A Study of Breast Cancer Cell Adhesion to Endothelium in Response to Cytokine Stimulus

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Karissa A. Henson

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This thesis titled

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by

KARISSA A. HENSON

has been approved for

the Department of Chemical and Biomolecular Engineering

and the Russ College of Engineering and Technology by

Monica M. Burdick
Assistant Professor of Chemical and Biomolecular Engineering

Dennis Irwin
Dean, Russ College of Engineering and Technology
ABSTRACT

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Molecules present in the bone marrow microenvironment contribute to attraction, adhesion, and infiltration of circulating breast cancer cells. Breast cancer stem cells, characterized as CD44+/CD24/low, are implicated in progression of the disease. However, the specific mechanism used by breast cancer cells to metastasize is unknown. This study thus aimed to determine the role of breast cancer stem cells and microenvironmental factors in bone marrow metastasis, specifically in the adhesion of migrating cancer cells to bone marrow endothelium. The focal points of the study were E-selectin, SDF-1α, and their binding partners because of their integral roles in the cell adhesion and migration of other cell types to bone. Both stem cell-like and non stem cell-like breast cancer cells were found to express CXCR4 which binds SDF-1α. SDF-1α consistently enhanced E-selectin related adhesion of breast cancer cells to endothelium. Conversely, TGF-β1 induced epithelial mesenchymal transition to make cancer cells stem-like appeared to have no effect on this process. These results indicate that the breast cancer stem cell phenotype may not be necessary for E-selectin mediated cancer homing to the metastatic site, but may play a role in a unique homing method to bone marrow or in the formation of a new tumor upon bone marrow infiltration.

Approved:

Monica M. Burdick

Assistant Professor of Chemical and Biomolecular Engineering
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CHAPTER 1. INTRODUCTION

1.1. Breast Cancer Metastasis

1.1.1. Description of disease

Breast cancer is the second most common cancer diagnosed in women in the United States. It frequently originates in the ductal areas of mammary tissue but can become invasive and migrate to surrounding tissue, lymph nodes, and distant organs\(^1\). When breast cancer is diagnosed at an early stage, it is largely considered curable. Women diagnosed with localized cancer such as ductal carcinoma \textit{in situ} have a 98% survival rate in the 5 years immediately following diagnosis and treatment. However, diagnosis at the invasive, metastatic stage results in a 71% decrease in 5-year survival rate, making breast cancer a leading cause of cancer death in women. The American Cancer Society reports that almost 200,000 new cases of invasive breast cancer were diagnosed in 2009, and over a lifetime, women have a 1 in 8 chance of getting this type of disease\(^1\). Therefore, breast cancer and subsequent invasion and metastasis, is a serious health problem for women.

Breast cancer metastasis is a result of an \textit{in situ} breast tumor becoming invasive and is the most important determining factor in prognosis\(^2\). It occurs when cancer cells break away from the primary tumor, move into circulation, and extravasate at a distant organ to set up a secondary colony. Most commonly, breast cancer metastases are found in the lungs and bone marrow tissue, exhibiting a tropism not explainable by circulation pattern alone\(^3\). Importantly, over 80% of metastatic breast cancer cases
result in skeletal tumor formation\(^4\). In a majority of cases, bone metastases are osteolytic resulting in bone loss. This event causes many of the symptoms seen in patients with bone metastasis, including: pain, anemia, hypercalcemia, and pathologic fractures\(^4\). Although much is known about the devastating effects of metastasis, the specific mechanisms promoting it remain elusive.

**1.1.2. Relationship between cell adhesion & metastasis**

Breast cancer metastasis is mediated by the interaction between adhesion-related molecules on the surface of migrating cells and their respective partners expressed at the metastatic site\(^5,6\). Breast cancer metastases often develop in bone marrow\(^7,8\), where adhesion molecule E-selectin is constitutively expressed\(^9,10\). E-selectin is a member of the selectin family that also includes L-selectin and P-selectin. E-selectin has an N-terminal lectin-like domain, an epidermal growth factor-like domain, consensus repeats, a transmembrane domain, and a short cytoplasmic tail in common with the other selectins. However, E-selectin is differentiated from the other selectins by the number of consensus repeats it contains and the carbohydrate structures it recognizes on potential ligands\(^5\). In addition, breast cancer cells are shown to present E-selectin ligands on their surface\(^11-13\). Interestingly, the normal physiological process of hematopoietic stem cell (HSC) migration into bone marrow, initiated by a tethering and rolling process of the cell on endothelium, is governed by selectin ligands and their respective selectin partners such as E-selectin\(^14\). It is logical that breast cancer migration
to bone may utilize similar molecules in a related process, rather than invent a new adhesion mechanism.

Notably, E-selectin concentration is elevated in the serum of cancer patients and the molecule in general has been found to facilitate colon, prostate, and pancreatic cancer metastasis\(^5\). Previous studies have illustrated the E-selectin dependence of binding interactions between commercially available breast cancer cell lines and human umbilical vein endothelial cells (HUVEC)\(^{12,15}\). Therefore, E-selectin is likely an important element of breast cancer metastasis \textit{in vivo}.

A secondary element in the adhesion cascade is Vascular Cell Adhesion Molecule-1 (VCAM-1) which plays an important role in the adhesion of rolling cells to endothelium. VCAM-1 can mediate cell adhesion to endothelium in a manner similar to E-selectin\(^{16,17}\). To facilitate this process, VCAM-1 on endothelial cells binds Very Late Antigen-4 (VLA-4) on migrating cells. The interaction between VCAM-1 and VLA-4 is an integral part of leukocyte adhesion and directs subsequent diapedesis \textit{in vivo}\(^9,18\). Additionally, stromal cell-derived factor-1\(\alpha\) present in the bone marrow milieu has been shown to activate VLA-4 on migrating cells in the blood stream\(^{16,19}\). The importance of VCAM-1 interaction with VLA-4 in leukocyte migration merits investigation into its possible function in breast cancer metastasis.

1.2. Importance of the CXCR4-SDF-1\(\alpha\) Axis

In addition to E-selectin, the interaction between stromal cell-derived factor-1\(\alpha\) (SDF-1\(\alpha\)) plays a necessary role in HSC homing and adhesion to bone marrow
endothelium\textsuperscript{20,21}. SDF-1\(\alpha\) is also constitutively expressed in the bone marrow microenvironment and interacts with its receptor CXCR4 to increase the binding affinity between cells in blood flow and the bone marrow endothelium\textsuperscript{21}. The presence of SDF-1\(\alpha\) in endothelial milieu also enhances the extravasation of leukocytes through endothelial walls\textsuperscript{22}. Interestingly, CXCR4 expression is a characteristic of malignant breast tissue, and is not normally expressed in mammary epithelial cells\textsuperscript{6}. Active CXCR4 is also expressed on many types of cancer cells, including breast cancer, prostate cancer, and glioblastoma, implicating CXCR4 in progression of disease\textsuperscript{20}.

