being accounted for.

2.6 Statistical Analysis

To test for statistical significance, assays were performed at least twice. The data is analysis is performed using the 2-sample 2-tailed t-test with unequal variances. Error bars shown in figures are standard error mean (SEM).

Chapter 3

RESULTS AND DISCUSSION

3.1 Results

Our research looks to characterize the role of Interleukin-8 (IL-8) and Monocyte Chemotactic Protein- 1 (MCP-1) on Matrix Metalloproteinase- 2 (MMP-2) levels in melanoma extravasation through the extracellular matrix. Specifically, it focuses on autocrine interaction between the tumor cells, which results in a cascade of events that causes subsequent degradation of the extracellular matrix. These events allow the melanoma cells to extravasate from the blood vessels into the surrounding tissues. For the purpose of our research, several melanoma cell lines available to the lab were evaluated for their metastatic potential, or the ability of cells to move from one organ or tissue to another (Table 3.1).

Table 3.1 Metastatic Potentials of melanoma cell lines (Khanna, 2010)

Cell Line	Metastatic Potential
WM35	-
A2058	++
Lu1205	+++

High metastatic potential is correlated to higher rate of extracellular matrix degradation, thus, Lu1205 cell line was chosen for further studies.

Previous studies have shown that IL-8 plays multiple roles in melanoma extravasation. Furthermore, previous studies indicate that endogenously produced IL-8

stimulated IL-8 secretion from granulocytes (PMN) (Slattery and Dong, 2003) and endothelial cells, (Kwang, 2008).

Studies measuring the levels of IL-8 and MCP-1 in Lu1205 supernatant via ELISA were performed by Chonghaw Kwang (Masters Candidate in Bioengineering, Pennsylvania State University) and reconstructed data is presented in (Figure 3.1)

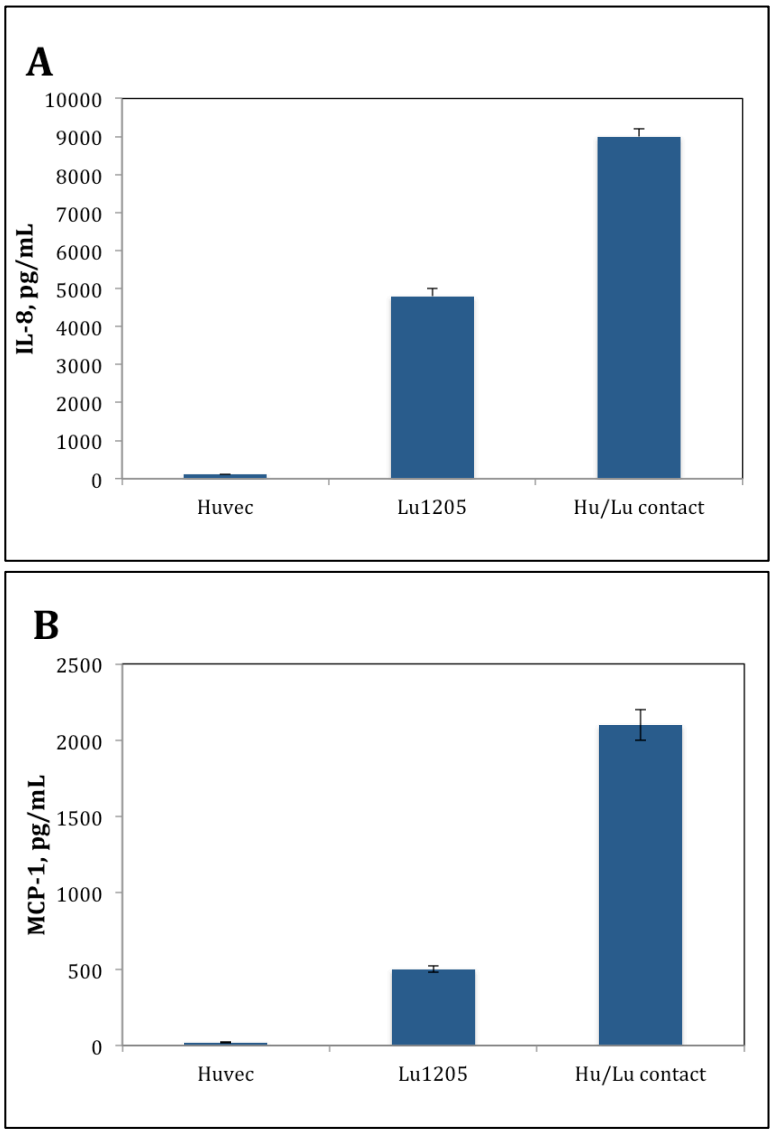


Figure 3.1 IL-8 and MCP-1 concentrations in Lu1205 supernatant (Kwang, 2008). Supernatant from Lu1205 culture is collected and assayed via ELISA A) Concentrations of IL-8 upon co-culture, P=0.11% B) Concentrations of MCP-1 upon co-culture, P=0.03%

Once it was established that Lu1205 microenvironment contained significant amounts of both chemokines, the effect of neutralizing Il-8 and MCP-1 on MMP-2 levels was measured by using human monoclonal antibodies to neutralize IL-8 and MCP-1 and assaying the change in MMP-2 levels via zymography (Figure 3.2)

