MECHANISTIC COUPLING OF CANCER CELL PROLIFERATION AND MIGRATION

By

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ABSTRACT

Following uncontrolled proliferation, a subset of primary tumor cells acquires additional traits/mutations to trigger phenotypic changes that enhance migration and are hypothesized to be the initiators of the metastatic cascade. This study reveals a novel adaptive mechanism that harnesses synergistic paracrine signaling via Interleukin 6 (IL-6) and Interleukin 8 (IL-8), which is amplified by cell proliferation and cell density, to directly promote cell migration. This effect occurs in metastatic human sarcoma and carcinoma cells - but not in normal or non-metastatic cancer cells-, through the downstream signaling proteins WASF3 and Arp2/3 as key intermediates. The transcriptional phenotype of high-density cells that emerges due to proliferation resembles to some extent that of low-density cells treated with a combination of IL-6 and IL-8. Simultaneous inhibition of IL-6/8 receptors significantly decreases the expression of Arpc2, an important subunit of the Arp2/3 complex, in a mouse xenograft model and consequently reduces metastasis.

While therapeutic intervention with Tocilizumab and Reparixin represses cell-density-dependent migration and accordingly decreases the metastatic burden of tumor cells, the combination of the drugs does not affect cell proliferation and thus tumor growth. Epidermal growth factor (EGF) and its family of receptors have been implicated in regulating uncontrolled proliferation in tumor cells. To simultaneously target cell proliferation and migration, receptors of IL-6, IL-8, and EGF were inhibited using Tocilizumab, Reparixin, and Cetuximab. The combination of these therapeutics significantly decreases tumor cell migration and proliferation. This study infers a new strategy to decrease tumor growth and metastatic capacity of tumor cells and thus and improve patient outcomes.

Thesis Advisor: Dr. Denis Wirtz
Thesis Committee Chair: Dr. Peter Searson
Thesis Committee: Dr. Jerry Lee, Dr. Sharon Gerecht, Dr. Daniele Gilkes, Dr. Rong Fan, and Dr. Ernesto Freire.
DEDICATION

This dissertation is dedicated to my parents Mr. Sarath Jayatilaka and Mrs. Shiranthi Jayatilaka who have taught me important lessons about dedication, discipline, and determination. Without their love, encouragement, and support, I could not have produced this work.
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CHAPTER 1: INTRODUCTION - DRIVERS OF METASTASIS

Metastasis, the spread of cancer cells from a primary organ and colonization at distal sites through the vascular and lymphatic systems, is responsible for 90% of cancer related deaths\textsuperscript{1,2}. Proliferation and migration are two key drivers of metastasis, that can either occur concurrently or divergently of each other. Here, I present recent work highlighting proliferation and migration as two important processes in the metastatic cascade and the role of the tumor microenvironment in metastasis.

1.1 Metastatic cascade

Metastasis is a complex, multistep process that is regulated by genetic factors, external environmental factors, and physical interactions with their microenvironment\textsuperscript{3,4}. The metastatic cascade includes detachment, intravasation, circulation and attachment, extravasation, and growth of secondary tumor\textsuperscript{5}. (Figure 1.1)

\textbf{Figure 1.1: Illustration of the metastatic cascade:} This cartoon depicts the multiple stages involved in the metastatic cascade. Adapted from Wirtz et al (2011). Nature Reviews Cancer, 11(7), 512-522.
1.1.1 Detachment

The detachment of cancer cells from the primary tumor involves the epithelial to mesenchymal transition (EMT) where a reduction in E-Cadherin (an intercellular adhesion molecule) and cytokeratins leads to a dramatic change in the physical and chemical properties of the cells, accompanied by cell morphology changes from cuboidal epithelial to mesenchymal. This process leads to the tumor cell adopting a motile phenotype and the secretion of matrix metalloproteinases (MMP), which promote the local digestion and remodeling of the basement membrane\(^\text{6,7,8}\). Following the detachment from the tumor, cancer cells encounter the architecturally complex extracellular matrix (ECM), which is rich in collagen I\(^\text{9}\). The cell digests the ECM through the secretion of MMPs and migrates through the collagen-rich environment by generating pseudopodial protrusions that are regulated by focal-adhesion proteins and the Arp2/3 complex and its activators\(^\text{10,11,12,13,14}\).

1.1.2 Intravasation, circulation, attachment, and extravasation

The cell enters the vascular system by undergoing dramatic shape changes that involve the remodeling of the cytoskeleton and deformation of the interphase nucleus. Deformation of the nucleus is regulated by the linkers of the nucleus and cytoskeleton (LINC) complexes, which are composed of nesprins and SUN proteins\(^\text{5}\). During their transit through the vascular system, tumor cells are subjected to shear and compressive forces and stresses that cause some of them to die or remain dormant. A tiny fraction of the circulating tumor cells survive to generate distant metastases.

Circulating tumor cells exit the circulatory system through either physical occlusion, tumor cell enters a vessel whose diameter is less than the circulating tumor cell, or cell adhesion, tumor cells adhere to the vessel wall through the formation of specific bonds\(^\text{15,16,17,18}\). Circulating tumor cells may associate with platelets to escape immune surveillance and promote their egress from the circulatory system through adhesion to the vessel walls. These platelets release an array of active compounds, such as vascular endothelial growth factor (VEGF), that promote vascular hyperpermeability and extravasation\(^\text{19,20,21}\).
1.1.3 Growth of secondary tumor

The site for metastases can be explained by two hypotheses. The ‘seed and soil’ hypothesis suggests that tumor cells will metastasize to site where the local environment is favorable\(^22\). The ‘mechanical’ hypothesis suggests that metastasis occurs at sites based on the pattern of blood flow\(^23\). Both these hypotheses are thought to have complementary roles in influencing the location of a metastatic site. Studies have also demonstrated that the active compounds secreted by the platelets can stimulate growth at metastatic sites\(^24\).

1.2 Tumor cell proliferation

Uncontrolled proliferation is a hallmark of cancer that leads to the development of primary tumors. Normal tissues carefully control the production and release of growth-promoting signals that instruct entry into and progression through the cell growth and-division cycle. This ensures homeostasis of cell number and thus maintenance of normal tissue architecture and function\(^26\). Tumor cells deregulate these signals and proliferate aggressively\(^25\). The enabling signals are conveyed in large part by growth factors that bind cell-surface receptors, which typically contain intracellular tyrosine kinase domains\(^26\).

1.2.1 The relationship between tumor cell proliferation and migration

Tumor cell proliferation and migration are two important processes in the metastatic cascade that are regulated by complex interactions of multiple pathways. Thus, these two processes can either occur concurrently or divergently of each other. Some studies have shown that these two processes occur simultaneously; proliferation and migration are both stimulated by secreted factors such as Fibroblast Growth Factors (FGFs)\(^27,28\). While other studies suggest that the two processes are mutually exclusive; primary tumor cells proliferate uncontrollably with tight cell-cell junctions and low mobility, while metastatic invasive tumor cells seem to delay proliferation during migration. Previous studies have also suggested that proliferative and migratory tumorigenic cells display two distinct transcriptional signatures\(^29,30,31,32,33\).
1.2.2 The role of epidermal growth factor in tumor cell proliferation

While different families of growth factors and growth factor receptors have been implicated in the autonomous growth of cancer cells, epidermal growth factor (EGF) has been shown to play a central role in the pathogenesis and progression of different types of cancers\textsuperscript{34,35}. Epidermal growth factor is part of a complex network of growth factors and receptors that together help to modulate the growth of cells. Many aggressive types of cancer have overactive signaling through the epidermal growth factor system. They either create excess amounts of the growth factor or develop mutant forms of the receptor that are unnaturally active\textsuperscript{36}.

EGF binds to its receptor EGFR (also known as ErbB-1 or HER-1) to stimulate a signaling cascade. The EGFR family of receptor tyrosine kinases consists of the EGFR (ErbB1), ErbB2/HER2/neu, ErbB3/HER3, and ErbB4/HER4. In cancer, EGFR is often observed to be perpetually stimulated due to the constant production of ligands in the tumor microenvironment or as a result of a mutation in the receptor itself that causes it to be constitutively active\textsuperscript{37,38}. Given its importance in hyperplastic proliferation, EGFR is a commonly targeted with cancer therapeutics. Currently there are several FDA-approved drugs that target EGFR that are used to treat cancer including breast, pancreatic, colorectal, and non-small cell lung cancer (NSCLC)\textsuperscript{39}. These therapeutics include:

- Gefitinib
- Erlotinib
- Lapatinib
- Cetuximab

1.3 Tumor cell migration

During detachment, following EMT, tumor cells adopt a migratory phenotype that allows them to digest to and move through the ECM\textsuperscript{40}. Conventional studies on the tumor cell migration have been conducted on 2D, flat surfaces, that do not mimic \textit{in vivo} conditions\textsuperscript{41,42,43,44}. Recent studies that have been conducted in 3D collagen I matrices
recapitulate the collagen rich environment that migrating cells are exposed to in vivo and provide new insights into cellular dynamics of migration\textsuperscript{13,14,45}.

### 1.3.1 Single-cell migration

Single-cell migration is the best studied mechanism of cell movement and has been implicated in many physiologically relevant processes such as development, immune surveillance and cancer metastasis. This migration allows cancer cells break away from the primary tumor and invade healthy tissue\textsuperscript{46}. During single-cell migration, tumor cells are predominantly exposed to the ECM which mainly consists of collagen I\textsuperscript{47}. Single-cell migration in 3D collagen I environments is directly influenced by the generation of pseudopodial protrusions that are readily modulated by the focal adhesion components. Focal adhesions are composed of clustered integrins that physically and dynamically connect the cellular actin network to ECM fibers\textsuperscript{13}.