A correlation between SDF-1\(\alpha\) expression and leukocyte migration across an endothelium has previously been demonstrated. This finding leads to the theory that CXCR4-expressing breast cancer cells would show enhanced adhesion and morphological alteration properties that facilitate extravasation into the bone marrow parenchyma\textsuperscript{23}. Consistent with their role in HSC migration to bone marrow, the adhesion regulators E-selectin and SDF-1\(\alpha\) are implicated in breast cancer metastasis\textsuperscript{24}. Hypothetically, migrating breast cancer cells possess the CXCR4 and E-selectin ligands in order to respond to SDF-1\(\alpha\) and E-selectin in the bone marrow microenvironment through coordinated tethering and rolling interactions as well as calcium mobilization and actin polymerization leading to pseudopodia formation\textsuperscript{6,25}. Overall, the bone marrow microenvironment is enriched in cytokines and chemokines that are believed to play a role in breast cancer metastasis to this site\textsuperscript{26}. 
1.3. Breast Cancer Stem Cells

1.3.1. Breast cancer stem cells may have a critical role in disease progression

An elegant study published by Al Hajj et al.\textsuperscript{27} detailed the discovery of a unique type of breast cancer cell that exhibited a much greater propensity for tumor formation than cells of a different phenotype. These cells were putatively characterized by the expression levels of two glycoproteins on the surface of the cell: high expression of CD44 and little to no expression of CD24\textsuperscript{27}. Although these molecules can be involved in adhesion to endothelial cells, for example: isoforms of CD44 bind E-selectin and CD24 is only known to bind P-selectin\textsuperscript{28-30}, for these purposes they are used only as markers of breast cancer cell phenotype. The study defining breast cancer stem cells demonstrated the ability of these cells to form heterogeneous tumors from a relatively small amount of cells. Specifically, only 200 CD44\textsuperscript{+}/CD24\textsuperscript{−/low} breast cancer cells, isolated from patient primary tumors, could regenerate and expand to form secondary tumors in as little as 12 weeks\textsuperscript{27}. As many as 20,000 cells of alternate phenotypes, but of the same origin as the CD44\textsuperscript{+}/CD24\textsuperscript{−/low} cells, were unable to form new tumors. Termed cancer stem cells, these cells are capable of self-renewal and differentiation, similar to the stem cells that regenerate normal tissue\textsuperscript{27,31,32}.

Although the clinical relevance of CD44\textsuperscript{+}/CD24\textsuperscript{−/low} cells is debatable, they have been shown to correlate with distant metastasis and poor survival\textsuperscript{33}. Breast cancer stem cells progress disease through an ability to survive traditional cancer treatments and an increased capacity for invasion as compared to other breast cancer cell populations\textsuperscript{34,35}.
Tumor cells may disseminate as early as 5 years prior to a breast cancer diagnosis, meaning these cells can survive in the body even when the primary tumor is removed at an early stage\textsuperscript{36}. Notably, clinical experiments have identified CD44\textsuperscript{+}/CD24\textsuperscript{low} breast cancer cells as major disseminated tumor cell inhabitants of bone marrow in patients with early breast cancer indicating that these cells have indeed migrated away from the primary tumor early in cancer development\textsuperscript{7}. However, it is important to note that the study did not investigate how the cells arrived in the bone marrow, and implications into the role of cancer stem cells in endothelial adhesion cannot be inferred. It is therefore no surprise that the presence of breast cancer stem cells at the time of diagnosis leads to poor prognosis\textsuperscript{35}. The dissemination of cancer cells from the primary tumor, and subsequent steps in metastasis, is facilitated by a phenomenon called epithelial mesenchymal transition that produces stem-like cells from epithelial tissue\textsuperscript{37}.

\textbf{1.3.2. Epithelial mesenchymal transition produces cancer stem cells}

Epithelial mesenchymal transition (EMT) is a normal physiological process that aids in embryogenesis, wound healing, and is implicated in the enhancement of invasive properties in cancer cells\textsuperscript{38}. Many of the properties it confers are helpful to development (motility and apoptosis resistance) but can contribute to cancer progression in adult tissue\textsuperscript{37}. Often it is found that the cancer cells present at the invasive-front of a primary tumor have a mesenchymal phenotype\textsuperscript{38}. Morel and colleagues demonstrated that EMT accelerates the formation of CD44\textsuperscript{+}/CD24\textsuperscript{−} cells from CD44\textsuperscript{low}/CD24\textsuperscript{+} human mammary epithelial cells that have been Ras-transformed\textsuperscript{39}. In
EMT enhances the in vivo metastatic capability of tumor cells\textsuperscript{40}. Therefore EMT may be necessary for epithelial cancer cells to become malignant and a contributor to the invasivity and dissemination of tumor cells\textsuperscript{38,41}. EMT is associated with a decrease in epithelial characteristics including loss of E-cadherin, a necessary component of cell-cell adhesion\textsuperscript{42,43}. The dissociation of epithelial cells from one another enables them to break away from the primary tumor, become more invasive, and potentially metastasize, contributing to the progression of breast cancer\textsuperscript{36,43}. Interestingly, in breast cancer patients with metastases, circulating tumor cells have been found to express markers of epithelial mesenchymal transition in addition to stem cell traits\textsuperscript{44}.

Considering the substantial involvement of epithelial mesenchymal transition in the progression of cancer, the factors initiating this event become crucial to understanding metastasis. Transforming growth factor-\(\beta\)1 (TGF-\(\beta\)1) is one of the most commonly described inducers of EMT in vitro\textsuperscript{41,45-47}, and is expressed by stromal cells in the bone marrow\textsuperscript{48}. TGF-\(\beta\)1 exhibits dual activity as a tumor suppressor and enabler of metastasis\textsuperscript{38}. Importantly, TGF-\(\beta\)1 can induce EMT in vitro for multiple cancer cell lines, which allows further study into the role it plays in cancer progression\textsuperscript{38,39,41,47,49}. However, the effect of TGF-\(\beta\)1 on E-selectin ligands for breast cancer cells, and therefore on adhesion to endothelium, is unknown. There are many biomarkers with which to distinguish EMT. Among them are cell surface markers such as the loss of E-cadherin, which promotes EMT, and the gain of N-cadherin in its place. Cytoskeletal filament vimentin is expressed mesenchymally, and transcription factors such as Snail,
Slug, and Twist are activated upon EMT\textsuperscript{42,50,51}. In particular, TGF-\(\beta\)1 directly activates Twist, effectively blocking E-cadherin expression while enhancing the levels of N-cadherin\textsuperscript{52}. In addition to molecular changes that occur throughout EMT, the morphology of the cell is subject to change. Epithelial cells are described as having a cobblestone appearance, but after treatment with TGF-\(\beta\)1, or a different method to induce EMT, these cells assume a long, spindle-like shape similar to fibroblasts\textsuperscript{47,50}. Figure 1 illustrates the process of EMT and important markers involved in the transition.

![Figure 1. Epithelial mesenchymal transition is characterized by the loss of epithelial markers and the gain of mesenchymal properties in a cell. Figure adopted from Drs. Kalluri and Weinberg, with kind permission\textsuperscript{38}.](image)

Separately, the potential roles of TGF-\(\beta\)1 and SDF-1\(\alpha\), particularly in cancer progression, are highly investigated. However, the link that brings together all the elements of breast cancer metastasis previously described remains elusive. Enlighteningly, there is interplay between TGF-\(\beta\)1 and SDF-1 in leukocyte adhesion and migration. TGF-\(\beta\)1 induces chemotaxis of T cells toward SDF-1\(\alpha\) in a concentration dependent manner. The growth factor is also capable of elevating CXCR4 expression on the surface of leukocytes and even increases actin polymerization in SDF-1\(\alpha\) stimulated
cells\textsuperscript{53}. Combined, these data show the importance of both molecules in the migration of leukocytes to endothelial cells. It is therefore possible that breast cancer cells exploit the interaction between TGF-\(\beta\)1 and SDF-1\(\alpha\) in order to facilitate adhesion to endothelium and therefore metastasis.

The following schematic, Figure 2, details the elements potentially involved in breast cancer metastasis. Understanding of the specific actions induced by each contributing molecule will provide a rational basis for the development of novel methods to inhibit breast cancer metastasis.