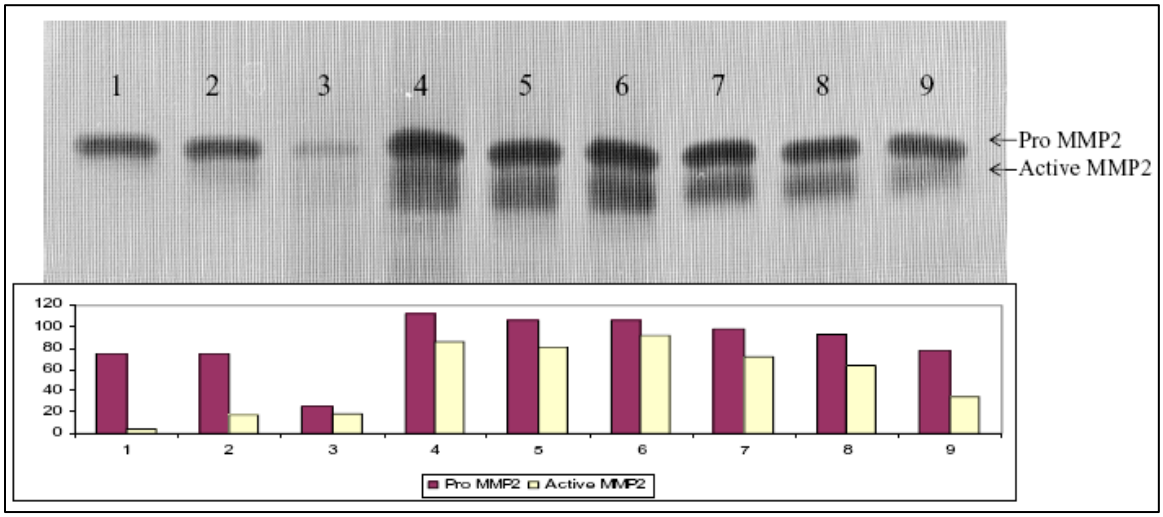


Figure 3.2 Co-culture with neutralizing IL-8 and MCP-1. Zymography assay is shown above with the relative intensity chart. Pro-MMP2 is 72 kDa and active MMP1 is 62 kDa. Lanes 1, 2: HUVEC only, 3: Lu1205, 4: HUVEC with Lu1205 contact co-culture, 5, 6: HUVEC with Lu1205 transwell co-culture, 7: HUVEC with Lu1205 transwell co-culture with anti IL-8, 8: HUVEC with Lu1205 transwell co-culture with anti MCP-1, 9: HUVEC with Lu1205 transwell co-culture with anti MCP-1 and anti IL-8 (Kwang, 2008)

Figure 3.2 shows that both pro- and active forms of MMP-2 is present in Lu1205 culture alone, although the levels of MMP-2 are higher in co-culture of Lu1205 and Human Umbilical Vein Endothelial cells (HUVEC). This is possibly due to tumor cell-endothelial cell interactions, which up-regulate MMP-2 levels. MCP-1 and IL-8 neutralization shows that the expression of MMP-2 in HUVEC/Lu1205 co-culture is

reduced compared to the control case. The reduction of MMP-2 levels suggests that IL-8 and MCP-1 play a role in MMP-2 up-regualtion. It is not clear yet whether this interaction has an autocrine component. In order to test whether Lu1205 cells use IL-8 and MCP-1 to produce an increase in MMP-2, and assess the extent of the resulting extracellular matrix degradation and melanoma migration, it was necessary to construct an appropriate assay that contains a layer of a substance that mimics the role of extracellular matrix. For this purpose, Matrigel (BD Biosciences, Franklin Lakes, NJ), a reconstituted basement membrane extracted from murine Engelbreth-Holm-Swarm sarcoma was chosen to simulate the desired environment. When developing the assay, it was important to choose an appropriate Matrigel volume, such that melanoma migration would be inhibited without the necessary secretion of matrix metalloproteinases and other digestive enzymes. On the other hand, if the extracellular matrix layer is too thin, the level of matrix degradation would be impossible to measure since melanoma cells would be able to migrate through the matrix without metalloproteinase secretion. Matrigel thickness assay was constructed, possessing a range of extracellular matrix volumes, and the migration rates of Lu1205 cells were determined (Figure 3.3).

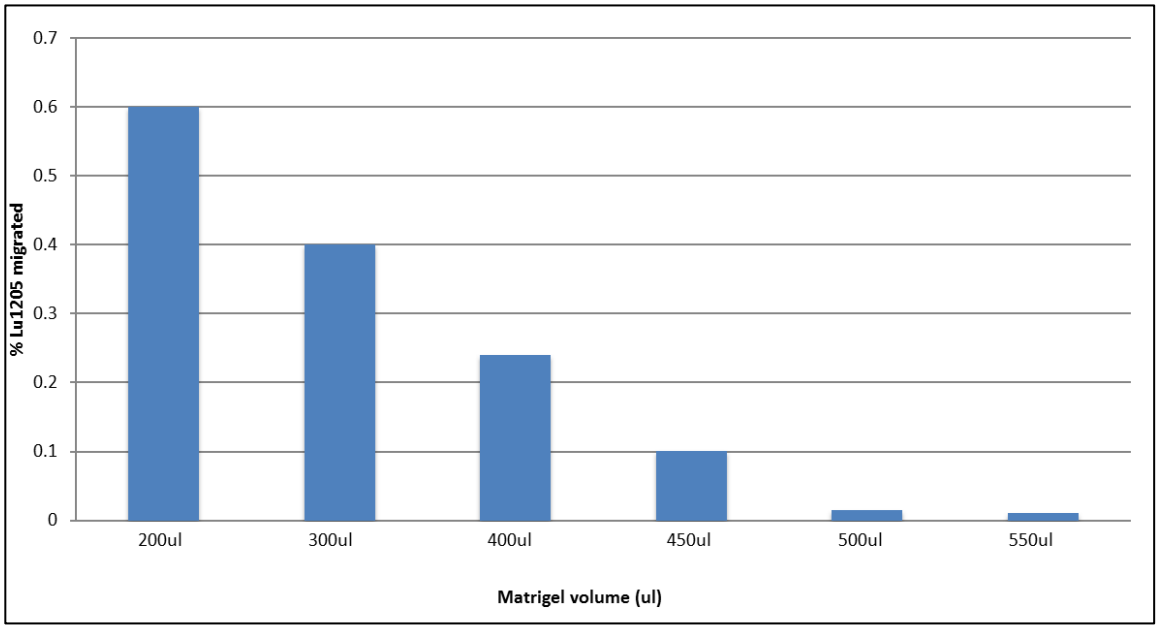


Figure 3.3 Effect of Matrigel volume on Lu1205 migration using 48-well Boyden chamber and 10um pore polycarbonate filters. Migration time is 4 hours.

When the volume of the deposited Matrigel is less than 200 ul, it fails to cover the filter entirely, and is therefore not viable for this assay. Based upon the results, an optimal volume of 200ul was chosen for further migration studies.