Furthermore, studies have shown that protrusions generated in 3D environments are multigenerational and dendritic and are structurally and functionally distinct from well-characterized invadopodia that stem from the basal surface of cancer cells placed on the surface of soft gels. Protrusions emerging directly from the cell body and prolonging the nucleus (which we call mother protrusions/0\textsuperscript{th} generation) are specifically regulated by focal adhesion proteins FAK, talin, and p130Cas, the formation of dendritic protrusions (daughter protrusions) that stem from mother protrusions are regulated by the Arp2/3 complex and associated proteins N-WASP, WAVE1, cortactin, Cdc42, and VASP. (Figure 1.2) The rate of generation (not the length) of daughter protrusions, and associated degree of branching from the mother protrusions, predicts cell speed in 3D matrices.\textsuperscript{14}
1.3.2. Collective cell migration

Collective cell migration is the coordinated migration of a group of cells during which cells are influenced by physical or biochemical interactions with their neighbors. Collective movements have been observed during development, wound healing, and invasion in cancer cells. This type of migration differs from single cell migration in that the cells remain physically and functionally connected to each other. Collective cell migration is also observed in bacterial cells that can produce and sense signal molecules, allowing the whole population to perform a cell-density-dependent concerted action. These bacterial cells do not need to be in contact to perform this concerted action.

1.4 Role of the tumor microenvironment on tumor cell migration

Cancer cells in the tumor microenvironment (TME) can secrete proteins, such as growth factors and cytokines, together with stromal and immune cells in a collagen-rich 3D extracellular matrix, mediate intercellular communications and collectively modulate pathophysiological processes, including cancer-induced angiogenesis, de-differentiation, and metastasis. For instance, the highly invasive nature of human brain tumors such as glioblastoma multiform has been attributed to its unique secretomic profile. However, secretomic profiles evolve as cancer cells proliferate and eventually progress to a higher grade (more invasive), suggesting a possible role for secreted paracrine...
factors to couple proliferation and migration. As the local concentration of secreted proteins increases with cell number, we speculate that the contribution of proliferation-induced local crowding (accompanied by increased local cell density) in the collagen-rich 3D TME, may be a significant, yet largely unidentified factor that directly alters tumor cell migration\textsuperscript{59}. 
CHAPTER 2: MECHANISTIC COUPLING OF TUMOR CELL PROLIFERATION AND MIGRATION

2.1 Introduction

Proliferation and migration, which are two key drivers of metastasis, can occur concurrently or divergently of each other. Considering previous findings on tumor cell migration and proliferation and the involvement of the tumor microenvironment, I hypothesized that as tumor cells proliferate and increase local cell density the secretomic profiles of tumor cells evolve which may in turn regulate tumor cell migration. This chapter explores the mechanistic coupling of tumor cell migration and proliferation through local cell density.

2.2 Materials and methods

2.2.1. Cell line and cell culture

Human fibrosarcoma HT1080 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone Laboratories), and 0.005% (w/v) gentamicin (Quality Biological). Human breast carcinoma MDA-MB-231 cells (ATCC) and MCF-7 cells (ATCC) were cultured in DMEM (Mediatech) supplemented with 10% FBS (Hyclone). Human glioblastoma U-87 MG cells (ATCC) were cultured in DMEM (Mediatech) supplemented with 10% FBS (Hyclone). Human diploid cell line, WI-38, (ATCC) were cultured in Eagle's minimal essential medium (EMEM, Mediatech) supplemented with 10% FBS (Hyclone). Human breast epithelial MCF10A cells (ATCC) and MCF12A cells (ATCC) were cultured in DMEM supplemented with 5% horse serum (Atlanta biologicals), 20 ng/ml Human epidermal growth factor (Sigma-Aldrich),100 ng/ml cholera toxin, (Sigma-Aldrich) 0.01 mg/ml bovine insulin (Life technologies), and 500 ng/ml hydrocortisone (Sigma-Aldrich). HT1080 cells transfected with shRNAs (see below) were grown in medium containing 1 µg/ml puromycin. The cells were maintained at 37°C and 5% CO₂ in a humidified incubator during cell culture and during live-cell microscopy.
2.2.2 3D collagen I matrix

HT1080 cells were embedded in 2mg/ml type I collagen gel as described previously by Fraley et al.\textsuperscript{13} Briefly, cell suspensions containing 5,000 to 75,000 cells in 1:1 (v/v) ratio of cell culture media and reconstitution buffer were mixed with appropriate volume of soluble rat-tail collagen I (Corning Inc.) to obtain a final collagen I concentration of 2 mg/ml. A calculated amount of 1M NaOH was added quickly and the final solution was mixed well to bring the pH to \( \sim 7 \). The cell suspension was added to a 24-well coverslip-bottom cell-culture dish and immediately transferred to an incubator maintained at 37°C to allow polymerization. Fresh medium was added 1 h before imaging. MDA-MB-231 and U-87 cells were embedded in 1mg/ml type I collagen matrix. The data displayed for each cell line was obtained from three independent experiments with a minimum of two replicates.

2.2.3 Speed and protrusion topology of matrix embedded cells

Phase-contrast images of matrix-embedded cells were recorded 2 min apart for 16.5 h using a Cascade 1K CCD camera (Roper Scientific) mounted on a Nikon TE2000 microscope with a 10X objective lens. Single cells were tracked using Metamorph imaging software. A custom MATLAB program calculated the velocity for each cell using the \( x \)- and \( y \)-coordinates obtained from tracking data using the following equation:

\[
\text{Speed} = \sqrt{\frac{\langle [x(t+\Delta t)-x(t)]^2 + [y(t+\Delta t)-y(t)]^2 \rangle}{t}}.
\]

For the characterization of protrusion topology, the movies were used to count the total number of mother protrusions, and the number of first-, second-, and third-generation protrusions generated by the cell (e.g. Fig. 1, C). The protrusions emanating directly from the cell body, even when split, were termed mother protrusions; protrusions originating from the mother protrusions were termed first-generation, and so on. Mitotic cells were not included in the measurements.
2.2.4 Proliferation assays

HT1080 cells were embedded in type 1 3D collagen matrices in increasing cell numbers from 5,000 to 60,000 cells. Phase contrast images of the cells were recorded 8 min apart for 48 h. The average doubling time was obtained by measuring the time between the 1st and 2nd divisions. Cell viability assay using Prestoblue (Invitrogen) was also conducted on the matrix-embedded cells of increasing cell number. Fluorescence was measured every 6h for 48h. The data displayed was obtained from three independent experiments with a minimum of two replicates for each experiment.

2.2.5 Collagen inter-fiber spacing and alignment

Matrix embedded cells with cell densities ranging from 10 cells/mm$^3$ to 150 cells/mm$^3$ were imaged and analyzed according to the methods highlighted in Fraley et al. to determine the inter-fiber spacing and alignment. The data displayed was obtained from three independent experiments with a minimum of two replicates for each experiment.

2.2.6 Condition medium and high throughput secretomic analysis

Matrix-embedded cells with cell densities ranging from 10 cells/mm$^3$ to 150 cells/mm$^3$ were incubated for 24h at 37°C in a humidified incubator. The conditioned medium from the cells was then collected and filtered through a 0.45-µm filter (Millipore) to remove cell debris. High throughput secretomic analysis was conducted on the condition medium collected as described previously by Lu et al.

Conditioned medium from HT1080 cells embedded in matrices with a cell density of 50 cells/mm$^3$ was added to freshly made matrices with a cell density of 10 cells/mm$^3$. These conditions were replicated when extracting conditioned medium from the HT1080 transfected cells. The data displayed was obtained from three independent experiments with a minimum of two replicates for each experiment.
2.2.7 Gain of function and antibody blocking assays

Recombinant IL-6 and IL-8 (R&D systems) reconstituted in DPBS (Life technologies) were added to matrix embedded cells with a cell density of 10 cells/mm³ and imaged as described above (See velocity and protrusion topology of matrix embedded cells). Matrix embedded cells with cell densities of 10 cells/mm³ and 50 cells/mm³ were exposed to specific IL-6 (Proteintech; catalog # 21865-1-AP) and IL-8 (Proteintech; catalog # 60141-2-Ig) functional antibodies, at a concentration of 0.5µg/mL. The data displayed was obtained from three independent experiments with a minimum of three replicates for each experiment.

2.2.8 Depletion of proteins with shRNAs

HT1080 cells were transfected as previously described in Giri et al. shRNA constructs targeting the Interleukin 6 and Interleukin 8 genes were purchased from Sigma Aldrich. After lentiviral-mediated transduction, Enzyme Linked Immunosorbent Assays (ELISA) and western blots were performed and only shRNAs showing more than 85% knockdown were used for subsequent studies. They include:

<table>
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</tr>
<tr>
<td>IL-8 sh232053</td>
<td>TGCGCCAACACAGAAATTATTCTCGAGAATAATTTCTGTGTTGGCGCA;</td>
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</tbody>
</table>

*Table 1: shRNA constructs targeting Interleukin 6 and Interleukin 8*

The transfected cells were embedded in type I collagen matrices and incubated overnight at 37°C and 5% CO₂ in a humidified incubator. The conditioned media from the cells were collected and filtered through a 0.45-µm filter (Millipore) to remove cell debris. The total quantity of IL-6 and IL-8 produced by the cells were measured using Human quantikine ELISA kits (R&D systems). The data displayed was obtained from three independent experiments with a minimum of three replicates for each experiment.
2.2.9 Statistics

The mean values ± SE were calculated and plotted using GraphPad Prism software (GraphPad Software). One-way ANOVA test was performed to determine statistical significance, which is indicated in the graphs using a Michelin grade scale ***p<0.001, **p<0.01, and *p<0.05.