![Figure 2. Factors such as SDF-1\(\alpha\) and TGF-\(\beta\)1 interact with migrating breast cancer cells that are captured by E-selectin. The captured cell can move into the bone marrow to develop a secondary tumor.](image-url)
1.4. Specific Aims

The cancer stem cell is believed to be responsible for metastasis and the subsequent generation of differentiated, heterogeneous tumors at metastatic sites\textsuperscript{27,33}. These cells are affected by cytokines, chemokines, and other soluble factors present in the microenvironments of tumors and potential metastatic sites\textsuperscript{6}. However, the specific mechanism utilized by breast cancer cells to metastasize is unknown. Further investigation will advance knowledge of breast cancer metastasis and potentially the development of novel therapeutics. \textit{Hypothesis: The capacity of breast cancer tumor cells to adhere to bone marrow endothelium can be correlated to their responsiveness to cytokines in the bone marrow microenvironment. The responsiveness of breast cancer cells to bone marrow resident cytokines can be correlated to cellular phenotype.} The specific aims of this study were (1) to determine if the breast cancer stem cell phenotype, more specifically the surface expression of CD44 and CD24, correlated to the capacity of a breast cancer cell to respond to chemokines such as SDF-1α and adhesion molecules present in the bone marrow microenvironment; and (2) to determine if breast cancer cells that bind to endothelium can be made stem-like through treatment with TGF-β1 for use in future determination of how breast cancer stem cells differ in adhesion properties.
CHAPTER 2. MATERIALS AND METHODS

2.1. Tissue Culture

Established breast cancer cell lines were used for all experiments, including MCF-7, MDA-MB-231, MDA-MB-468, BT-20, Hs578t, T-47D, and ZR-75-1. MCF-7 and MDA-MB-231 cells were a generous gift from Dr. Robert Sackstein at Brigham and Women’s Hospital, Boston, Massachusetts. As described in the results section, later experiments used MCF-7 cells directly purchased from ATCC. All other cell lines were directly purchased from ATCC. MCF-7 and MDA-MB-231 cells were cultured in Dulbecco’s Minimum Essential Medium (DMEM, Hyclone) with 15% heat-inactivated fetal bovine serum (hiFBS, Hyclone) and 1x antibiotic-antimycotic (Gibco). MDA-MB-468 cells were cultured in Leibovitz’s L-15 Medium (ATCC), 10% hiFBS, and 1x penicillin-streptomycin (Hyclone). BT-20 cells were maintained in Minimum Essential Medium (MEM, Hyclone) supplemented with 10% hiFBS and 1x penicillin-streptomycin (Invitrogen). Hs578t cells were kept in DMEM supplemented with 10% hiFBS, 0.01 mg/ml bovine insulin and 1x penicillin-streptomycin. T-47D and ZR-75-1 cells were cultured in RPMI 1640 (Hyclone) with 10% hiFBS and 1x penicillin-streptomycin. Unless otherwise stated, all cells were harvested for experiments using 0.025% trypsin EDTA (Gibco) in Dulbecco’s phosphate buffered saline (DPBS, Hyclone) without calcium or magnesium. A low concentration of trypsin EDTA was necessary to prevent aberrant cleavage of the surface proteins being analyzed. Due to the need for a single-cell suspension of viable cells, it was not possible to use EDTA alone to avoid protein cleavage during cell harvest. EDTA incubation causes
clumps of cells to form, making it difficult to obtain a single-cell suspension without vigorous mixing. However, mixing can significantly decrease the viability of the cells. Excepting MDA-MB-468, which were grown in air with no additional CO₂, all cells were cultured at 37°C and 5% CO₂.

Human umbilical vein endothelial cells (HUVEC) and human bone marrow endothelial cells (hBMEC) were used in adhesion assays. HUVEC were purchased from Lonza and cultured in Media 199 (Lonza), 10% hiFBS, 1% glutamine, 0.1 mg/ml heparin (Sigma-Aldrich), 0.05 mg/ml endothelial cell mitogen (Biomedical Technologies), and 1x penicillin-streptomycin. hBMEC were established by Ellen van der Schoot, Sanquin Research & Academic Medical Center, Amsterdam, and a generous gift of Dr. Charles Dimitroff, Brigham and Women’s Hospital, Boston, Massachusetts. Culture conditions for hBMEC were Media 199 with HEPES and glutamine, 10% hiFBS, 10% human serum, 5 units/ml heparin, 100 μg/ml G418, 1 ng/ml fibroblast growth factor, and 1% penicillin-streptomycin. A concentration of 0.005% trypsin EDTA in DPBS (no calcium or magnesium) was used to dissociate endothelial cells.

### 2.2. Characterization of Surface Molecules

The characterization of cell surface molecules CD44, CD24, CXCR4, and α4 (CD49d) and β1 (CD29) segments of Very Late Antigen-4 (VLA-4) expression on breast cancer cell lines was performed using monoclonal antibodies and flow cytometry. Anti-human CD44 (clone G44-26, BD Pharmingen) and anti-human CD24 (BD Pharmingen) were used to label breast cancer cell lines MCF-7, MDA-MB-231, MDA-MB-468, BT-20,
Hs578t, T-47D, and ZR-75-1. Anti-mouse IgG-fluorescein isothiocyanate (FITC, BD Pharmingen) and anti-mouse IgG-phycoerythrin (PE, BD Pharmingen) were used as secondary antibodies for CD44 and CD24, respectively. Isotype controls were mouse IgG2a for CD24 and mouse IgG2b for CD44. Alternatively, cells were labeled in a one-step process using anti-human CD44-FITC (clone G44-26, BD Pharmingen) and anti-human CD24-PE (BD Pharmingen) to detect concurrent CD44 and CD24 expression on cells.

Additionally, all breast cancer cell lines were incubated with anti-human CXCR4 antibodies recognizing different epitopes (clone 12g5, BD Pharmingen, clones 44708, 44716, 44717, R&D Systems). Clone 44717 is the most general for recognition of any CXCR4 on the surface of cells\textsuperscript{54}. The isotype control for all clones was mouse IgG\textsubscript{1} (BD Pharmingen). Anti-mouse IgG-biotin was added after primary antibody incubation, followed by streptavidin-PE (BD Pharmingen). Fluorescently labeled cells were then detected using a FACSort (BD Biosciences).

Characterization of the expression of α4 and β1 segments of VLA-4 used anti-human CD49d and anti-human CD29 as primary monoclonal antibodies, respectively (BD Pharmingen). Isotype controls were anti-rat IgG\textsubscript{2A} and anti-mouse IgG\textsubscript{1} for CD29 and CD49d, respectively. Anti-mouse IgG-FITC was used as the secondary antibody.

Analysis of the expression of E-selectin on endothelial cells following stimulation was carried out using monoclonal antibody mouse anti-human CD62E (BD Pharmingen) as a primary label, followed by anti-mouse IgG-FITC. EDTA at a concentration of 5 mM in DPBS without calcium or magnesium was used to harvest the endothelial cells. E-
selectin is known to be susceptible to cleavage by trypsin, and therefore trypsin EDTA could not be used for cell harvest. Anti-mouse IgG₁ was used as an isotype control.

All antibody and cell-labeling molecule concentrations for the above experiments were 10 μg/ml in DPBS with calcium and magnesium unless otherwise stated. All cells were harvested, using either trypsin EDTA or EDTA alone in DPBS without calcium or magnesium as specified, prior to incubation with the primary antibody. 20 μl of primary antibody was added to 200,000 cells in suspension and incubated for 40 minutes on ice. Secondary, and tertiary if applicable, labeling molecules were added to cells for 20 minutes on ice in a dark environment to prevent photobleaching of the fluorescent molecules. Between antibody incubations, cells were washed using 0.1% bovine serum albumin (BSA, Sigma-Aldrich) in DPBS with calcium and magnesium. After final antibody incubation, cells were washed once in 0.1% BSA in DPS with calcium and magnesium and once in DPBS with calcium and magnesium. Cells were kept on ice in the dark after labeling, and run on the flow cytometer within an hour after the final incubation was complete. A FACSort (BD Biosciences) was used to analyze fluorescence intensity for all labeled cells.