The developed assay was used to investigate the effect of the MCP-1 and IL-8 on melanoma migration through the extracellular matrix. The goal of the experiment was to establish if there was a synergistic effect between IL-8 and MCP-1 on Lu1205 migration. To assess the effect of MMP-2 on Lu1205 migration, human monoclonal antibody against MMP-2 was used as well. The combined data from the experiments described above is presented in (Figure 3.4)

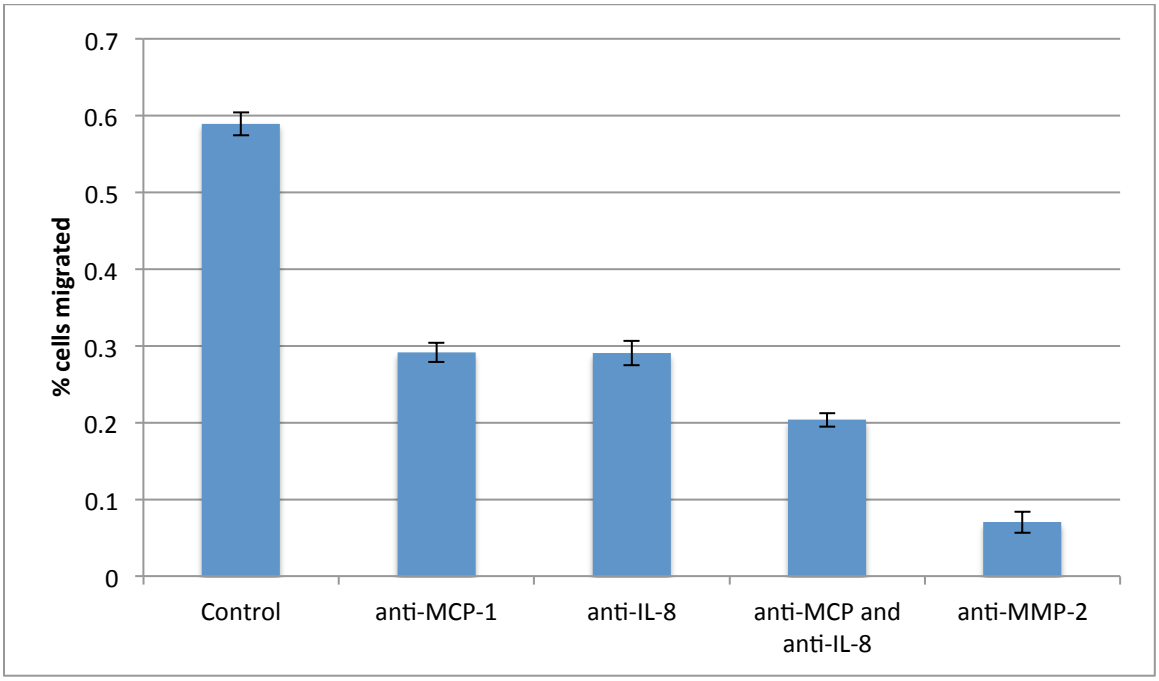
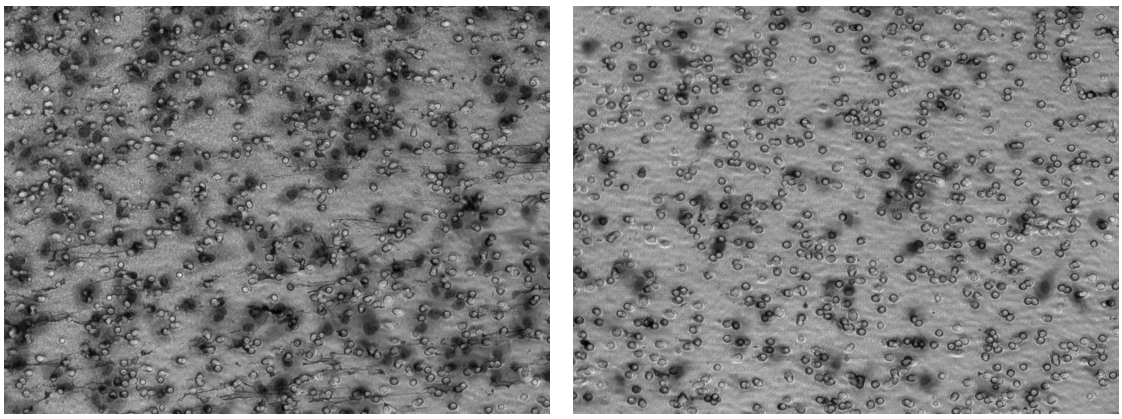


Figure 3.4 Effect of MCP-1, IL-8, and MMP-2 on Lu1205 migration. Matrigel migration assay is shown above, human monoclonal antibodies used in concentrations of 1.5ng/mL for MCP-1, 2ng/mL for Il-8, and 20ng/mL for MMP-2. Experiments done in triplicate, and cells migrated counted by hand. All p-values<0.05.

The cells on the underside of the filter were not difficult to count and were easily distinguishable from the filter pores and cell debris. (Figure 3.5) shows a frame of view for the control and anti-MCP-1 and anti-IL-8 case and the difference in migrated cells is apparent.



Control field-of-view anti-IL-8 and anti-MCP-1 field-of-view

Figure 3.5 Comparison of field-of-view of Lu1205 Matrigel migration assay after cell attachment and staining. Cells stained with 3-step HEMA stain, fields of view taken at 10x using bright field microscopy. Stained nuclei are clearly visible, as well as the dark-grey cytoplasm and cell membranes.

The Matrigel migration assay data from (Figure 3.4) shows that neutralizing either or both of the chemokines significantly reduces migration rate of Lu1205. It also shows that MMP-2 plays a significant role in Lu1205 migration, and it can be inferred that IL-8 and MCP-1 have a role in the autocrine interaction in Lu1205, affecting MMP-2 levels. In order to establish whether the effect of IL-8 and MCP-1 is synergistic in nature, (Figure 3.6) compares the decrease in migration to the control case to assess whether the decrease due to neutralizing both IL-8 and MCP-1:

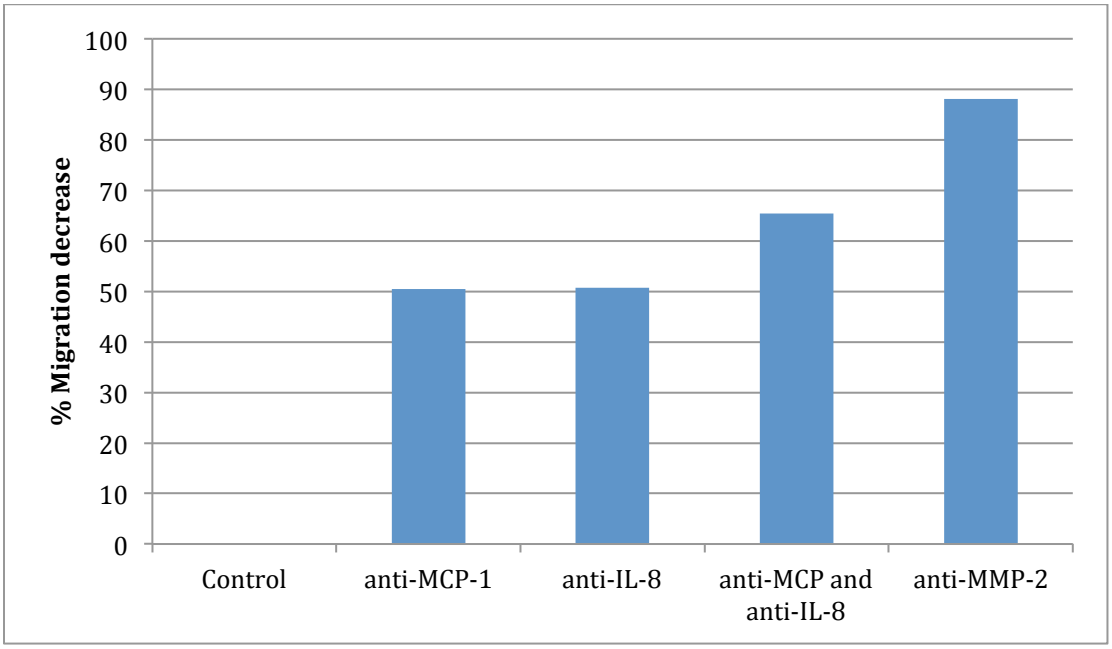


Figure 3.6 Decrease in migration compared to control as a result of antibody neutralization.

The decrease in migration due to IL-8 and MCP-1 blocking is close to 50%, while inhibition of both chemokines reduces migration levels by 65%. Therefore it can be concluded that the functions of IL-8 and MCP-1 on increasing the rate of Matrigel digestion by Lu1205 cells are not synergistic in nature. Additionally, inhibition of MMP-2 reduces migration by 88%, which indicates that MMP-2 plays a crucial role in extracellular matrix digestion, as previously confirmed by other studies.

3.2 Discussion

Previous research done in our lab has established that Lu1205 secretes both IL-8 and MCP-1 in significant amounts, confirmed by ELISA and presented in Figure 3.1, which is not unexpected. Literature provides strong evidence for autocrine IL-8 synthesis by melanoma cell lines, as well as its significance in extravasation (Nurenberg, 1999).

Expression of MCP-1 in melanoma cell lines has been documented as well (Diaz-Valdes, 2011). Both chemokines have been investigated for their role in tumor invasion and formation, having an effect on endothelial cells, as well as inflammatory cells (Hsu, 2002). It is hypothesized that tumor cells secrete these chemokines to recruit host cells in assisting in extravasation and tumor formation process. Since our research focuses on isolating the effect of MCP-1 and IL-8 on Lu1205 MMP-2 secretion, it was needed to be established that Lu1205 does secrete MMP-2. Zymography data, column 3 in Figure 3.2, indicates the presence of both pro- and activated forms of MMP-2 in Lu1205 culture. Although the levels are comparatively less than endothelial (HUVEC) and Lu1205/HUVEC co-cultures, MMP-2 are nevertheless present in significant quantities to digest the extracellular matrix, as Figures 3.4 and 3.5 have shown: inhibiting MMP-2 in Lu1205 migration assay significantly reduced the number of cells migrated.

Determining the optimal Matrigel volume and concentration to assess Lu1205 digestive and migratory capabilities resulted in finding it to be 200ul (Figure 3.3) and a concentration of 0.33 mg/mL. This is consistent with a chemoinvasion protocol published in *Nature Journals* (Albini, 2007), which suggests using Matrigel concentrations from 0.33 mg/mL to 0.20 mg/mL, and a Volume/filter area ratio of approximately 45ul/cm^2, as well as migration time of 4-6 hours, depending on the invasive potential of the cell line tested. The total area of the middle 12 wells in the 48-well Boyden chamber is 4cm^2, and the experimentally determined volume/filter area ratio is 50ul/cm^2, which agrees with previously-published literature.

It was discovered that IL-8 and MCP-1 play a role in Lu1205 migration, as neutralizing either chemokine reduced the number of cells migrated by approximately

50% (Figure 3.6). This finding suggests that Lu1205 secrete IL-8, which binds a receptor located on the same or adjacent melanoma cell, and triggers an intercellular mechanism which aids melanoma migration, and a possible secretion of MMP-2. Previous research suggests that melanoma cells express CXCR1 and CXCR2 receptors, which bind IL-8(CXCL-8) (Varney, 2012), although the presence of other, IL-8-specific receptors in Lu1205 cell line is possible.

Although there is little evidence in literature of MCP-1(CCL2) receptors (CCR2) being expressed by melanoma cell lines, CCR2 expression has been confirmed in various tumor types, such as breast cancer cell lines (Conti, 2004). It is possible that MCP-1, secreted by Lu1205 interacts with neighboring cells to either directly or indirectly aid in tumor migration.

Due to the fact that neutralizing each chemokine, IL-8 and MCP-1 separately decreased the number of migrated cells by 50%, it would be impossible to determine whether the two chemokines acted synergistically. Synergy is defined as the effect arising from two or more factors is greater than the sum of their individual effects. In order for the effect of IL-8 and MCP-1 to be considered synergistic, Lu1205 migration will need to be decreased by more than 100%, which is not possible to determine. In an attempt to neutralize both chemokines, the migration was decreased by 65% (Figure 3.6), which suggests that the roles IL-8 and MCP-1 play in melanoma migration may share a common, although not identical pathway.