2.3 Results

2.3.1 Proliferation of tumor cells enhances their motility

To assess the potential effect of cell proliferation – and associated increase in local cell density - on cancer cell migration *in vitro*, human fibrosarcoma HT1080 cells, a cell line commonly used in studies of cell migration\textsuperscript{60,61,62,63}, were embedded in three dimensional (3D) type I collagen matrices. Collagen I is not only the main extracellular matrix component of connective tissues, but is also enriched in the vicinity of carcinoma and sarcoma tumors\textsuperscript{9}. Cell migratory patterns within the matrix were monitored for 16.5h using live-cell phase-contrast microscopy at a rate of a 30 frames/h every other day for 5 days. This analysis revealed that fibrosarcoma cells became progressively more motile as cells proliferated and increased local cell density (Figure 2.1, A - C).
To investigate the role of increased cell density on cancer cell migration, we analyzed how increasing cell density in matrices would modulate cell migration. The initial cell densities used in the experiments, ranging from 10 cells/mm$^3$ to 120 cells/mm$^3$, corresponded to average cell-to-cell distances from 470 to 130 µm in the 3D matrix, which were all significantly larger than the average cell size (10-20 µm in diameter)$^{64}$. The data reveals that cells became progressively more motile as cell density increased. This enhanced motility cannot be attributed to repulsive cell-cell interaction as the tracked cells did not come in contact with any other cells. Cell speed eventually plateaued for cell densities higher than 100 cells/mm$^3$. Similar trends were observed for migration parameters such as persistence and invasive distance (Figure 2.2, A - D).

Figure 2.1: Proliferation of tumor cells enhances their motility. A. Phase contrast micrographs demonstrate confluence of human fibrosarcoma cells (HT1080WT) days after initial seeding. Scale bar, 100 µm. B. Cell speed measured at a time lag of 2min days after initial seeding. C. Average distance to nearest cell (dR) relates density at different days to initial seeding density. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA)
We have previously shown that cell motility in 3D matrices is predicted by the ability of cells to form dendritic protrusions\(^{14}\). Consistent with these observations, we found that the total number of main and dendritic protrusions generated per unit time by tumor cells steadily increased and then plateaued with cell density. The cell-density-dependent number of protrusions generated by the cells in 3D matrix strongly correlated with the cell-density-dependent cell velocity (Figure 2.3, A-C).

Figure 2.2: Effect of cell density on tumor cell migration. A. Randomly selected trajectories of human fibrosarcoma cells (HT1080WT) under different seeding densities of 10, 50, 120 cells/mm\(^3\) embedded in a 3D collagen matrix. Phase contrast micrographs demonstrate the confluence at each density. Scale bar, 100 µm. B-D. Cell speed, persistence distance, and invasive distance measured at a time lag of 2 min at different seeding densities. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001.
This remarkable relationship between tumor cell density and cell migration was also found in human metastatic carcinoma cells (MDA-MB 231) and human metastatic glioblastoma cells (U-87). (Figure 2.4 A-C) Similar to fibrosarcoma cells, the migration of these two tumorigenic, metastatic/invasive cell lines increased with cell density. In contrast, cell-density-dependent migration was not observed in tumorigenic, non-metastatic carcinoma cells (MCF7) and non-tumorigenic cell lines, including WI-38 human lung fibroblasts and MCF10A human epithelial cells. (Figure 2.4 D-F)

**Figure 2.3: Cell-density-dependent protrusion activity.** A. Topology of protrusions for cells embedded in 3D collagen matrices: 0th generation protrusions (N₀) originate from the cell body, 1st generation protrusions (N₁) stem from N₀ and 2nd generation protrusions (N₂) stem from N₁. B. Relationship between protrusion frequency and cell density: Protrusion frequency increases with cell density and plateaus at a threshold density of 50 cells/ mm³. C. Cell speed and protrusion frequency are highly correlated. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA)
It is noteworthy that enhanced migration mediated by cell density in cancer cells did not occur when cells were placed on two-dimensional (2D) collagen-coated substrates (Figure 2.5). Moreover, in contrast to 3D cell migration, the proliferation of cells in 3D matrices was unaffected by cell density (Figure 2.6), i.e. cells continued to proliferate at a constant rate regardless of cell density. These results indicate that cancer cell density enhances cancer cell migration, but not proliferation, and that cell-density-dependent migration could be unique to tumorigenic, metastatic cells in 3D microenvironments.
2.3.2. Cell migration enhancement is not caused by extracellular matrix remodeling

Enhancement of cell migration through increased cell density could be mediated by the collagen matrix and cell-induced matrix remodeling. We investigated if cell density modulated the microstructural properties of the 3D collagen I matrix, such as interfiber spacing (effective pore size) and local fiber alignment. Using reflection confocal microscopy, we determined that local fiber alignment showed poor correlation with cell density.
density and speed. Average interfiber spacing showed a poor correlation with cell speed as well. Interestingly, a strong negative correlation was identified between average interfiber spacing and cell density, which is expected since cell density increases the forces exerted on the collagen fibrils by the cells, causing the space between collagen fibrils to decrease. (Figure 2.7) Based on this result alone, we would have expected cell speed to decrease with increasing cell density in the matrix. Since we observed that cancer cells move faster as cell density increases, this physical property of the collagen matrix cannot modulate cell-density-dependent migration. In sum, our observed enhanced cell migration for increasing cell density cannot be attributed to changes in the physical properties of the matrix.

**Figure 2.7: Physical cues do not contribute to cell-density-dependent migration:** A. Reflection confocal micrographs of 3D collagen matrices Scale bar, 10 µm B. Correlation plot of fiber alignment vs. cell density. C. Correlation plot of cell speed vs. fiber alignment. D. Correlation plot of average interfiber spacing vs. cell density. E. Correlation plot of cell speed vs. average interfiber spacing. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA)

### 2.3.3 Secretomic profiles of matrix-embedded cells and recapitulation

Based on these results, we hypothesized that cell-density-dependent migration was regulated by soluble molecules secreted by the cells in a cell-density-dependent manner. To test this hypothesis, we introduced conditioned medium collected from a matrix containing a high density of HT1080 cells (50 cells/mm³) into a matrix containing a low density of HT1080 cells (10 cells/mm³). We found that enhanced cell velocity observed at high cell density could be recapitulated at a low cell density by adding conditioned medium collected from high cell density matrices (Figure 2.8 A,B). This result indicated
that soluble molecules secreted by cancer cells in the matrix are sufficient to promote enhanced cell migration.

To identify the soluble factor(s) driving enhanced motility, we measured and analyzed the secretomic profiles of HT1080 and MDA-MB 231 cells embedded at low and high densities in 3D matrices, using a multiplex antibody microarray assay\textsuperscript{66}, which simultaneously measures the concentration of 24 soluble molecules in ~20uL of medium. We found that cytokines IL-6 and IL-8 were both secreted in relatively high concentrations and increased linearly with cell density for both HT1080 and MDA-MB 231 cells. Remarkably, all other secreted proteins that were assayed including vascular endothelial growth factor (VEGF), which is a key mediator of angiogenesis in cancer\textsuperscript{67}, and hepatocyte growth factor (HGF), which is known to contribute to tumor progression and tumor metastasis in several cancers\textsuperscript{68}, were not elevated at higher cell densities during our experimental time window. (Figure 2.8 C,D) Using ELISA, we confirmed our results and determined the precise concentrations of IL-6 and IL-8 at specific cell densities of matrix-embedded HT1080 and MDA-MB 231 cells. (Figure 2.8 E-H) Together, this result suggests that IL-6 and IL-8 drive density-dependent cell migration in 3D matrices.
Figure 2.8: Identification of biochemical cues: A. Method to prepare condition medium: medium is incubated for 24h with a collagen matrix containing a high density of cells, 50 cells/mm$^3$ (HD), which is then filtered using a 0.45-µm filter, and added to a matrix containing a low density of cells, 10 cells/mm$^3$ (LD). B. The addition of conditioned medium (CM) from a matrix containing a high cell density (HD) increases the speed of cells in a matrix containing a low cell density (LD). The HD cell speed in the presence of fresh medium (FM) is recapitulated in LD when using CM. C. Secretomic analysis of CM harvested from human fibrosarcoma cells indicates that levels of interleukin 6 (IL-6) and interleukin 8 (IL-8) increase as a function of HT1080 cell density in the matrix, while levels of other major cytokines do not significantly change. D. Secretomic analysis of conditioned medium from human breast carcinoma cells (MDA-MB-231) confirms our observations with HT1080 cells. E and F. Increasing density of human fibrosarcoma cells in the matrix increases the concentrations of secreted IL-6 (A) and IL-8 (B), as analyzed by ELISA. G and H. Increasing cell density of human carcinoma cells in the matrix increases the concentrations of secreted IL-6 (A) and IL-8 (B), as analyzed by ELISA. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA).
2.3.4. IL-6 and IL-8 together are necessary and sufficient to induce enhanced cell migration

Next, we systematically assessed whether IL-6 and IL-8 were required to drive cell-density-enhanced migration by conducting gain-of-function and loss-of-function experiments. We exposed HT1080 cells seeded at a low density in the matrix to controlled concentrations of human recombinant IL-6 and IL-8. We found that IL-6 or IL-8 alone had no effect on cell migration, even at high concentrations. In contrast, IL-6 and IL-8, when combined at the prescribed concentrations found at the high density of 50 cells/mm$^3$ in the precise stoichiometric ratio of 5:2, induced cells at low density to move at the high velocity observed at high cell density and also detected for cells at low density exposed to conditioned medium. (Figure 2.7 A-C) Strikingly, other stoichiometric ratios of IL-6 and IL-8 did not induce the enhanced migration (Figure 2.7 D-F). These results indicate that a mixture of IL-6 and IL-8 is sufficient to recapitulate the enhanced migration of cells embedded at high densities in matrices.
To verify that both cytokines were required to enhance cell-density-dependent migration, we conducted experiments with conditioned medium from HT1080 cells depleted of IL-6 or IL-8 via shRNA induction. Depleting either IL-6 or IL-8 prevented the conditioned medium from high density matrices to enhance cell migration of low density matrices. (Figure 2.8 A) Similar results were obtained when we utilized specific neutralizing antibodies to block secreted IL-6 and IL-8. The loss-of-function assays conducted with matrix-embedded cells at low and high cell densities exposed to specific neutralizing antibodies and with HT1080 cells depleted of IL-6 and IL-8 through shRNA (over 70% depletion) and embedded in 3D matrices demonstrated that the cell-density-dependent migration patterns observed previously were no longer detected. (Figure 2.8 B-E)