2.3. Calcium Mobilization

Calcium mobilization was detected by labeling MCF-7, MDA-MB-231, Hs578t, and BT-20 breast cancer cells at 10⁶ cells/ml with Fluo-3am at a concentration of 5 μM (Invitrogen) in DPBS without calcium and magnesium for 1 hour at 37°C to detect calcium mobilization. Cells were washed twice in DPBS without calcium and magnesium
after incubation with Fluo-3am and resuspended in DPBS with calcium and magnesium. Upon binding to free calcium, Fluo-3am fluoresces in the 520-530 nm range. When cells are incubated with Fluo-3am, it moves into the cytoplasm of the cell. Specific stimulation, by SDF-1α for example, releases calcium from intracellular compartments into the cytoplasm where it binds to Fluo-3am causing fluorescence emission and enabling detection. SDF-1α (R&D Systems) in 0.1% BSA in DPBS with calcium and magnesium was added to labeled cells resulting in a final concentration of 50 or 100 ng/ml. Ionomycin (Sigma-Aldrich), which causes the release of intracellular calcium, was used as a control at a final concentration of 1 to 5 μg/ml in DPBS. SDF-1α and ionomycin were added immediately before analysis which observed the levels of intracellular calcium using a Spectramax M2 (Molecular Devices) fluorescent spectrophotometer at an excitation wavelength of 488 nm and emission wavelength of 530 nm over a period of 10 minutes. Prior to experimentation, cells were incubated with Fluo-3. Fluo-3am labeled cells unstimulated by ionomycin or SDF-1 were used to define basal fluorescence. Prior to and during analysis, cells were kept at 37°C. This protocol was adapted from Hallam et al and Princen et al\textsuperscript{55,56}.

**2.4. Actin Polymerization**

Breast cancer cells MCF-7, MDA-MB-231, and BT-20 at 10^6 cells/ml in 0.1% BSA in DPBS with calcium and magnesium were treated with 100 ng/ml of SDF-1α in 0.1% BSA in DPBS in suspension and fixed in time increments of 15, 30, 60, 120, 300, and 600 seconds. Cells were fixed for 15 minutes in a 1:1 ratio of cell suspension to 3.7%
paraformaldehyde for a final concentration of 1.8% paraformaldehyde, permeabilized for 5 minutes in 0.1% Triton-X (Fisher Bioreagents) in DPBS without calcium or magnesium. This protocol was derived from a similar experiment performed by Voermans et al. Between fixation and permeabilization steps, cells were washed in DPBS without calcium and magnesium. Subsequent to fixation and permeabilization, cells were incubated with (concentration) Alexa Fluor 488 phalloidin (Molecular Probes) for 20 minutes. Phalloidin binds to F-actin and Alexa Fluor 488 fluoresces around 525 nm to allow visualization of actin filaments in the cell. Cells were washed and resuspended with DPBS without calcium or magnesium. Approximately 300,000 cells were mounted per slide with Pro-long Gold (Molecular Probes) to preserve fluorescence and a cover slip was added prior to visualization using a filter for 490 nm excitation and 525 nm emission detection on a Leica 6100B microscope (Leica Microsystems). Cell images were pseudocolored using SimplePCI software (Hamamatsu Corporation). A similar study was conducted by Müller, et al showing actin polymerization in breast cancer cells.

2.5. Epithelial Mesenchymal Transition

To induce epithelial mesenchymal transition (EMT), breast cancer cells were treated with transforming growth factor-β1. BT-20 cells were seeded in 100 mm x 20 mm polystyrene tissue culture dishes at 900,000 cells per dish, MCF-7 and MDA-MB-231 cells were seeded at 700,000 cells per dish. To optimize the transition, cells were incubated in varying concentrations of TGF-β1 (R&D Systems) for periods of 24, 48, or
72 hours. It was necessary to perform optimization of treatment protocol because literature reports varied in the TGF-β1 concentrations and incubation times in order to induce EMT\textsuperscript{37,39,47,58}. Optimal transition properties were observed at 48 hours. Experiments shown in results utilized 5 mM EDTA in DPBS without calcium or magnesium for 5 minutes and a cell scraper to remove cells because E-cadherin is vulnerable to cleavage by trypsin\textsuperscript{59}. Subsequent experiments using TGF-β1 treated cells were optimally treated for 48 hours with 5 ng/ml TGF-β1 in normal growth medium. E-cadherin levels were measured via flow cytometry, cell morphology changes were visualized using phase contrast microscopy, and changes in vimentin were observed using immunofluorescence microscopy.

### 2.5.1. Detection of EMT markers via flow cytometry

To characterize mesenchymal-like alterations in breast cancer cells as induced by TGF-β1, cell surface E-cadherin levels were analyzed via flow cytometry. Per the manufacturer’s protocol, TGF-β1 was reconstituted at 10 μg/ml in a solution of 4 mM hydrochloric acid (HCl). A final concentration of 2 μM HCl, 5 ng/ml TGF-β1 in medium was used to treat cells. To control for possible effects of HCl in the TGF-β1 solution, the cell samples analyzed included: untreated cells grown in normal growth medium, cells treated with 5 ng/ml reconstituted TGF-β1 in normal growth medium, and cells treated with a volume of 4 mM HCl resulting in final concentration of 2 μM in medium. Cells were treated for 48 hours with 5 ng/ml TGF-β1 prior to harvesting for analysis, an optimized protocol from previous studies\textsuperscript{37,39,53,58}. Anti-human E-cadherin (Santa Cruz
Biotechnology) was added to 200,000 cells for 40 minutes at a final concentration of 20 μg/ml in 0.1% BSA in DPBS with calcium and magnesium, followed by incubation with anti-mouse IgG-FITC at 20 μg/ml for 20 minutes. The isotype control for E-cadherin was mouse IgG1.

Morphology changes signifying EMT in cells treated with TGF-β1 were observed via microscopy using a Leica 6100B microscope and SimplePCI software. This change included a shift in cellular morphology from an epithelial shape to a spindle-cell like, fibroblastic shape. Phase contrast images were taken of the adherent cells after 48 hour treatments and immediately prior to harvest for E-cadherin expression analysis.

**2.5.2. Detection of EMT markers via microscopy**

Immunofluorescence microscopy was used to visualize vimentin in BT-20 and MDA-MB-231 breast cancer cell lines to compare expression levels in untreated versus TGF-β1 treated cells. Adherent cells were incubated in normal growth medium or medium with 0.25 ng/ml TGF-β1 for 24 hours. DPBS without calcium or magnesium was used to wash cells prior to fixation in 3.7% paraformaldehyde in DPBS without calcium or magnesium for 10 minutes at room temperature. Permeabilization of fixed cells was achieved using 0.2% Triton-X in DPBS without calcium or magnesium for 5 minutes at room temperature. Cells were then incubated with anti-human vimentin primary monoclonal antibody at 10 μg/ml in 10% hiFBS in DPBS (blocking buffer) overnight at 4°C. Cells were incubated in 10 μg/ml anti-mIgG Alexa Fluor 568 as the secondary antibody for 2 hours at 4°C. DAPI mounting medium (Vectashield 11-1200, Vector
Laboratories) was used to label cell nuclei. Between all incubation steps, fixation, permeabilization, primary antibody, secondary antibody, and after DAPI treatment, cells were washed in 10% hiFBS in DPBS without calcium or magnesium to block non-specific binding interactions.