Chapter 4

Development of 2D PDMS migration Chamber

4.1 Introduction

Assays to assess tumor invasion through the extracellular matrix have been widely employed in a variety of studies. Boyden pioneered a two-chamber chemotaxis assay, which uses a micro-pore polycarbonate filter to separate the chamber containing chemoattractant from the cell suspension (Albini, 2007). Variations of this assay continue to be used today. One such variation, the parallel plate concept was combined with the multi-well Boyden chamber, was developed in our laboratory. The top well, designed to deposit the cells was replaced with a recirculating cell suspension in flow conditions, requiring the cells to adhere to the endothelial layer coving a polycarbonate filter before they are allowed to migrate. Such modification is one step closer to the physiological model of tumor cell invasion, as tumor cells extravasate from blood flow, which is constantly in motion (Slattery and Dong, 2003). Data collection of this type of assays employs end-point determination, and does not account for the partial cell migration through the extracellular matrix, nor does it allow for the visualization of the migration process (Chaw, 2007), although the visualization of cellular adhesion processes have been extensively studied in our laboratory, using another device, the side-view particle imaging velocimetry flow system (Leyton-Mange and Dong, 2006).

When using a standard Boyden chamber migration assay, such as one employed for the purposes of this paper, collagen IV is used as a chemoattractant in the lower well of the Boyden chamber, due to the fact that it is a prevalent component of the basement

lamina and intima under the endothelium, and would thus encourage cells to migrate along its well-established concentration gradient. Collagen IV is meant to evoke a chemotactic response from the migrating cells, or the tendency of the cells to move toward the area of higher concentration of a certain chemokine. (Hodgson and Dong, 2003). However, the migrating cells also display a chemokinetic response to various components of Matrigel, which represents an increased movement in random directions. Both of these responses are detected by the Boyden chamber assay, but only the assessment of the chemotactic response is desirable.

The difficulty of observing cell migration lead to the development of a variety of two-dimensional, as well as 3-dimentional migration assays. These assays employ time-lapse video microscopy to track cell movement, and consist of an artificial barrier the cell must digest in order to migrate. Some of the materials used for such barrier include different collagen types, which are predominant in the organ of tumor extravasation study. Studies of a more general nature use Matrigel, described above (Entschladen, 2005). 3D assays that involve a Matrigel layer and flow conditions have been developed as well. One such assay, described by (Chaw, 2007) uses PDMS and microfabrication techniques to create channels of varying widths from 3um to 15um, filled with Matrigel to track single-cell ECM digestion, as well as incorporating flow conditions into the assay. (Figure 4.1)

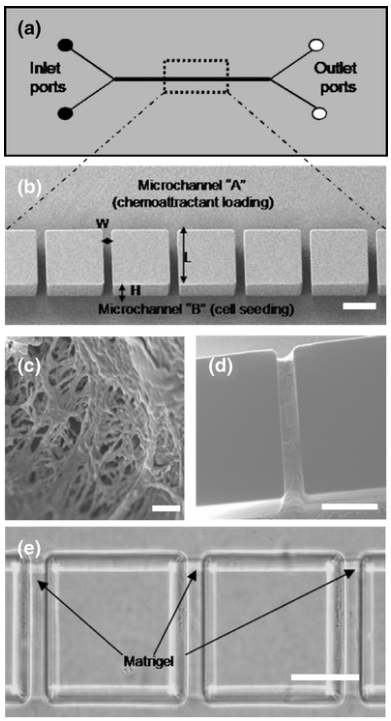


Figure 4.1 (**a**) A schematic diagram (not to scale) of the device layout. (**b**) Layout of the three-dimensional cell migration device. It comprises of two side microchannels sandwiching a row of microgaps measuring 100 μm (L) by 50 μm (H) by 15 μm (W). Microchannel A is used for placing the cells and microchannel B is used for loading the chemoattractant (20% FBS) (**c**) ×50,000 magnification of Matrigel pores within the microgaps. The sizes of the pores are in the nanometer range. (**d**) Scanning electron micrographs of the coated microgaps indicates a 3-D coating of the Matrigel. (**e**) Top view of three Matrigel-coated microgaps which mimics the basement membrane. Scale bars: 500 nm for panel (**c**), 50 μm for panel (**b**), (**d**) and (**e**) (Chaw, et. al 2007)

The 2D PDMS migration chamber developed and described in our paper takes the idea of incorporating flow conditions into migration, but does away with the microchannel concept due to the complicated nature of manufacturing such structures. Instead, it uses a continuous Matrigel layer, which is closer to physiological conditions.

One important difficulty in accurately assessing the migration potential of melanoma cells through the extracellular matrix in our paper is that even without neutralizing chemokines, only 0.6% of the cells deposited in the Boyden chamber

completely migrate and attach to the underside of the filter. It is possible that some of the cells that have partially migrated through the Matrigel layer and have expressed MMP-2 remain unaccounted for, since wiping the top of the filter removes them.

These difficulties led to the development of a two-dimensional migration chamber, which would introduce flow conditions to intravasation measurements and allow to observe melanoma migration through the Matrigel layer in real-time while saving on reagents and generating better quality results.

4.2 Flow chamber manufacture

Polydimethysiloxane (PDMS) chamber was manufactured by combining in a 10:1 ratio Sylgard® 182 silicone elastomer and polymerizing compound (DOW Corning, Midland, MI). The mixture was degassed for 15 minutes and poured into a pre-manufactured aluminum mold. The mold was cured at 80^{o} C for 1 hour.

4.3 Results and further development

The resulting design takes advantage of polydimethylsiloxane (PDMS) properties of flexibility, fast curing time and waterproof adhesion to glass parts. PDMS has also been shown to adhere well to Matrigel (Grafton, 2011) and is often used in surgical implants due to its biological inertness (Jabbari, 2010). The flow chamber has a compartment for Matrigel and melanoma cell deposition, as well as grooves for capillaries used to introduce flow conditions to the intravasation environment (Figure 3.7)