Figure 2.9: Determination of functional influence using gain of function assays: A and B. The addition of recombinant IL-6 alone or recombinant IL-8 alone do not increase cell speed. C and D. The addition of high concentrations of recombinant IL-6 alone or recombinant IL-8 alone do not increase cell speed. E. The addition of recombinant IL-6 and IL-8 in combination at the precise concentrations found in a matrix containing a high density of 50 cells/mm$^3$ (RM) recapitulates the high speed observed of human fibrosarcoma cells at high densities. F. The addition of recombinant IL-6 and IL-8 in combination at ratios other than 5:2 does not induce the high speed observed at high densities. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA).
Figure 2.10: Determination of functional influence using loss of function assays: A. Decreased speed at LD ($\rho =10$) where cells are exposed to conditioned medium produced by IL-6 and IL-8 knockdown cells and conditioned medium obtained from a matrix containing a high cell density (HD) following exposure to specific IL-6 and IL-8 functional antibodies compared to control cells exposed to conditioned medium from wild type cells at HD ($\rho =50$). B. Decreased velocity of cells exposed to specific IL-6 and IL-8 functional antibodies at LD ($\rho =10$) and HD ($\rho =50$) compared to untreated control cells. C. Decreased speed of the IL-6 and IL-8 knockdown cells at LD ($\rho =10$) and HD ($\rho =50$). D and E. Decreased expression of IL-6 and IL-8 in shRNA induced knockdowns. In all panels, data is represented as mean ± s.e.m. *$P<0.05$; **$P<0.01$; ***$P<0.001$(ANOVA).

These results were confirmed with MDA-MB-231 cells embedded in 3D matrices (Fig. 3F). Interestingly, as with HT1080 cells, the enhanced cell migration of MDA-MB-231 cells was observed when IL-6 and IL-8 were present in the stoichiometric ratio of 5:2 (Fig S3D). In marked contrast, tumorigenic, non-metastatic carcinoma cells (MCF7) and non-tumorigenic cells MCF10A human epithelial cells exposed to both IL-6 and IL-8 did not exhibit enhanced migration. (Fig. S3E and F) These results suggest that IL-6 and IL-8 are each individually required but only sufficient in combination to induce enhanced migration in tumorigenic, metastatic cells. (Fig. 3G).
2.3.5 IL-6 and IL-8 is sensed by the cells via a paracrine pathway

Our findings indicate that enhanced migration through the synergistic signaling of IL-6 and IL-8 is sensed by the cells via a paracrine pathway through the receptors of IL-6 (IL-6R) and IL-8 (IL8R1/CXCR1 or IL8R2/CXCR2). Matrix-embedded cells are exposed to a gradient of secreted proteins that can readily build up around a cell and consequently paracrine signaling can occur as the inter-cellular distance is decreased with an increase in cell density. As a result, the paracrine signaling could trigger a response in cellular behavior (e.g., enhanced migration) or the production of a second wave of cytokines to modulate cell density. Our results demonstrated that the expression of IL-6R and CXCR2...
indeed increased as cell density increased, indicating that signaling pathway is paracrine (Figure 2.12).

![Figure 2.12: Paracrine pathway activated through the receptors of IL-6R and IL-8R: A and B. The expression of IL-6R and CXCR2 (IL-8R) is upregulated under high density conditions. The In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA).]

2.4 Discussion

The results of this study suggest that as cancer cells proliferate and local cell density increases accordingly, the secretion profile of cancer cells in the TME may be dynamically altered and this may play an important role in metastasis. Increased local cell density directly enhances cell migration in metastatic cells embedded in 3D matrices by increasing IL-6 and IL-8 levels. Interestingly, cell-density-dependent migration is unique to tumorigenic, metastatic cells exposed to 3D microenvironments, not 2D flat surfaces, which reconstitute features of tissues that enable in vitro recapitulation of in vivo function including spatiotemporal gradients of biochemical cues such as cytokines, chemokines and growth factors.

This study identified a novel synergistic mechanism between the cytokines IL-6 and IL-8 required to promote cell-density-dependent migration. IL-6 is a pleiotropic cytokine that has been associated with tumor progression and metastasis in different types of cancers, including prostate and breast cancer. IL-8 has also been implicated in promoting angiogenesis and tumorigenicity, promoting the cancer stem cell phenotype, and enhancing metastasis in multiple cancer types. Unlike Interleukin 1(IL-1) and
Tumor Necrosis Factor (TNF), clinical data indicate that both IL-6 and IL-8 are found at high concentrations in serum of patients with lung and liver metastases\textsuperscript{76}, which suggests that they may play a critical role in metastasis. Previous clinical studies have also shown that the serum concentrations of these two cytokines strongly correlate with the stage of cancer\textsuperscript{77}. The results of this study explain the underlying mechanisms that account for these observations in three-dimensional (3D) type I collagen-rich microenvironments that simulate \textit{in vivo} conditions, where tumor cells rapidly proliferate and increase local cell density\textsuperscript{57,42,78}.

Our findings also indicate that metastatic cancer cells are able to independently produce IL-6 and IL-8 and do not require other cells in the TME such as fibroblastic stromal cells to supply them with the secreted proteins required to metastasize\textsuperscript{70}. This data also suggests that the presence of a feedback signaling mechanism that is able to regulate many secreted molecules keeping them in check. However, the production of IL-6 and IL-8 is not regulated by this feedback signaling mechanism and continues to be produced as cell density increases similar to a cell-autonomous process to develop invasive tumors although that interestingly occurs through paracrine signaling.

\textbf{2.5 Summary}

Tumor cell proliferation and migration can be mechanistically coupled in the tumor microenvironment through local cell density by the synergistic paracrine signaling mechanism of Interleukin 6 and Interleukin 8. This coupling is observed in metastatic sarcoma, carcinoma, and glioblastoma cells but not in normal and non-metastatic cells.
CHAPTER 3: NOVEL SYNERGISTIC MECHANISM AND THERAPEUTIC INTERVENTION

3.1. Introduction

Considering that Interleukin 6 (IL-6) and Interleukin 8 (IL-8) together enhance tumor cell migration, we sought to map out the specific signaling pathway using RNA sequencing, PCR techniques, and inhibitory studies. RNA sequencing uses next-generation sequencing to reveal the presence and quantity of RNA in a biological sample at a given moment in time. We also explored possible therapeutic targets to decrease metastatic capacity by inhibiting this cell-density-dependent signaling pathway. The receptors of IL-6 (IL-6R) and IL-8 (IL-8R) on tumor cell membranes are also important drivers in such pathways and thus could be targeted to inhibit paracrine signaling.

Tocilizumab is a recombinant humanized anti-human IL-6 receptor monoclonal antibody currently used in the treatment of Rheumatoid Arthritis and has been clinically studied in its efficacy against recurrent ovarian cancer. Reparaxin is an inhibitor of the IL-8 receptor which is currently being evaluated for safety, tolerability, pharmacokinetics, and detection of early signs of antitumor activity in breast cancer patients. However, the potential of these agents as anti-metastasis therapeutics in highly invasive cancers has not been previously studied in vivo systems. This chapter explores a combination of pharmacological agents for potential therapeutic interventions against cell-density-dependent migration.

3.2 Materials and methods

3.2.1 Cell lines and culture

Human fibrosarcoma HT1080 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone Laboratories), and 0.005% (w/v) gentamicin (Quality Biological). Human breast carcinoma MDA-MB-231 cells (ATCC) were cultured in DMEM (Mediatech) supplemented with 10% FBS (Hyclone). HT1080 and MDA-MB-231 cells transfected
with shRNAs (see below) were grown in medium containing 1 µg/ml puromycin. The cells were maintained at 37°C and 5% CO₂ in a humidified incubator during cell culture and during live-cell microscopy.

### 3.2.2 Depletion of receptors with shRNAs

HT1080 and MDA-MB-231 cells were transfected as previously described in Giri et al. shRNA constructs targeting the Interleukin 6 Receptor and Interleukin 8 Receptor genes were purchased from Sigma Aldrich. After lentiviral-mediated transduction, western blots were performed and only shRNAs showing more than 85% knockdown were used for subsequent studies. They include:

<table>
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<th>Receptor</th>
<th>shRNA Name</th>
<th>Sequence</th>
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<td>sh289773</td>
<td>CCGGCCAGTCCAGATATTTCACATTCTCGAGAATGTGAAATATCTGGACTGG;</td>
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<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-8R</td>
<td>sh378365</td>
<td>CCGGGAAGCGCTACTTGGTGCAATTCTCGAGAATTTGACCAAGTACGCTTCTT</td>
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</table>

**Table 2:** shRNA constructs targeting the receptors of Interleukin 6 and Interleukin 8

The data displayed was obtained from three independent experiments with a minimum of two replicates for each experiment.

### 3.2.3 3D Collagen I matrix

HT1080 cells were embedded in 2mg/ml type I collagen gel as described previously by Fraley et al. Briefly, cell suspensions containing 5,000 and 25,000 cells in 1:1 (v/v) ratio of cell culture media and reconstitution buffer were mixed with appropriate volume of soluble rat-tail collagen I (Corning Inc.) to obtain a final collagen I concentration of 2 mg/ml. A calculated amount of 1M NaOH was added quickly and the final solution was mixed well to bring the pH to ~7. The cell suspension was added to a 24-well coverslip-bottom cell-culture dish and immediately transferred to an incubator maintained at 37°C to allow polymerization. Fresh medium was added 1 h before imaging. MDA-MB-231 cells were embedded in 1mg/ml type I collagen matrix with cell suspensions containing...
5,000 and 50,000 cells. The data displayed for each cell line was obtained from three independent experiments with a minimum of two replicates.