Cells labeled for vimentin were visualized using a Texas Red filter (approximately 596 nm excitation and 615 nm emission detection) for vimentin and a DAPI filter (350 nm excitation and 470 nm emission detection) on a Leica 6100B microscope. Cell images were pseudocolored and merged using SimplePCI software.

2.6. CD24 Knock-down via siRNA

Positive transduction control lentiviral particles with Turbo-GFP (SHC003V, Sigma-Aldrich) were used to determine the MOI for siRNA particles. MCF-7 and BT-20 breast cancer cells were seeded at 30% confluency in a 96 well plate and left to adhere overnight. A range of 0.5, 1.0, 2.0, and 5.0 MOI was used in growth medium. Polybrene was added to cells at 8 μg/ml to enhance cellular uptake of viral particles. Two days after the viral constructs were added, puromycin was added to the cells at 2 μg/ml to select for transduced cells. All constructs, vector control, scramble control, Clone A, and Clone B, were lentiviral particles that contained a gene for puromycin resistance. Lentiviral particles provide an advantage over other transducing vectors because they stably integrate into the genome and can infect non-dividing cells. The inclusion of the puromycin resistance gene allowed for specific selection of transduced cells. The vector control did not include a hairpin RNA insert that would stimulate the RNA-induced
silencing complex (RISC), and was used to ensure that the viral vector was not responsible for any changes in cells. The scramble control contains a shRNA sequence that does not react with any known human or mouse genes, but will interact with RISC. This vector is useful to guarantee that any changes observed after transduction are due to the specific silencing of your chosen gene, in our case CD24. Clones A and B, as designated for laboratory use, contain shRNA that becomes siRNA once transcribed and effectively silences CD24 expression. The sequence comprising CD24 shRNA is given below in Table 1. Briefly, each lentiviral particle contained a central polypurine tract, human phosphoglycerate kinase eukaryotic promoter, puromycin resistance gene for mammalian selection as mentioned above, 3' self inactivating long terminal repeat, f1 origin of replication, ampicillin resistance gene for bacterial selection, pUC origin of replication, 5' long terminal repeat, RNA packaging signal, Rev response element, and an shRNA construct, excepting vector control particles. All information describing lentiviral constructs was obtained from the vendor, Sigma-Aldrich.

<table>
<thead>
<tr>
<th>Transduction Particle</th>
<th>Nucleotide Sequence</th>
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<tbody>
<tr>
<td>Clone A (shRNA CD24)</td>
<td>CCGTCCAACTAATGCCACCACCACTCGAGTTGGTGGCGATTAGTTGGATTTTG</td>
</tr>
<tr>
<td>Clone B (shRNA CD24)</td>
<td>CCGAATTTAAATGCCGATATACCTCGAGGTATATCGGCATTAAATTAGTTG</td>
</tr>
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To transduce CD24 siRNA constructs, MCF-7 and BT-20 cells were seeded at 50% confluency in a 96 well plate and left to adhere overnight in normal growth medium. The following treatments were added to the cells the following day: 1 well as a
polybrene control, 1 well treated with the vector control (SHC001V, Sigma-Aldrich), 1 well treated with a scramble control (SHC002V, Sigma-Aldrich), or 2 wells treated with CD24 siRNA clones TRCN0000057674 and TRCN0000245105, or A and B respectively. In addition, untreated cells were used as kill controls upon the addition of puromycin. An MOI of 2.0 was used for all transductions based on results from the pilot experiment using Turbo-GFP lentiviral particles. After 24 hours, transduction medium was removed and new growth medium was added to cells. Growth medium with 2 μg/ml puromycin was added to transduced cells 48 hours after initial transduction. Selection of transduced cells in medium with puromycin lasted 5 days, and cells were subsequently grown in normal growth medium. The level of CD24 knock-down was observed using the CD24 mAb and flow cytometry as described above.

2.7. Flow Chamber Adhesion Assays

The following experiments used a flow chamber adhesion assay to determine the adhesion effects of different conditions on breast cancer cell binding to endothelium and the importance of surface proteins, specifically CD24, in the adhesion cascade. This technique involves perfusing cells in suspension over a plate coated with adherent endothelial cells or immobilized substrate. The advantage of this system is that it can produce physiologically relevant shear stresses on both flowing and immobilized cells, providing a more realistic analysis of adhesion behavior. In this technique, a syringe pump creates shear flow by pulling the cell suspension across an immobilized substrate or cells in a petri dish. A flow chamber is placed inside the petri dish, creating a parallel
plate flow set-up. Adhesion activities between flowing cells and the stationary content of the petri dish are observed using an inverted microscope connected to a monitor, which allows better visualization and recording of the process. Protocols used for the following assays were adapted from Burdick et al. The following diagram (Figure 3) illustrates the components of the flow chamber adhesion assay.

![Diagram of flow chamber adhesion assay set-up.](image)

**Figure 3. Diagram of flow chamber adhesion assay set-up.**

**2.7.1. CD24 antigen capture for breast cancer cells**

Monoclonal antibodies, anti-CD24 or anti-CD44 for control experiments, were added at a volume of 20 μl to 5 mm diameter cloning chambers placed in a petri dish at a concentration of 20 μg/ml in 0.1% BSA in DPBS with calcium and magnesium overnight at 4°C. 50 μl of lysate (from approximately 1 million cells) was then added onto the antibody spots and incubated overnight at 4°C. Spots formed by the cloning chambers
were blocked using 0.1% BSA in DPBS with calcium and magnesium after antibody and cell lysate incubations. Cloning chambers were removed prior to the assay, leaving captured proteins on the surface of the petri dish. Breast cancer cell lysates were obtained by incubating $10^7$ cells per ml lysate buffer in suspension overnight at 4°C. Lysate buffer contained distilled water, 150 mM NaCl, 50 mM Tris-Base, 1 mM EDTA, 20 mg/ml phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor), 0.02% NaN₃, and 1 protease inhibitor cocktail tablet per 50 ml lysis buffer. After incubation in lysis buffer, the solution was centrifuged to separate supernatant from precipitant, and the supernatant was isolated and used in subsequent experiments. The day of the experiment, CHO-E cells were harvested using 5 mM EDTA in DPBS without calcium or magnesium, resuspended at $10^6$ cells/ml, and perfused over the antibody spots at shear rates of 79.3 s⁻¹ and 107.4 s⁻¹. Anti-CD44 was used to capture antigen from the lysate of Ls174T colon carcinoma cells which served as a positive control for adhesion. CD44 on Ls174T cells is known to be an E-selectin ligand. Therefore, CD44 isolated from Ls174T cells would provide a positive control for the experiment by demonstrating how E-selectin binding to captured antigen would appear. To ensure that adhesion interactions were specifically between CD44 or CD24 and E-selectin, isotype controls mouse IgG₁ and mouse IgG₂a were used in place of anti-CD44 and anti-CD24, respectively in control experiments.

An additional control experiment was performed to ensure that the CD24 antibody being used to capture antigen did not block binding interactions between
flowing CHO-E cells and CD24 from breast cancer cell lysate. Through personal communication with Venktesh Shirure at Ohio University, it was known that the antibody used to capture CD44 did not block cancer cell adhesion to endothelial cells. This experiment perfused breast cancer cells incubated with 10 μg/ml anti-human CD24 for 30 minutes over an activated HUVEC monolayer. Breast cancer cells not incubated with the antibody were used as a reference for typical binding behavior.