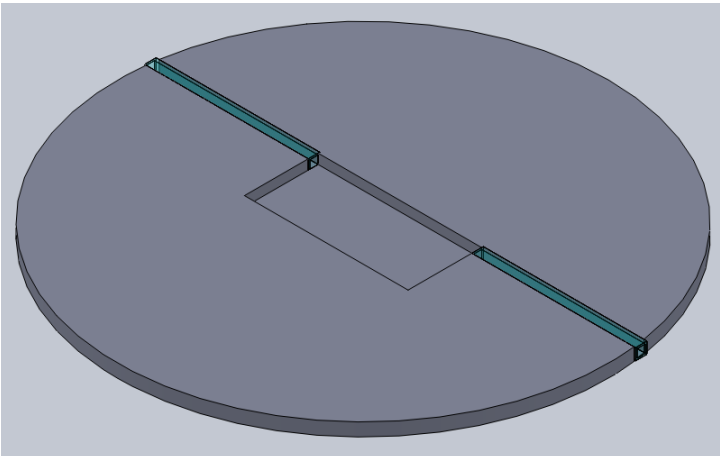


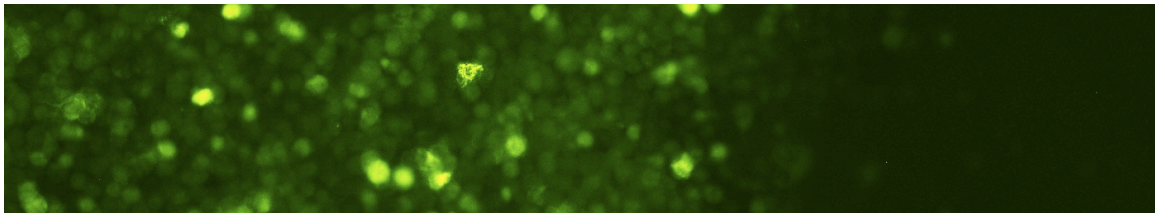
Figure 4.2 Flow chamber design, 3D view. More detailed description of device dimensions and implementation is presented in (Appendix A).

The experimental concept for introducing flow conditions takes into account the shear stress due to flow of 3 dyne/cm^2 (Shin, 2011) and is calculated using the following equation:

$$\tau_w = 6\eta Q/(wh^2)$$

The channel width and height is 0.5mm, the viscosity of flow media, η, is 0.7 cp at 37^o C, while the flow rate produced by the syringe pump (not shown) should be 320 ul/hour. Taking into account the small volume of the circulating media, it becomes easier to quantify the amount of cells migrated into flow and various chemokines can be added to the medium to induce cell migration through the Matrigel into flow. The chamber dimensions were chosen for it to be compatible with an on-stage temperature-control system (Harvard apparatus, Holliston, MA), allowing migration to be observed in real time.

The PDMS chamber's vertical height allows the cells to be spread out horizontally, and allow for superior resolution (Figure 3.8):



Direction of cell Migration 

Figure 4.3 1/5th field-of-view under 10x magnification of Cell-tracker green-stained Lu1205 cells in PDMS migration chamber after 4 hours incubation.

At this point, only trials in static conditions were performed, and a reliable control should be established before the PDMS chamber can be used to accurately assess melanoma migration distance through the Matrigel Layer.

Chapter 5

CONCLUSIONS

5.1 Further Directions

While our research identified the effect of Monocyte Chemotactic Protein 1 (MCP-1) and interleukin-8 (IL-8) on melanoma migration, the exact distribution of tumor cells in the Matrigel layer at the end of 4 hours remains unclear due to instrumentation and assay limitations. The utilization of the PDMS flow migration chamber to characterize the migration distance of tumor cells is necessary and would provide the results needed to accurately quantify the extracellular matrix digestion capabilities of tumor cells that cannot be shown by traditional invasion assays.

Our research focused on Lu1205 autocrine stimulation by IL-8 and MCP-1 to up-regulate MMP-2, future research should incorporate endothelial monolayer into the migration assay to assess the effect of paracrine tumor cell/endothelial cell interactions and their role in tumor cell extracellular matrix digestion capabilities.

Lastly, the PDMS flow chamber should be improved to allow for maintenance of 5% CO_2 levels, as dictated by physiological conditions and an accurate measurement strategy should be developed to standardize the migration results.

5.2 Conclusions

In general, our research explored and developed methods for assessing the intravasation potential of a highly metastatic tumor cells line, Lu1205 by determining the correct amount and consistency of Matrigel. It was shown that a Matrigel volume of

200ul and concentration of 0.33mg/mL provides adequate conditions to assess the extracellular matrix digestive capabilities of Lu1205 melanoma cell line in static conditions.

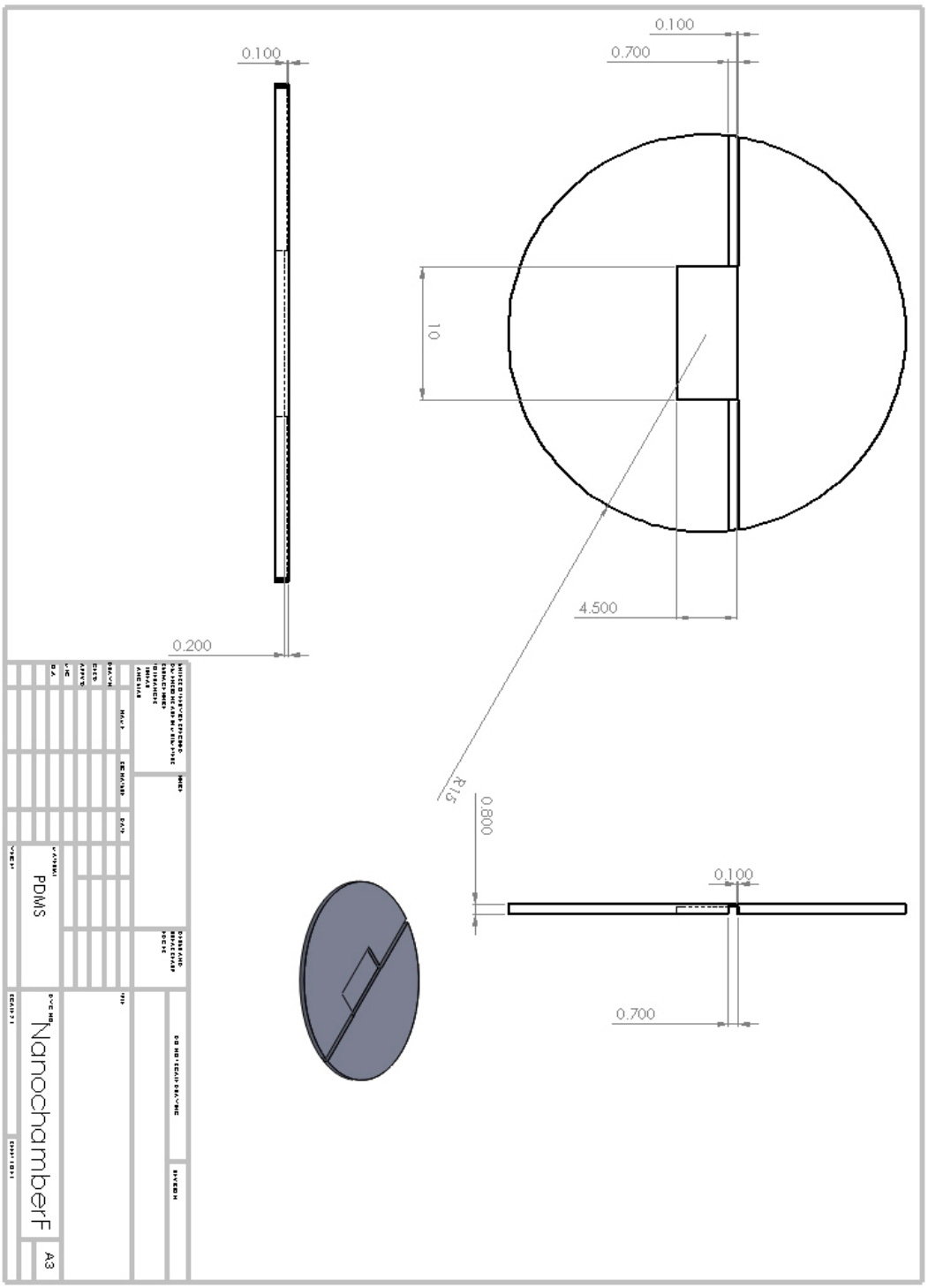
It was shown that blocking MCP-1 decreases the digestion rate of the extracellular matrix and the migration rate of tumor cells in the absence of an endothelial cell monolayer.