3.2.4 STAT3 and WASF3 activity

Matrix embedded cells with cell densities of 10 cells/mm$^3$ and 50 cells/mm$^3$ were incubated for 24h at 37°C in a humidified incubator. The matrices were exposed to cell lysis buffer and mechanically broken down using a syringe. The suspension was centrifuged and the supernatant was measured for STAT3 and PhosphoSTAT3 using an ELISA kit (Abcam). WASF3 expression was measured using qRT-PCR for the cell densities stated previously and matrix embedded cells with a cell density of 10 cells/mm$^3$ exposed to IL-6 and IL-8 alone and in combination at the precise concentrations found at a high cell density of 50 cells/mm$^3$. Total RNA isolation was performed with RNA MiniPrep kit (Zymo research). cDNA synthesis was carried out as previously described by Gilkes et al$^{83}$. The sequence for the cDNA primers that were used during PCR are found in the table below. The data displayed was obtained from three independent experiments with a minimum of three replicates for each experiment.

<table>
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<th>HS-18S-FOR</th>
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</tr>
</tbody>
</table>

*Table 3: Sequence of the cDNA primers of 18s, WASF3, and ARPC2*
3.2.5 Transcriptome analysis by RNA sequencing and analysis of RNA-Seq data

Total RNA isolation was performed as described in the previous section. cDNA synthesis, sequencing and analysis of RNA-Seq data were conducted as previously described by Guo, Shangqin, et al⁸⁴

3.2.6 Speed and protrusion topology of matrix embedded cells

Phase-contrast images of matrix-embedded cells were recorded 2 min apart for 16.5 h using a Cascade 1K CCD camera (Roper Scientific) mounted on a Nikon TE2000 microscope with a 10X objective lens. Single cells were tracked using Metamorph imaging software. A custom MATLAB program calculated the velocity for each cell using the x- and y-coordinates obtained from tracking data using the following equation:

\[
\text{Speed} = \frac{\sqrt{\langle [x(t + \Delta t) - x(t)]^2 + [y(t + \Delta t) - y(t)]^2 \rangle}}{t}
\]

For the characterization of protrusion topology, the movies were used to count the total number of mother protrusions, and the number of first-, second-, and third-generation protrusions generated by the cell (e.g. Fig. 1, C). The protrusions emanating directly from the cell body, even when split, were termed mother protrusions; protrusions originating from the mother protrusions were termed first-generation, and so on. Mitotic cells were not included in the measurements.

3.2.7 Inhibitor assays

Matrix embedded cells with low and high cell densities were exposed to IL-8R inhibitor, Reparixin (Cayman Chemical), IL-6R inhibitor, Tocilizumab (Genentech), JAK2 inhibitor, AG-490 (Santa Cruz Biotechnology), STAT3 inhibitor, S3I-201, (Santa Cruz Biotechnology) and Arp2/3 complex inhibitor, CK 666, (Sigma-Aldrich) for 1h before cells were imaged as described above. (See velocity and protrusion topology of matrix embedded cells) Cell viability assays using Prestoblue (Invitrogen) were also conducted on matrix embedded cells exposed to Reparixin and Tocilizumab. The data displayed was obtained from three independent experiments with a minimum of three replicates for each experiment.
3.2.8 *In vivo* mouse work

Studies using 5-7 week-old NSG (NOD SCID Gamma) mice were carried out according to protocols approved by the Johns Hopkins University Animal Care and Use Committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All mice were housed at a temperature of 25°C under a 12-hr dark/light cycle. NSG mice were obtained from Johns Hopkins Medical Institution. Tocilizumab and saline for injection were obtained from the research pharmacy of The Johns Hopkins Hospital. Reparixin was obtained from Cayman Chemical and Med Chem Express.

MDA-MB-231 cells were harvested by trypsinization, resuspended at $10^7$ cells/mL in a 1:1 mix of PBS:Matrigel and $1\times10^6$ cells were injected into the mammary fat pad (MFP) of the mouse. After 10 days, mice received a subcutaneous injection of either 100 μL Tocilizumab alone (25 mg/kg), 300 μL Reparixin (30 mg/kg) alone or a combination of Tocilizumab and Reparixin. The control mice received 100 μL of a saline solution. Primary tumors were measured in two dimensions (a and b), and volume was calculated as $4/3\pi \times ((a \times b)/2)^3$. Mice were sacrificed after 6 weeks. Tumors were excised, weighed and processed for RNA isolation and tissue lysate preparation. Expression of STAT3, WASF3, and Arpc2 were measured using PCR methods.

Lungs were perfused with agarose. One lung was inflated for formalin fixation and paraffin embedding; the other lung was used to isolate genomic DNA for qPCR. Genomic DNA was isolated from the livers. The livers, tumors, and lymph nodes were sectioned and stained with Vimentin (Sigma; catalog # V2258) and ARPC2 (Protein tech; catalog # 10922-1-AP). Images were acquired by Nikon Eclipse NI-U and analyzed using ImageJ. Data represented for each condition was obtained from five animals.

3.2.9 Statistics

The mean values ± SE were calculated and plotted using GraphPad Prism software (GraphPad Software). One-way ANOVA test was performed to determine statistical significance, which is indicated in the graphs using a Michelin grade scale: ***p<0.001, **p<0.01, and *p<0.05.
3.3 Results

3.3.1 RNA-sequencing: distinct transcriptional phenotype is induced solely by cell density and can be recapitulated by adding recombinant IL-6 and IL-8 proteins

To confirm the formation of a more invasive and migratory phenotype solely induced by cell proliferation and increase of cell density, we performed global transcriptional phenotype analysis by RNA sequencing (RNA-seq)\(^\text{86}\). The transcriptomes of fibrosarcoma cells at low density (LD) and high density (HD) were sequenced and compared for differential gene expression. They were also compared to the transcriptomes of fibrosarcoma cells at a low density exposed to recombinant IL-6 alone (IL-6), IL-8 alone (IL-8), and IL-6 and IL-8 found in the precise concentrations at the high density of 50 cells.mm\(^{-3}\) (RM). To identify the sources of transcriptional variations caused by different conditions, we performed an ANOVA-like test to detect the genes most variable among multiple groups. To study the relationship of global transcriptomes, principle component analysis (PCA) of the top 500 most significant genes was performed. Our PCA showed that the transcriptomes of LD, IL-6, and IL-8 cluster in close proximity in the first quadrant while the high-density (HD) cells shows phenotypical shift toward the synergistic IL6/IL8 treatment (RM) in the second quadrant. RM cluster together, indicating their phenotypic similarity between cell-proliferation-induced migratory phenotype and the phenotype generated by adding IL-6 and IL-8. The shift of transcriptional phenotype induced solely by cell density increase is confirmed with differential gene expression analysis. (Figure 3.1 A)

Next, we performed Ingenuity Pathway Analysis (IPA) to investigate the biological mechanisms underlying transcriptional phenotypes by analyzing the functional annotation of differential expression gene clusters and pathway enrichment. The most enriched gene ontology category of the genes highly expressed in RM was “cell movement”. Another group of genes, which were highly expressed in IL-6, IL-8, and LD but downregulated in RM, was enriched in cellular metabolism and division-related pathways. This result suggests that RM induces a strong phenotype with enhanced cell movement. Other biological pathways contributing to the change of LD to HD cell
phenotype includes cell death and survival, cell metabolic activity, cell cycle and division. Although HD is closer to LD, IL6 and IL8 cells, there exists a phenotypic alteration towards RM, or in other words, the shift to the negative side of PCA, follows the direction of HD cells. Relatively, HD resembles this phenotype more closely than LD cells or the cultures with only IL-6 or IL-8 added (Figure 3.1 B and C).

Figure 3.1: Distinct transcriptional phenotype is induced solely by cell density and can be recapitulated by adding recombinant IL-6 and IL-8 proteins: A. Principle component analysis (PCA) of the top 500 most significant genes to determine the relationship of global transcriptomes. B and C Heat maps demonstrating the differences between gene ontology categories and tables describing biological functions affected.

3.3.1 Mechanism: protein signaling transduction pathways linking IL6/IL8 to cell migration

Signal transducer and activator of transcription 3, STAT3, is a transcription factor that is a common downstream effector in the individual pathways of IL-6 and IL-8. Therefore, we hypothesized that STAT3 could regulate cell-density-dependent migration. We found that the activity of STAT3 in HT1080 cells seeded in matrices at a high density was 2-fold higher than that of cells seeded at a low density. (Figure 3.2 A)
The Arp2/3 complex nucleates F-actin assembly and mediates dendritic protrusions required for 3D cancer cell migration\textsuperscript{14}. Further, previous studies and clinical annotations of Arp2/3 associated with genomic expression data from breast cancer patients also demonstrates that patients (n=3557) who expressed higher levels of the Arp2/3 had a lower survival rate than those who expressed lower levels of the complex\textsuperscript{89,90,91,92,93,94}. Thus, we reasoned that cell-density-dependent migration may be regulated by the Arp2/3 complex through the Janus kinase JAK/STAT3 pathway. Thus, we examined the migration of cells at low and high cell densities exposed to the specific JAK2 inhibitor AG-490\textsuperscript{95}, STAT3 inhibitor S3I-201\textsuperscript{96}, or Arp 2/3 complex inhibitor CK666\textsuperscript{97}. Through these inhibitor studies, we determined that JAK2, STAT3, and the Arp2/3 complex were indeed required for cell-density-dependent migration. Treatment with either of the three inhibitors prevented cell-density-dependent migration. (Figure 3.2 B)