2.7.2. E-selectin dependence of breast cancer cell binding to hBMEC

A flow chamber adhesion assay was used to determine the role of E-selectin in breast cancer cell binding to bone marrow endothelial cells. IL-1β (R&D Systems) at a concentration of 50 ng/ml for 4 hours in growth medium was used to stimulate E-selectin expression in the human bone marrow endothelial cells. Breast cancer cells MCF-7 and MDA-MB-231 were harvested using 0.025% trypsin EDTA in DPBS without calcium or magnesium, and resuspended at 10^6 cells/ml in 0.1% BSA in DPBS with calcium and magnesium. Subsequently, hBMEC were untreated or treated with 10 μg/ml anti-CD62E for 30 minutes prior to the perfusion of breast cancer cells at a shear rate of 91.6 s⁻¹, which is representative of the shear rate observed in bone marrow microvessels.

2.7.3. Effect of SDF-1α on adhesion to endothelium under shear stress

To evaluate breast cancer cell adhesion to endothelium under shear flow in response to SDF-1α, MCF-7 and MDA-MB-231 cells were perfused at a concentration of 10^6 cells/ml in 0.1% BSA in DPBS with calcium and magnesium over endothelial cells.
following a short incubation with SDF-1α. Sample groups for our experiment included:
(1) breast cancer cells (untreated with SDF-1α) perfused over SDF-1α untreated HUVEC,
(2) breast cancer cells treated with 100 ng/ml SDF-1α for 2 minutes over untreated
HUVEC, (3) untreated breast cancer cells over HUVEC treated with SDF-1α for 2 minutes,
and (4) SDF-1α treated breast cancer cells over SDF-1α treated HUVEC for 2 minutes. In
every experiment, HUVEC were incubated with 1 ng/ml IL-1β for 4 hours prior to use in
adhesion assays in order to stimulate the expression of E-selectin. All SDF-1α
incubations were at 37°C and a concentration of 100 ng/ml for 2 minutes. A shear rate
of 91.6 s⁻¹ was used, which is similar to that observed in bone marrow
microvasculature.  

2.7.4. Adhesion of TGF-β1 and CD24 siRNA treated cells to HUVEC

MCF-7 and BT-20 cells were treated as described above with TGF-β1 for 48 hours
at 5 ng/ml, and harvested using 5 mM EDTA in DPBS without calcium and magnesium
and removed via cell scraper. MCF-7 and BT-20 cells were also transduced with CD24
siRNA, as previously described, to knock-down CD24 expression. After transduction,
these cells were harvested using 0.025% trypsin EDTA in DPBS without calcium or
magnesium. Subsequent to harvest, both TGF-β1 and CD24 siRNA treated cells were
resuspended in 0.1% BSA in DPBS with calcium and magnesium at 10⁵ cells/ml. The cells
were then perfused over activated HUVEC and adhesion behavior was observed. A
shear rate of 91.6 s⁻¹ was used for these experiments.
2.8. E-selectin Affinity of TGF-β1 Treated Cells

MCF-7 and BT-20 breast cancer cells were incubated with mouse recombinant E-selectin Fc chimera (R&D Systems) or human recombinant E-selectin-Fc chimera (R&D Systems) in chimera buffer (DPBS with 5 mM HEPES, 2 mM CaCl₂, and 5 % hiFBS) at 20 μg/ml for 30 minutes on ice. The chimera is a compilation of mouse (or human) E-selectin and the human Fc region of an antibody. As a control, one of the wells of E-selectin chimera treated cells was incubated for 20 minutes in 40 mM EDTA in calcium free buffer on ice to release the E-selectin bond from the cells. Human IgG was used as an isotype control⁶³. Cells were then labeled consecutively with anti-human IgG–biotin (Thermo Scientific) and streptavidin-PE for 20 minutes on ice.

2.9. Statistical Methods

Statistical analysis of the E-selectin dependence of breast cancer cell binding to hBMEC (Figure 9) was performed using one-tailed Students t test, an asterisk denotes significance (*p< 0.05). The statistical significance of the effect of SDF-1α on adhesion (Figure 14) was determined using ANOVA and Tukey with a family error rate of 0.05.
CHAPTER 3. SDF-1α AND ADHESION OF BREAST CANCER CELLS

The microenvironment surrounding a tumor cell plays a substantial role in its behavior and potentially affects its metastatic capacity. The ability of cancer cells to respond to their microenvironment to metastasize may be due to the stemness of the cancer cell. However, current studies have not investigated a link between the cancer stem cell phenotype (CD44+/CD24⁻/low for breast cancer) and the adhesive capacity of cancer cells to the endothelium of a metastatic site such as bone marrow. The contribution of the cancer stem cell phenotype to the metastatic propensity of a cancer cell can be investigated in part by observing the response of stem-like or non-stem-like cancer cell phenotypes to cytokines in the bone marrow. Of particular interest is the gradient of chemokine stromal cell-derived factor 1α (SDF-1α) expressed constitutively in the bone marrow parenchyma and surrounding milieu²¹,⁶⁴. SDF-1α plays a role in the adhesion of migrating cells to endothelium and therefore may affect the metastatic ability of disseminated breast cancer cells. The following experiments investigate the influence of SDF-1α and CXCR4 on breast cancer and endothelial cells.

3.1. Breast Cancer Cells Display Varying CD44 and CD24 Expression

The expression or absence of glycoproteins CD44 and CD24 on the surface of a breast cancer cell can identify cells of the breast cancer stem cell phenotype (CD44⁺/CD24⁻/low). A comparison of non-stem-like and stem cell like breast cancer cells will provide insight into the involvement of the breast cancer stem cell in bone marrow metastasis. In addition, to observe the potential role that CD24 plays in breast cancer
cell adhesion to endothelium, it is necessary to know whether the cells do or do not express CD24 prior to experimentation. Flow cytometry was used for this analysis. Figure 4 demonstrates the expression levels of these glycoproteins in the seven cell lines observed: BT-20, Hs578T, MCF-7, MDA-MB-231, MDA-MB-468, T47-D and ZR-75-1. The cell lines could be differentiated by expression profile, specifically: CD44+/CD24+, CD44+/CD24−/low, and CD44−/CD24+, with some overlap. They were characterized by the expression of the majority of the population, but some heterogeneity in expression was observed. The cell lines that most resembled breast cancer stem cells in phenotype were Hs578T and MDA-MB-231 because they had positive CD44 expression and relatively low levels of CD24. Expression levels of CD44 were similar between the two cell types, but Hs678t cells exhibited slightly more cells that were CD44+/CD24+. Above basal levels of both CD44 and CD24 were observed in BT-20, MCF-7, and MDA-MB-468 cells. BT-20 cells showed very high CD24 expression, but CD44 levels were similar to MCF-7 cells. MDA-MB-468 cells had by far the highest expression of both glycoproteins. A profile opposite of the cancer stem cell phenotype, CD44 negative and CD24 positive, was seen in the T47-D and ZR-75-1 cells. T47-D cells exhibited CD24 levels similar to those seen in MCF-7 and BT-20 cells, but ZR-75-1 showed extremely high CD24 expression. CD44 expression was almost completely absent in both cell lines. The average expression levels of CD44 and CD24 can be seen in Figure 5.
Figure 4. MDA-MB-231 and Hs578T cells are putative cancer stem cells (CD44+/CD24−/low). T47-D and ZR-75-1 are CD44+/CD24−, and MCF-7, MDA-MB-468, and BT-20 cells express both CD44 and CD24. Flow cytometry and immunofluorescence was used to label CD44 and CD24 on various breast cancer cell lines. Percentage of cells found in the majority population of CD44/CD24 expression is indicated in the upper right quadrant. Plots are representative of the average population distribution for at least n=3 independent experiments.