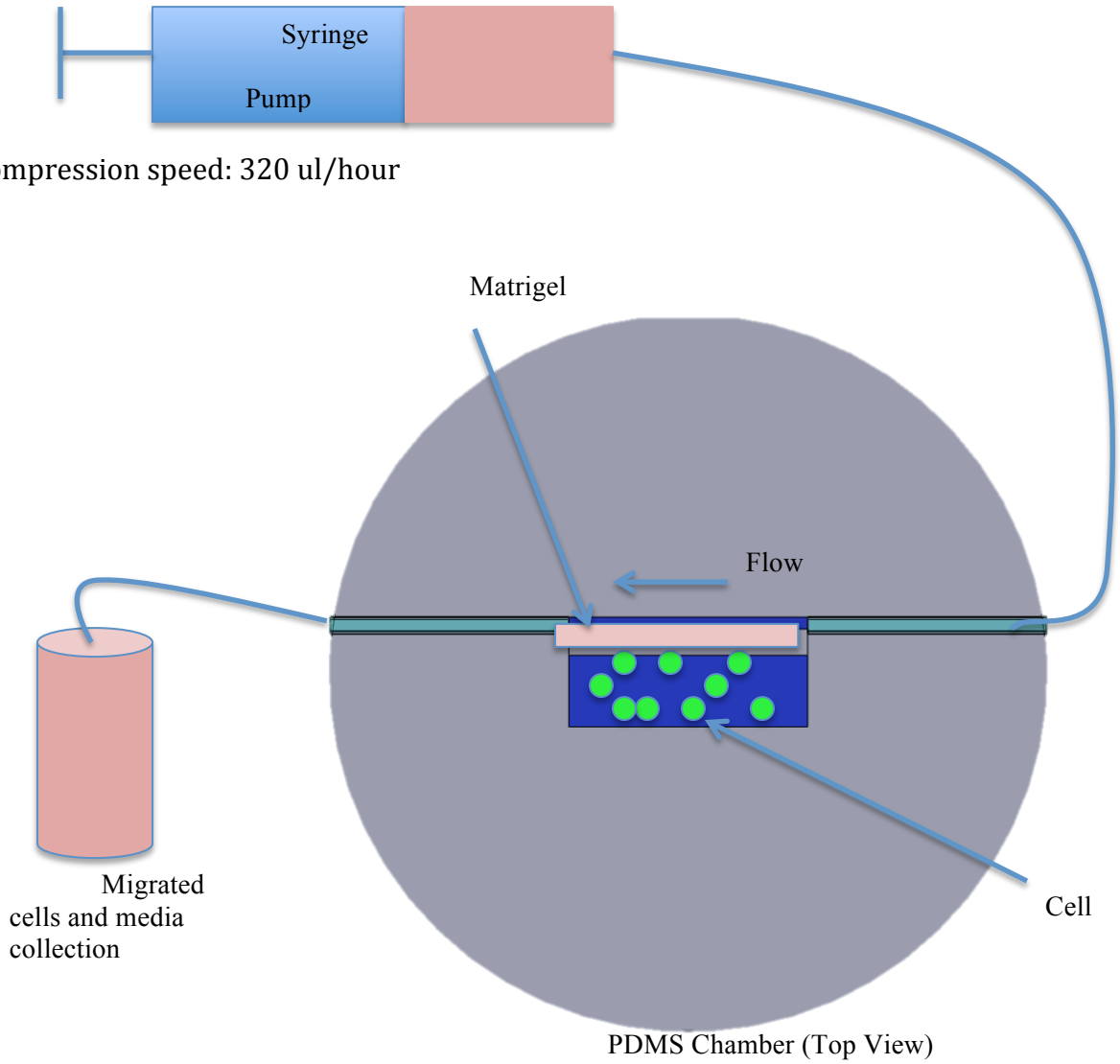
The synergistic effect of IL-8 and MCP-1 on Matrigel digestion by Lu1205 was assessed, and it was concluded that the two chemokines do complement each other in increasing the digestive capabilities of the Lu1205 but not significantly enough to consider the effect being of synergistic nature.

Lastly, in order to accurately quantify the migration distance, a novel two-dimensional flow chamber was designed to serve this purpose.

APPENDIX A

PDMS Flow Chamber design and Implementation





Static Migration Assessment procedure:

Matrigel was diluted in 2:1 DMEM, and 400 ul was deposited inside the well. It was incubated at 37° C for 40 minutes. Lu1205 cells were labeled with cell tracked green (Invitrogen, Carlsbad, CA) and deposited in the cleared area. The chamber was covered with a flat transparent button to allow air access to cells and incubated for 4 hours at 37° C, 5% CO_2.

REFERENCES

Alberts B, Bray D, Hopkin K, Johnson A, Lewis J, *et al.* Essential Cell Biology, *Garland Science.* **3**rd edition: 720-721, (2004).