Because Wiskott-Aldrich syndrome protein family member 3 (WASF3) is involved in the regulation of actin cytoskeleton dynamics through the recruitment of Arp2/3 complex\textsuperscript{98,99,100}, we hypothesized that WASF3 is an important intermediate between STAT3 and Arp2/3. As a first step, we quantified the expression of WASF3 using PCR methods and found that the mRNA level of WASF3 was increased in cells cultured at a high relative to a low cell density. We also observed that WASF3 expression of cells at low density exposed to RM was comparable to those detected at a high cell density. (Figure 3.2 C)

To further determine the role of Arp2/3 on cell-density-dependent migration, we measured protrusions and branching frequency for LD, HD, IL-6, IL-8, and RM conditions. The introduction of recombinant IL-6 or IL-8 alone did not increase protrusion frequency or branching frequency. However, the introduction of RM significantly increased protrusion frequency and branching frequency. (Figure 3.2 D and E) These results suggest that WASF3 together with the Arp2/3 complex are important regulators in the pathway that controls cell-density-dependent migration. (Figure 3.2 F)

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Interestingly, when the expression of STAT3, WASF3, and Arp2 (an important subunit of ARP2/3) were measured for differing stoichiometric ratios of IL-6 and IL-8 in HT1080WT cells, we observed that the expression of these three intermediates were maximally stimulated under ratio of IL-6: IL-8 of 5:2.
3.3.3 In vitro targeting of IL-6R and IL-8R

Fibrosarcoma and breast carcinoma cells depleted of IL-6R and IL-8R via shRNA were embedded in 3D collagen matrices. We found that the depletion of IL-6R had no effect on 3D cell migration at a low cell density. Instead, this molecular intervention suppressed cell velocity at elevated cell densities. Interestingly, cells depleted of IL-8R displayed a reduction in cell velocity at both low and high cell densities. (Figure 3.3)

Figure 3.3: Maximal stimulation of STAT3, WASF3, and ARPC2: A-C. Key intermediates in the pathway; STAT3, WASF3, ARPC2 are maximally stimulated under the IL-6: IL-8 ratio of 5:2. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001 (ANOVA).
Figure 3.4: Depletion of IL-6R and IL-8R: A and B. Decreased speed of the IL-6R and IL-8R knockdown fibrosarcoma and carcinoma cells at LD (ρ =10) and HD (ρ =50). C-F. Decreased expression of IL-6R and IL-8R in shRNA-induced knockdowns in HT1080 fibrosarcoma and MDA-MB-231 carcinoma cells. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA).

In order to determine pharmacological agents for potential therapeutic interventions, inhibitors of IL-6R (Tocilizumab) and IL-8R (Reparixin) were added to matrix-embedded cells at low and high densities; we observed that Tocilizumab induced a small decrease in fibrosarcoma cell velocity at low cell density, but induced a more visible decrease in cell velocity at high cell density. Reparixin decreased fibrosarcoma cell velocity at both low and high cell densities, with notable reduction in cell velocity at higher Reparixin concentrations. (Figure 3.4 A and B). The combination of the two inhibitors showed a decrease in fibrosarcoma cell velocity at both low and high
fibrosarcoma cell densities. (Figure 3.4 C) We observed similar effects of the inhibitors on the velocity of breast carcinoma cells. (Figure 3.4 D-F)

**Figure 3.5: Targeting IL-6R and IL-8R using pharmacological agents:** A. Cartoon depicts that Tocilizumab and Reparixin can be used to block the cognate receptors of IL-6 and IL-8. B. Individually, Tocilizumab and Reparixin decreased cell speed of human fibrosarcoma cells embedded in a 3D matrix at LD ($\rho =10$) and HD ($\rho =50$) compared to cells exposed to fresh medium (0). C. Tocilizumab and Reparixin in combination greatly decrease cell speed of fibrosarcoma cells embedded in a 3D matrix at LD ($\rho =10$) and HD ($\rho =50$) compared to cells exposed to fresh medium (0). D and E. Individually, Tocilizumab and Reparixin decreased cell speed of human carcinoma cells embedded in a 3D matrix. F. Tocilizumab and Reparixin in combination greatly decrease cell speed of carcinoma cells embedded. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA).
3.3.4 Animal models and *in vivo* experiments to test potential therapeutic strategies

The effect of these drugs on metastasis was examined by generating an animal model through the introduction of MDA-MB-231 carcinoma breast cancer cells into the mammary fat pad of NSG (NOD SCID Gamma) mice and injecting four sets of mice with saline, Tocilizumab alone (25mg/mL), Reparixin alone (30mg/mL), and Tocilizumab and Reparixin in combination every three days for 6 weeks. As predicted from our *in vitro* results, we observed that the treatment had no effect on rate of tumor growth. As also predicted from our *in vitro* results, we found that the metastases to the lungs, liver, and lymph nodes were suppressed in the treated group. Specifically, the combination of the two drugs was the most effective in repressing metastatic burden on the liver and the lymph nodes. (Figure 3.5)

![Figure 3.6: Reduction of metastatic burden using Tocilizumab and Reparixin: A. Tumor volume measured over time. B and C. Human genomic DNA content in mouse lungs and livers were quantified using qPCR to determine the metastatic burden. D. Vimentin staining of lymph nodes quantified by image analysis. E. Images of mice lungs that were stained with hematoxylin and eosin. F. Images of lymph nodes that were stained with vimentin. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001 (ANOVA).](image-url)
Moreover, the expression of the key intermediates in the synergistic pathway, STAT3, WASF3, and Arp2/3 (ARPC2 subunit), were significantly decreased in the treated group suggesting that the cell-density-dependent paracrine signaling pathway was responsible for the decreased metastases observed in the lungs, liver, and lymph nodes. Immunohistochemical staining for Arp2/3 confirmed expression in control tumors and markedly decreased expression in the tumors from mice treated with the combination of drugs. (Figure 3.6)

Figure 3.7: Cell-density-dependent paracrine signaling pathway regulates metastasis: . A, B, and C. Decreased expression of STAT3, WASF3, and Arp2/3 in treated group compared to the control group. D. Immunohistochemical staining of primary tumor sections for arp2/3. E. Arp 2/3 staining of primary tumor sections quantified by image analysis. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA).
3.4 Discussion

The results of this study further emphasize the necessity of 3D cultures in pharmaceutical studies as monolayer cell culture methods remain the de facto prevalent testing platform\textsuperscript{69}. Cells cultured on dishes adopt physiologically irrelevant morphology and signaling patterns\textsuperscript{101}. For instance, the cell-density-dependent migration seen in matrix embedded cells is not observed in cells placed on 2D substrates. In addition, there are currently no therapeutics in the market that specifically target metastasis which is responsible for 90\% of cancer related deaths\textsuperscript{102}. This study suggests that by concurrently inhibiting the identified pathway with Tocilizumab and Reparixin, metastasis can be directly targeted and decreased. Tocilizumab is a recombinant humanized anti-human IL-6 receptor monoclonal antibody currently used in the treatment of Rheumatoid Arthritis\textsuperscript{79} and has been clinically studied in its efficacy against recurrent ovarian cancer\textsuperscript{80}. Reparaxin is an inhibitor of the IL-8 receptor which is currently being evaluated for safety, tolerability, pharmacokinetics, and detection of early signs of antitumor activity in breast cancer patients\textsuperscript{81,82}. However, the potential of these agents as anti-metastasis therapeutics in highly invasive cancers has not been previously studied in vivo systems. This study suggests that simultaneous use of Tocilizumab and Reparixin could greatly decrease the metastatic capacity of tumors, thereby potentially improving cancer patient outcomes similar to Denosumab which is a human monoclonal antibody that was initially developed to treat osteoporosis\textsuperscript{103}, but later found to be effective in the treatment of bone metastases, multiple myeloma, and giant cell tumor of the bone\textsuperscript{104}.

Inflammatory signals released from infiltrating immune cells have been implicated in tumor progression, invasion and metastasis. However, our work revealed a new mechanism for tumor-secreted IL-6 and IL-8, but not common inflammatory cytokines such as TNF\(\alpha\) and IL-1\(\beta\) secreted by immune cells, to promote tumor cell migration and metastasis. These cytokines work uniquely in a cell-autonomous manner through paracrine signaling amongst the same population of cancer cells. Through this study, we have shown that blocking the activation of synergistic IL-6/IL-8 signaling pathway reduces metastatic burden in mice, suggesting a new strategy to prevent or treat cancer metastasis through the inhibition of tumor cell migration.
3.5 Summary

Interleukin 6 and Interleukin 8 together enhance tumor cell migration through a synergistic paracrine signaling mechanism with Wiskott-Aldrich syndrome protein family member 3 (WASF3) and the Arp2/3 complex as important intermediates in the pathway. Simultaneous inhibition of this pathway by blocking the receptors of Interleukin 6 and 8 using Tocilizumab and Reparixin decreases metastatic capacity of cells in a mouse xenograft model with significantly reduced metastases to the lungs, liver, and lymph nodes.
CHAPTER 4: SIMULTANEOUS TARGETING OF PROLIFERATION AND MIGRATION

4.1. Introduction

Uncontrolled proliferation is a common phenotype associated with tumor cells. While different growth factors are implicated in regulating tumor cell proliferation, epidermal growth factor (EGF) and its cognate receptor EGFR (also known as ErbB-1 or HER-1) have been shown to play a central role in the pathogenesis and progression of different types of cancers\textsuperscript{34,35}. EGFR is a common therapeutic target for several types of cancer including breast, pancreatic, colorectal, and non-small cell lung cancer (NSCLC)\textsuperscript{39}. Cetuximab is a monoclonal antibody directed against the EGFR, which is currently used in the treatment of head, neck, and colon cancer. It is also currently in multiple clinical trials to determine its efficacy against esophageal, gastric, and pancreatic cancer\textsuperscript{105,106}.