Figure 5. Average expression of CD44 (a) and CD24 (b) on breast cancer cells determined by flow cytometry and immunofluorescence. Expression level is representative of the mean fluorescence intensity (MFI). Average MFI for isotype controls was 2.97 for mlgG2b (for CD44) and 1.22 for mlgG2a (for CD24). Data are mean values +/- SEM for at least n=3.
3.2. Breast Cancer Cells Express CXCR4

The interaction of chemokine receptor CXCR4 and its ligand SDF-1α is increasingly implicated in the ability of cancer cells to migrate to bone marrow endothelium\(^6\). CXCR4 binds exclusively to SDF-1α indicating that the normal presence of SDF-1α in the bone marrow environment may facilitate the adhesion of migrating cells expressing CXCR4 to endothelial cells\(^20\). Furthermore, SDF-1α enhances the migratory and invasive properties of aggressive breast cancer cells\(^65\). The first step to determine the role of the SDF-1α – CXCR4 axis in cancer metastasis was the characterization of CXCR4 expression on the surface of breast cancer cells.

Analysis of four different CXCR4-specific monoclonal antibodies was performed in order to characterize the surface expression of this important adhesion regulator on breast cancer cells. The antigen identified by anti-CXCR4 clone 44717 showed the highest levels of expression compared to the other clones, and was used for the remainder of experiments that observed CXCR4 levels. Figures 6 and 7 illustrate the levels of CXCR4 binding found in the breast cancer cells. Given that MDA-MB-231 and MCF-7 cells showed the highest levels of expression, these two cell lines were used in subsequent experiments to determine the effect of SDF-1α on the adhesion behavior of breast cancer cells. Almost all cell lines observed showed relatively high expression of CXCR4 on the cell surface (approximately 50% or more cells were positive for CXCR4); BT-20, Hs578T, and ZR-75-1 were the exception.
Figure 6. Breast cancer cells express varying levels of CXCR4. Cancer stem cell like MDA-MB-231 cells and non stem-like MCF-7 cells express the highest levels of CXCR4 indicating they may be the most responsive to SDF-1α. CXCR4 expression on breast cancer cell lines was detected via flow cytometry and anti-CXCR4 monoclonal antibody (clone 44717), shown with isotype control. Plots are representative of the average CXCR4 expression for at least n=3 independent experiments. Percentage of cells expressing CXCR4 as determined by the number of cells with greater fluorescence intensity than 98% of the isotype control is indicated in the top right corner.

Figure 7. Average expression of CXCR4 on breast cancer cell lines determined by flow cytometry and immunofluorescence. Anti-CXCR4 antibody clone 44717 was used to label cells. Expression level is representative of the mean fluorescence intensity. Data are representative of mean values +/- SEM for at least n=3.
3.3. E-selectin Dependence of Adhesion to Endothelial Cells

3.3.1. **IL-1β stimulated bone marrow endothelial cells express E-selectin**

In adhesion analysis, it is optimal to use multiple types of endothelial cells as they are known to produce varying expression levels of different adhesion molecules and are in general physiologically distinct, even when stimulated similarly\(^6\). The use of human bone marrow endothelial cells (hBMEC) in flow chamber adhesion analyses would ideally provide a more physiologically relevant model of cancer cell adhesion to bone marrow *in vivo* as compared to HUVEC adhesion. Therefore, in addition to HUVEC, adhesion assays were performed using hBMEC. For the sake of being able to compare hBMEC results with previous results using HUVEC, it was important to obtain comparable E-selectin expression in the two types of cells upon stimulation prior to further experimentation. Flow cytometry was used to determine the expression of E-selectin of the two types of endothelial cells under normal conditions and after stimulation with Interleukin-1β (IL-1β). A concentration of 50 ng/ml of IL-1β was used for hBMEC stimulation based on previous studies by Dimitroff et al\(^6\) and pilot studies in our laboratory to determine the optimal concentration for flow adhesion analysis. HUVEC were stimulated with 1 ng/ml IL-1β, a concentration that results in E-selectin expression on these cells\(^6\). The cells were incubated in stimulation medium for 4 hours prior to analysis. As expected, HUVEC and hBMEC expressed similar levels of E-selectin expression upon stimulation. From the data shown in Figure 8, it can be
inferred that any difference in adhesion behavior between the two cell types during flow adhesion analysis is due to some factor other than E-selectin levels on the cells.

**Figure 8.** HUVEC and hBMEC express similar levels of E-selectin upon stimulation with IL-1β. This data allows for the comparison of HUVEC and hBMEC in adhesion assays to determine specific molecules potentially involved in bone marrow metastasis. Flow cytometry and immunofluorescence were used to determine E-selectin expression in unstimulated and IL-1β stimulated HUVEC (a) and hBMEC (b) compared to isotype control. Data are the result of n=1 independent experiment.

3.3.2. Breast cancer cell binding to hBMEC is dependent on E-selectin

It has been previously shown that breast cancer cell binding to HUVEC is an E-selectin dependent process. From the experiment shown in Figure 8, we also know that hBMEC express E-selectin upon stimulation with IL-1β. This finding led to experimentation to observe if breast cancer cell binding to hBMEC utilizes a similar mechanism dependent upon E-selectin expression. In order to use hBMEC in future experiments to determine adhesion behavior, it is crucial to know the components involved in the adhesion process. An experiment similar to the HUVEC flow adhesion assay was used to determine the E-selectin dependence of binding interactions between breast cancer cells in flow and hBMEC. The results of this study detailed in Figure 9
show that E-selectin plays an integral role in the adhesion between MCF-7 breast cancer cells and hBMEC. In addition, the level of cell interactions resembled those found using HUVEC. MDA-MB-231 cells did not exhibit significant binding interaction with hBMEC, a behavior similar to that seen in HUVEC experiments (personal communication with Venktesh Shirure, Ohio University).

Figure 9. Adhesion of MCF-7 cells to hBMEC is dependent on E-selectin because adhesion does not occur when E-selectin is blocked. The adhesion behavior of MCF-7 and MDA-MB-231 breast cancer cells to human bone marrow endothelial cells (hBMEC) was determined by a flow chamber adhesion assay. (a) MCF-7 adhesion to hBMEC is significantly inhibited when E-selectin on hBMEC is blocked via anti-E-selectin monoclonal antibody (*p<0.05). (b) MDA-MB-231 cells do not adhere to hBMEC regardless of E-selectin blocking. hBMEC were stimulated with IL-1β to express E-selectin 4 hours prior to use in the adhesion assay. Binding interactions are the number of breast cancer cells/minute-mm² that bind to hBMEC. No mAb group refers to breast cancer cells perfused over hBMEC without blockage of E-selectin, the E-selectin mAb group used hBMEC incubated with a function blocking E-selectin antibody (anti-CD62E). Data are mean values +/- SEM for n=3.
3.3.3. **VLA-4 is not involved in breast cancer cell binding to endothelium**

Three cell lines (MCF-7, MDA-MB-231, T47-D) were analyzed for expression of the α4 and β1 subunits of Very Late Antigen-4 (VLA-4) to confirm that VLA-4 – VCAM-1 interactions were not responsible for the binding interaction between breast cancer cells and HUVEC. Combined, the α4β1 molecule is VLA-4 and a ligand for Vascular Cell Adhesion Molecule -1 (VCAM-1). Flow cytometry analysis of the expression of α4 (CD49d) and β1 (CD29) on the surface of breast cancer cells allows the determination of the involvement of VCAM-1 in adhesion. The resulting data (Figure 10) showed that breast cancer cell lines MCF-7, MDA-MB-231, and T-47D do express the β1, but not α4, subunit of VLA-4. Therefore, VLA-4 does not play a role in the adhesion of these breast cancer cells to endothelial cells. Figure 11 shows the average α4 and β1 expression of the breast cancer cells. β1 expression on T47-D cells varied across testing much more than on the other cell lines.
Figure 10. Breast cancer cell lines express the β1 but not the α4 subunit of adhesion molecule VLA-4. This indicates that the interaction between VLA-4 and endothelial VCAM-1 is not likely involved in the adhesion of untreated breast cancer cells to endothelium. Flow cytometry and immunofluorescence were used to analyze the expression of α4 and β1 on breast cancer cells. The percentage of cells with positive expression for each molecule is listed in the top right corner. Values represent the average of n=3 independent experiments.