Alberts B, Johnson A, Lewis J, *et. Al*, Molecular Biology of the Cell, *Garland Science.* **5**th edition: 1194-1222, (2008).

Albini, A., Benelli, R., The chemoinvasion assay: a method to assess tumor and endothelial cell invasion and its modulation *Nature Protocols* **2**, 504-511 (2007).

Chaw, K.C., Manimaran, F., Tay, H., Swaminathan, S., Matrigel coated polydimethylsiloxane based microfluidic devices for studying metastatic and non-metastatic cancer cell invasion and migration *Biomed Microdevices* **9**;597-602 (2007)

Conti, I., Rollins, B., CCL2 (monocyte chemoattractant protein-1) and cancer *Seminars in Cancer Biology*, **14**(3):149-154 (2004)

Diaz-Valdez, N., Basagoiti, M., Dotor, J., et al. Induction of Monocyte Chemoattractant Protein-1 and Interleukin-10 by TGFb1 in Melanoma enhances Tumor infiltration and Immunosuppression. *Cancer Research,* **72**(7): 812 (2011)

Dittmar T, Heyder, C, Gloria-Maercker E, Hatzmann W, and Zanker KS. Adhesion molecules and chemokines: the navigation system for circulating tumor (stem) cells to metastasize in an organ-specific manner. *Clin Exp Metastasis.* **25**: 11-32, (2008).

Entschladen, F., Drell, T., Lang., K., Masur, K., Palm, D., Bastian, P., Niggermann, B., Zaenker, K., Analysis methods of human cell migration, *Exp Cell Res* **307**(2): 418-426 (2005).

Fidler IJ, The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revistited. *Nature Reviews: Cancer.* **3**: 1-6, (2002).

Fidler IJ, The organ Microenvironment and cancer metastasis, *Differentiation*, **70**(9-10):498-505, (2002).

Grafton, M., Wang., L., Vidi, P., Leary, J., Lelievre, S., Breast on-a-chip: mimicry of the channeling system of the breast for development of theranostics. *Integr. Biol.* **3**:451-459, (2011).

Hodgson, L., Henderson, A., Dong, C., Melanoma cell migration to type IV collagen requires activation of NF-kB. *Oncogene* **22:**98-108, (2003).

Hofmann U, Westphal P, van Muijen G, Ruiter D. Matrix Metalloproteases in Human Melanoma. *Journal of Investigative Dermatology.* **115**: 337-344, (2000).

Hoskins M and Dong C. Kinteics analysis of binding between melanoma cells and neutrophils. *MCB.* **3**(2): 79-87, (2006).

Hsu M, Meier F, Herlyn M, Melanoma development and progression: a conspiracy between tumor and host. *Differentiation* **70**, 522-36, (2002).

Jabbari, E., Khademhosseini, A., Biologically-responsive hybrid biomaterials, *World Scientific Publishing Co.* (2010).

Jerant A, Johnson J, Sheridan C, Caffrey T, Early Detection and treatment of skin cancer. *American Family Physician* 357: 07/15, (2000).

Khanna P., Yunkunis T., Muddana, H., Peng, H., August, A., Dong, C., p38 MAP kinase is necessary for melanoma-mediated regulation of VE-cadherin disassembly. *Am J Physiol Cell Physiol.* **298**(5), C1140-1150, (2010).

Kwang, C., Chemokines from melanoma upregulate MMP2 within the tumor microenvironment. *A thesis in Bioengineering,* Pennsylvania State University, (2008).

Lance L, Stetler-Stevenson W. Tumor Invasion and Metastasis: An Imbalance of Positive and Negative Regulation. *Cancer Research,* **51**: 5054-5059, (1991).

Leyton-Mange, J., Yang, S., Hoskins, M., Kunz, R., Zahn, J., Dong, C., Design of a Side-view particle imaging velocimetry system for cell-substrate adhesion studies, *J. of Biomech. Eng.* **128:**271-278, (2006).

Liang, S., Dong, C., "Integrin VLA-4 enhances sialyl-Lewis$^{x/a}$- negative melanoma adhesion to and extravasation through the endothelium under low flow conditions" *Am J Physiol Cell Physiol* **295**: C701-C807, (2008).

Liang, L., Slattery, M., Wagner, D., Simon, S., Dong, C., Hydrodynamic Shear Rate Regulates Melanoma-Leukocyte Aggregation, Melanoma Adhesion to the endothelium, and Subsequent Extravasation, *Annals of Biomedical Engineering,* **36**(4): 661-671, (2008).

Luca, M., Huang, S., Gershenwald, J. E., Singh, R. K., Reich, R. and Bar-Eli, M. Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. *Am J Pathol* **151**, 1105-13 (1997).

Lucas R, McMichael T, Smith W, Armstrong B, Global Burden of disease from solar ultraviolet radiation. *Environmental Burden of Disease* **13**: 23, (2006).

Melnikova V, Bar-Eli M, Bioummunotherapy for melanoma using fully human antibodies targeting MCAM/MUC18 and IL-8, *Pigment Cell Research* **19**(5): 395-405, (2006).

Mukaida, N., Harada, A. and Matsushima, K., Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. *Cytokine Growth Factor Rev* **9**, 9-23. (1998).

National Cancer Institute, Defining Cancer, National Institutes of Health, (2011).

Nurenberg, W., Tobias, D., Otto, F., Henz, B., Schadendorf, D., Expression of interleukin-8 detected by *in sutu* hybridization correlates with worse prognosis in primary cutaneous melanoma. *J. Pathol.* **189**: 546-551 (1999)

Ria R, Reale A, Castrovilli A, Mangialardi G, Dammacco F, Ribatti D, Vacca A. Angiogenesis and Progression in human melanoma. *Dermatology Res Pract,* 2010:185687, (2010).

Shin, M., Kim, S., Jung, H., Integration of intra- and extravasation in one cell-based microfluidic chip for the study of cancer metasthasis *Lab Chip,* **11**, 3880, (2011).

Slattery, M., and Dong, C., Neutrophils influence melanoma adhesion and migration under flow conditions., *Int J Cancer* **106**, 713-722, (2003)

Varney, M., Johansson, S., Singh, R., Distinct expression of CXCL8 and its receptors CXCR1 and CXCR2 and their association with vessel density and aggressiveness in Malignant Melanoma, *Anatomic Pathology*, **137**(4): 209-216, (2012)

World Health Organization. Cancer. (2010).

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