The studies described in chapter 3 demonstrate that through the inhibition of IL-6R and IL-8R using Tocilizumab and Reparixin, respectively, cell-density-dependent migration can be repressed which leads to a decrease in the metastatic burden in the lungs, liver, and lymph nodes in a mouse xenograft model. However, these results also demonstrate that this combination of therapeutics does not affect the growth rate of tumors. In this study, we simultaneously target tumor cell migration and proliferation, two key drivers of metastasis, by using a combination of Tocilizumab, Reparixin, and Cetuximab to target the receptors of IL-6, IL-8, and EGF.

4.2. Materials and methods

4.2.1 Cell lines and culture

Human fibrosarcoma HT1080 cells (ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Mediatech) supplemented with 10\% (v/v) fetal bovine serum (FBS, Hyclone Laboratories), and 0.005\% (w/v) gentamicin (Quality Biological). Human breast carcinoma MDA-MB-231 cells (ATCC) were cultured in DMEM (Mediatech)
supplemented with 10% FBS (Hyclone). The cells were maintained at 37°C and 5% CO₂ in a humidified incubator during cell culture and during live-cell microscopy.

### 4.2.2 3D Collagen I matrix

HT1080 cells were embedded in 2mg/ml type I collagen gel as described previously by Fraley et al. Briefly, cell suspensions containing 5,000 and 25,000 cells in 1:1 (v/v) ratio of cell culture media and reconstitution buffer were mixed with appropriate volume of soluble rat-tail collagen I (Corning Inc.) to obtain a final collagen I concentration of 2 mg/ml. A calculated amount of 1M NaOH was added quickly and the final solution was mixed well to bring the pH to ~7. The cell suspension was added to a 24-well coverslip-bottom cell-culture dish and immediately transferred to an incubator maintained at 37°C to allow polymerization. Fresh medium was added 1 h before imaging. MDA-MB-231 cells were embedded in 1mg/ml type I collagen matrix with cell suspensions containing 5,000 and 50,000 cells.

### 4.2.3 High-throughput secretomic analysis of condition media

Matrix-embedded cells with cell densities ranging from 10 cells/mm³ to 150 cells/mm³ were incubated for 48h at 37°C in a humidified incubator. The conditioned medium from the cells was then collected and filtered through a 0.45-µm filter (Millipore) to remove cell debris. High-throughput secretomic analysis was conducted on the condition medium collected as described previously by Lu et al. The data displayed was obtained from one experiment with three replicates.

### 4.2.4 Inhibitor assays

Matrix embedded cells with low and high cell densities were exposed to IL-8R inhibitor, Reparixin (Cayman Chemical), IL-6R inhibitor, Tocilizumab (Genentech), Cetuximab (Bristol Meyers Squibb) for 1h before cells were imaged as described above. (See velocity and protrusion topology of matrix embedded cells) Cell viability assays using PrestoBlue (Invitrogen) were also conducted on matrix embedded cells exposed to Reparixin, Tocilizumab, and Cetuximab. The data displayed was obtained from three independent experiments with a minimum of two replicates for each experiment.
4.2.5 Proliferation assays

HT1080 cells were embedded in type I 3D collagen matrices in increasing cell numbers from 5,000 to 60,000 cells. Cell viability assay using Prestoblue (Invitrogen) was also conducted on the matrix-embedded cells of increasing cell number. Fluorescence was measured at 48h and 72h. The data displayed was obtained from three independent experiments with a minimum of two replicates for each experiment.

4.2.6 Speed and protrusion topology of matrix embedded cells

Phase-contrast images of matrix-embedded cells were recorded 2 min apart for 16.5 h using a Cascade 1K CCD camera (Roper Scientific) mounted on a Nikon TE2000 microscope with a 10X objective lens. Single cells were tracked using Metamorph imaging software. A custom MATLAB program calculated the velocity for each cell using the $x$- and $y$-coordinates obtained from tracking data using the following equation:

$$\text{Speed} = \frac{\sqrt{\langle [x(t + \Delta t) - x(t)]^2 + [y(t + \Delta t) - y(t)]^2 \rangle}}{t}.$$ 

For the characterization of protrusion topology, the movies were used to count the total number of mother protrusions, and the number of first-, second-, and third-generation protrusions generated by the cell (e.g. Fig. 1, C). The protrusions emanating directly from the cell body, even when split, were termed mother protrusions; protrusions originating from the mother protrusions were termed first-generation, and so on. Mitotic cells were not included in the measurements.

4.2.8 Expression of Ki67 and WASF3

Matrix embedded cells with cell densities of 10 cells/mm$^3$ and 50 cells/mm$^3$ were incubated for 24h at 37°C in a humidified incubator. The matrices were exposed to cell lysis buffer and mechanically broken down using a syringe. The expression of Ki67 and WASF3 was measured using qRT-PCR. Total RNA isolation was performed with RNA MiniPrep kit (Zymo research). cDNA synthesis was carried out as previously described by Gilkes et al$^{83}$. The sequence for the cDNA primers that were used during PCR are
found in the table 2 and 3. The data displayed was obtained from three independent experiments with a minimum of three replicates for each experiment.

<table>
<thead>
<tr>
<th>HS-KI67-FOR</th>
<th>GCCTGCTCGACCCTACAGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS-KI67-REV</td>
<td>GCTTGTCAACTGCGGTTGC</td>
</tr>
</tbody>
</table>

Table 4: Sequence of the cDNA primers of Ki67

4.2.9 Statistics

The mean values ± S.E.M were calculated and plotted using GraphPad Prism software (GraphPad Software). One-way ANOVA test was performed to determine statistical significance, which is indicated in the graphs using a Michelin grade scale ***p<0.001, **p<0.01, and *p<0.05.

4.3 Results

4.3.1 EGF secretion decreases with increased cell density

Epidermal growth factor (EGF) is part of a complex network of growth factors and receptors that has shown to be unnaturally active in aggressive forms of tumors. To determine the expression of EGF of cells embedded at low and high densities in 3D matrices, where the expression of IL-6 and IL-8 increases linearly with increasing cell density, we used a multiplex antibody microarray assay, we measured the concentration of the protein in 20uL of medium following a 48H incubation period. The assay demonstrated that the secretion of EGF decreased with increasing cell density. Strikingly, this assay also demonstrates that the secretion of other critical growth factors involved in regulating tumor cell proliferation such as HGF and TNFα are unaffected by increasing cell densities (Figure 4.1). These observations suggest that EGF may play a role in regulating cell-density dependent migration.
4.3.2 Effect of Cetuximab on cell-density-dependent migration

We next investigated the effect of inhibiting the binding of EGF to its cognate receptor by using Cetuximab, a monoclonal antibody that is directed against the epidermal growth factor receptor (EGFR), on cell-density-dependent migration. Human fibrosarcoma cells, HT1080WT, and breast carcinoma cells, MDA-MB 231, were embedded in a 3D type I collagen matrix and cell migratory patterns within the matrix were monitored for 16.5h using live-cell phase-contrast microscopy at a rate of 30 frames/h. This analysis revealed that the single-cell migration of both fibrosarcoma and carcinoma cells were mostly unaffected by increasing concentrations of Cetuximab at a low cell density of 10 cells/mm$^3$. However, at a high density condition of 50 cells/mm$^3$ for fibrosarcoma cells and 100 cells/mm$^3$ for carcinoma cells cell speed significantly decreased when Cetuximab was introduced into the 3D collagen I matrix. (Figure 4.2)
Based on this result, we hypothesized that a combination of Tocilizumab, Reparixin, and Cetuximab could suppress cell-density-dependent migration more effectively than a combination of Tocilizumab and Reparixin. To test this hypothesis, we exposed matrix-embedded fibrosarcoma and carcinoma cells to different drug combinations. Our data demonstrated that the combination of the three inhibitors was as equally or more effective, as the combination of Tocilizumab and Reparixin in low and high density conditions. (Figure 4.3)

Figure 4.2: Effect of Cetuximab on 3D cell migration at low and high density conditions: A and B. In both fibrosarcoma and carcinoma cell lines, cell speed is mostly unaffected by increasing concentrations of Cetuximab at a low cell density (ρ =10). However, at a high density (ρ =50, for HT1080WT and ρ =100, for MDA MB 231) cell speed was significantly decreased.
4.3.3 Inhibition of proliferation

We next explored the effect of Cetuximab individually and in combination with Tocilizumab and Reparixin on the rate of proliferation. The rate of proliferation was assessed using Prestoblue viability assays. Matrix embedded fibrosarcoma and carcinoma cells with cell densities ranging from 10 cells/mm$^3$ to 120 cells/mm$^3$ were exposed to the inhibitors and fluorescence readings were measured at 48 and 72h time points. The

Figure 4.3: Combination of Tocilizumab, Reparixin, and Cetuximab repress 3D cell migration: A and B. In both fibrosarcoma and carcinoma cell lines, cell speed greatly decreased when treated with a combination of Tocilizumab, Reparixin, and Cetuximab.
proliferation of fibrosarcoma and carcinoma cells in 3D matrices was unaffected by Reparixin and Cetuximab treatments. Surprisingly, the rate of proliferation increases with treatment with Tocilizumab. (Figure 4.2 A and B)

![Figure 4.2: Effects of proliferation with Tocilizumab, Reparixin, and Cetuximab alone: A and B. Prestobluviability assays with fibrosarcoma and carcinoma cells demonstrate that Reparixin and Cetuximab have no effect on rate of proliferation. Tocilizumab increases the rate of proliferation. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA).]

The combination of Tocilizumab, Reparixin, and Cetuximab repressed tumor cell proliferation more effectively than the combination of Tocilizumab and Reparixin where the rate of proliferation was comparable to the rate of proliferation of the control condition. (Figure 4.3 A and B) This data combined with the data from the migration assays suggests that the combination of Tocilizumab, Reparixin, and Cetuximab can simultaneously inhibit tumor cell proliferation and migration.
To further validate these observations, we used PCR techniques to determine the expression of Ki67, a common proliferation marker, and WASF3, an important intermediate in the cell-density-dependent paracrine signaling pathway (See Chapter 3). Our assays confirmed that both Ki67 and WASF3 were down regulated when fibrosarcoma and carcinoma cells were exposed a combination of Tocilizumab, Reparixin, and Cetuximab. This data strongly suggesting that the three inhibitors in combination can simultaneously inhibit tumor cell proliferation and migration.

**Figure 4.5: Inhibition of proliferation with drug combination:**
Prestobluvi viability assays with fibrosarcoma and carcinoma cells demonstrate that a combination of Tocilizumab, Reparixin, and Cetuximab decrease the rate of proliferation. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA).

**Figure 4.6: Down regulation of WASF3 and Ki67:** PCR experiments with fibrosarcoma cells demonstrate that a combination of Tocilizumab, Reparixin, and Cetuximab decrease the expression of WASF3 and Ki67. In all panels, data is represented as mean ± s.e.m. *P<0.05; **P<0.01; ***P<0.001(ANOVA).
4.4 Discussion

The results of this study infer a novel therapeutic combination that simultaneously suppresses tumor cell proliferation and migration through the inhibition of the receptors of IL-6, IL-8, and EGF. IL-6 is a cytokine that has been frequently linked with tumor growth, progression, and relapse\(^\text{107}\). IL-8 has been implicated in multiple signaling pathways that are known to promote angiogenic responses, increase proliferation and survival, and potentiate the migration of tumor cells\(^\text{87}\). EGF and its family of receptors have been associated with aggressive behavior in tumor cells through the modulation of cell proliferation, survival, adhesion, migration and differentiation\(^\text{108}\). Individually these three molecules have been targeted by therapeutics to improve cancer patient outcomes using Tocilizumab, Reparixin, and Cetuximab. However, the potential of these agents as anti-cancer therapeutics that can simultaneously target tumor growth and metastasis in highly invasive cancers has not been previously studied \textit{in vivo} systems. This study suggests that simultaneous use of Tocilizumab, Reparixin, and Cetuximab could greatly decrease tumor growth and metastatic capacity of tumor cells.

This \textit{in vitro} work further emphasizes the necessity of testing the three agents; Tocilizumab, Reparixin, and Cetuximab, in combination in mouse xenograft models. Data from the mouse xenograft model could further validate this combination of therapeutics as a new strategy to prevent or treat cancer through the inhibition of tumor cell proliferation and migration.

4.5 Summary

Inhibition of cell-density dependent migration by blocking the receptors of Interleukin 6 and 8 using Tocilizumab and Reparixin decreases the metastatic capacity of cells but does not affect tumor growth. The simultaneous targeting of the receptors of Interleukin 6, Interleukin 8, and Epidermal growth factor suggests that this drug combination can decrease both metastasis and tumor growth through inhibition of tumor cell proliferation and migration.
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EDUCATION

Johns Hopkins University  
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Concentration: Molecular and Cellular Bioengineering

DOCTORAL THESIS WORK

Johns Hopkins University
Department of Chemical and Biomolecular Engineering  
PhD. Candidate, PI: Denis Wirtz, PhD.

• **Project 1**: Mechanistic coupling of cancer cell proliferation and migration through the synergistic paracrine signaling of Interleukins 6/8 – This study functionally connects cell proliferation to tumor cell migration through a novel, synergistic paracrine signaling pathway and demonstrates that inhibiting this pathway through the targeting of IL-6 and IL-8 receptors has the potential to decrease the metastatic capacity of cancer cells.

• **Project 2**: Concurrent targeting of cell-density dependent migration and tumor proliferation – This study investigates the effect of simultaneous inhibition of IL-6, IL-8, and EGF binding to their cognate receptors on tumor growth and metastasis

RESEARCH EXPERIENCE/COLLABORATIONS

• **Yale University, Department of Biomedical Engineering**  
Collaborating PI: Rong Fan, PhD.

  Project 1: Mechanistic coupling of cancer cell proliferation and migration through the synergistic paracrine signaling of Interleukins 6/8

  Project 2: Concurrent targeting of cell-density dependent migration and tumor proliferation

• **Johns Hopkins Medical Institution**  
The Sidney Kimmel Comprehensive Cancer Center  
Collaborating PI: Daniele M. Gilkes, PhD.

  Project: Mechanistic coupling of cancer cell proliferation and migration through the synergistic paracrine signaling of Interleukins 6/8

• **Johns Hopkins University**  
Department of Chemical and Biomolecular Engineering  
Undergraduate research assistant, PI: Denis Wirtz, PhD.

  Project 1: The role of the ARP2/3 complex on three-dimensional cancer cell migration

  Project 2: Investigation of the role of EB1 and cytoplasmic dynein in protrusion dynamics for efficient three-dimensional cell migration

• **Washington University, Department of Biomedical Engineering**  
Collaborating PI: Greg Longmore, M.D.

  Project: The role of the ARP2/3 complex on three-dimensional cancer cell migration
WORK EXPERIENCE

Genentech, Department of Molecular Oncology 06/16 - 08/16
Graduate intern. PI: Fred de Sauvage, PhD.
• Conducted a screening for Wnt targets that prevent differentiation in colon cancer

Johns Hopkins University, Whiting School of Engineering 09/15 – 12/15
The Hopkins Engineering Applications and Research Tutorials – Instructor
• Planned and conducted weekly seminars for undergraduate students to develop their knowledge and understanding on cancer cell migration and its role in metastasis

University of Colombo
Institute of Biochemistry, Molecular biology, and Biotechnology 01/15 – Present
Guest lecturer
• Conduct seminars for graduate students on current cancer research
• Mentor Master’s students on applying for PhD programs

Johns Hopkins University
Department of Chemical and Biomolecular Engineering 08/14 – 12/14
Graduate Student Teaching Assistant
• Taught senior students majoring in Chemical and Biomolecular Engineering methods in gas absorption, distillation, bacterial cell culture, membrane separations, and biocatalysis

Johns Hopkins University, Whiting School of Engineering 08/10 – 12/13
Peer Lead Tutoring Program (PLTL) – Instructor
• Taught undergraduate students chemical engineering concepts such as mass balances and separation techniques
• Facilitated study groups to solve problem sets for the course: Chemical and Biomolecular Process Analysis
• Proctored exams and graded homework assignments, midterms and finals.

PUBLICATIONS

2. Giri, A., Jayatilaka, H., Trenton, N., & Wirtz, D. EB1/LIC2-mediated microtubule dynamics promotes efficient three-dimensional cell migration. (In review at Nature Communications)

PATENT APPLICATIONS

RESEARCH PRESENTATIONS

1. Mechanistic coupling of tumor cell migration and proliferation, University of Moratuwa, Moratuwa, Sri Lanka, November 2016 (Invited talk)
2. Functional coupling of cancer cell proliferation and migration through the synergistic paracrine signaling of Interleukins 6/8, 2015 American Society for Cell Biology meeting, San Diego, December 2015 (Poster)
3. Influence of cell density on three-dimensional cancer cell migration, Institute of Biochemistry, Molecular Biology and Biotechnology, Colombo, Sri Lanka, January 2015 (Invited talk)
4. Influence of cell density on cancer cell motility, 2014 Institute for NanoBiotechnology annual research symposium, Baltimore, Maryland, April 2014 (Poster)
5. The role of microtubule-binding proteins in cell cancer cell motility in 3D matrices, 2013, AACR Annual Undergraduate Student Caucus and Poster Competition, Washington DC, April 2013 (Poster)

HONORS AND AWARDS

- Award for best poster presentation, Johns Hopkins University Institute for NanoBiotechnology annual research symposium. (2016)
- ASCB Immigrant Travel Award, American Society for Cell Biology (ASCB) (2015)
- Joseph L. Katz award, Whiting School of Engineering Johns Hopkins University (2013) - *Awarded for academic excellence in the Chemical and Biomolecular Engineering Senior Lab course*
- Paul A.C. Cook award, Whiting School of Engineering Johns Hopkins University (2012) - *Awarded to an outstanding junior majoring in Chemical and Biomolecular Engineering, who is the best all-round student*
- Provost undergraduate research award, Johns Hopkins University (2011)
- MAPP Rookie of the year, Office of Multicultural Affairs, Johns Hopkins University (2011)
- Dean’s List, Johns Hopkins University
- International Students Scholarship, Johns Hopkins University (2009 – 2013)

ACTIVITIES

- Member, American Society of Cell Biology (09/15 – present)
- Member, Society of Women Engineers (09/14 – Present)
- Volunteer, JHU-GSLC Science, Technology, Engineering and Math (STEM) (01/14 – Present)
- Foster, Baltimore Animal Rescue and Care Shelter (09/13 – 12/14)
- Student member, American Association for Cancer Research (09/12 – 05/13)
- Founder, Re-build (nonprofit organization), Sri Lanka (03/11 – Present)
- President (09/11-09/12), Treasurer (09/10-09/11), Johns Hopkins University AIDS Alliance
- Mentor, Mentoring Assistance Peer Program (MAPP) (09/10 – 09/11)
SKILLS

- **Broad knowledge of engineering principles and life sciences**
  Worked on collaborative, multidisciplinary teams in research encompassing areas such as cancer, cell biology, biophysics, and bioengineering.
  Experienced in first principles of engineering and natural sciences.

- **Cell culture**
  2D, 3D, primary cell culture, single cell proteomics, ELISA, real time and quantitative PCR, western blotting, hypoxia, immunofluorescence staining, transformation and transfection of DNA/RNA using lentivirus.

- **In vivo Mouse models**
  Orthotopic transplantation, administration of agents, necropsy, immunohistochemistry.

- **Microscopy**
  Phase contrast, fluorescence, confocal.

- **Data analysis and visualization**
  MATLAB, Metamorph, NIS Elements, Microsoft Office Suite, Adobe Illustrator, Graph Pad Prism.

- **Foreign language**
  Sinhala (native), French.

REFERENCES

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