Figure 11. Average expression of α4 (a) or β1 (b) subunits of VLA-4 on breast cancer cell lines determined by flow cytometry and immunofluorescence. Expression level is representative of the mean fluorescence intensity. Data are mean values +/- SEM for n=3 independent experiments.
3.4. CD24 is not an E-selectin Ligand on Breast Cancer Cells

The breast cancer stem cell phenotype is commonly characterized by a reduced level of CD24 expression on the cell surface. Interestingly, CD24 on breast cancer cells is a ligand for P-selectin expressed on inflamed endothelium, but its reactivity with E-selectin in terms of breast cancer adhesion is unknown. In order to determine if CD24 plays a role in E-selectin mediated adhesion, an antigen capture assay was used with four breast cancer cell lines (BT-20, MCF-7, MDA-MB-468, and ZR-75-1). E-selectin expressing CHO-E cells were perfused over CD24 antigen spots to determine if CD24 is an E-selectin ligand. CD44 captured from Ls174T colon carcinoma cells was used as a control for positive adhesion interactions to verify assay functionality, because CD44 on Ls174T is a known ligand for E-selectin. As the results from Figure 12 indicate, CD24 does not appear to be a ligand for E-selectin in any of the breast cancer cells analyzed. To ensure the antibody used to capture CD24 from cell lysates was not blocking adhesion, control experiments were performed. A high level of E-selectin expression (mean fluorescence intensity of 147 +/- SEM of 16.7) on the surface of the CHO-E cells was observed prior to use in flow adhesion analysis. To ensure that the CD24 antibody was not blocking adhesive interactions between other molecules in the breast cancer cells with E-selectin, an experiment perfused breast cancer cells incubated with anti-CD24 over IL-1β activated HUVEC. Binding interactions between anti-CD24 treated breast cancer cells and HUVEC were similar to those seen when no antibody was used, indicating that the CD24 antibody was not interfering with E-selectin dependent
adhesion. The binding interactions for CD24 treated or untreated breast cancer cells to HUVEC are given in Figure 13.
Figure 12. **CD24 is not an E-selectin ligand for breast cancer cells.** E-selectin on the surface of CHO-E cells does not interact with CD24 from breast cancer cells to facilitate adhesion. A flow chamber adhesion assay was used with shear rates of 79.3 s⁻¹ (a) and 107.4 s⁻¹ (b) to determine if CD24 captured from breast cancer cell lysates is a ligand for E-selectin. CD44 captured from LS174t cells serves as a positive control for binding interaction and assay utility because CD44 on Ls174T cells is a known E-selectin ligand. Binding interactions are the number of CHO-E cells/minute-mm² that bind to captured CD24 from breast cancer cell lysates. Values are +/- SEM for at least n=3 independent experiments.
Figure 13. **CD24 capture antibody does not block adhesion interactions between breast cancer cells and HUVEC.** A flow chamber adhesion assay was used to determine if the antibody used to capture CD24 from breast cancer cell lysates (in Figure 12) blocked the E-selectin binding epitope on CD24. Whole breast cancer cells were incubated with CD24 capture antibody or untreated and perfused over HUVEC stimulated by IL-1β to express E-selectin. Binding interactions are the number of breast cancer cells/minute-mm² that bind to HUVEC. Values are representative of n=1 experiment.

### 3.5. Breast Cancer Cells Show Enhanced Adhesion after SDF-1α Treatment

It is reported in literature that CXCR4 and its exclusive ligand SDF-1α are involved in the transition of a cell from rolling to firm adhesion on endothelium	extsuperscript{20,21}. SDF-1α is constitutively expressed in the bone marrow microenvironment; therefore we sought to determine if short-term exposure to this chemokine affects the adhesion capacity of breast cancer cells to IL-1β activated endothelial cells. Previous studies have treated only endothelial cells with SDF-1α and perfused untreated cells over them	extsuperscript{26}. However, by incubating both the endothelial cells and the cancer cells in flow with this chemokine, a more physiologically representative milieu may be obtained. Sample groups for our experiment included: (1) breast cancer cells (untreated with SDF-1α) perfused over SDF-
1α untreated HUVEC, (2) breast cancer cells treated with 100 ng/ml SDF-1α for 2 minutes over untreated HUVEC, (3) untreated breast cancer cells over HUVEC treated with SDF-1α for 2 minutes, and (4) SDF-1α treated breast cancer cells over SDF-1α treated HUVEC for 2 minutes. HUVEC were used in these experiments because they are relatively easy to grow and are established in adhesion assays\textsuperscript{69,70}. In all experiments, endothelial cells were activated with IL-1β prior to use. Figure 14 displays the number of binding interactions per minute per area that occurred for each sample group. There was not a significant difference in binding observed between any of the treatment groups. However, when breast cancer cells were incubated with SDF-1α, regardless of HUVEC treatment, they showed a consistent increase in adhesion to HUVEC indicating a trend for SDF-1α involvement in cancer cell adhesion to endothelium.
**Figure 14. SDF-1α enhances the adhesion between MCF-7 cells and HUVEC.** A flow chamber adhesion assay was used to determine the effect of SDF-1α on the adhesion between breast cancer cells and endothelial cells. (a) MCF-7 cells treated with SDF-1α show a non-statistically significant increase in adhesion to HUVEC, independent of HUVEC treatment, compared to the untreated and HUVEC only treated samples. (b) SDF-1α does not have an effect on the adhesion behavior MDA-MB-231 cells. Binding interactions are the number of breast cancer cells/minute-mm² that bind to HUVEC. HUVEC were stimulated with 1 ng/ml IL-1β 4 hours prior to use in adhesion assays. BCC is breast cancer cells, EC is endothelial cells, UnTr is untreated with SDF, and SDF group is treated with SDF. Data are mean values +/- SEM for n=3 independent experiments.

**3.6. Intracellular Changes in Breast Cancer Cells in Response to SDF-1α**

**3.6.2. Actin polymerization occurs in response to SDF-1α**

In addition to calcium mobilization, actin polymerization at the edge of the cell is a common response to chemokine stimulation. Further analysis of the behavior of breast cancer cells in response to SDF-1α was observed via actin polymerization. Cells were incubated with SDF-1α and labeled for F-actin while in suspension to produce a scenario more similar to that observed in vivo than adherent cell treatment would provide. Incubation times for SDF-1α ranged from 15 to 600 seconds, with an untreated sample to provide baseline actin expression. Figures 15-17 show the microscopy images
from this experiment for MCF-7, MDA-MB-231, and BT-20 cells. It can be observed that MCF-7 and BT-20 breast cancer cells show actin cluster formation at the cell periphery in the form of pseudopodia. This response appears to increase with incubation time, stabilizing around 2 to 5 minutes. However, for MDA-MB-231 cells no actin polymerization was visualized. Actin polymerization experiments were performed concurrently with calcium mobilization. Therefore, the formation of actin pseudopodia in response to SDF-1α indicates that the SDF-1α used in both calcium mobilization and actin polymerization is biologically active. To ensure that the actin polymerization was not an artifact of cells being in suspension for an extended period, a validation study was performed that exactly resembled the SDF-1α treatment analysis in time and protocol except that no SDF-1α was added. Figure 18 shows these results for BT-20 cells. In this experiment cells did not exhibit pseudopodia to the same extent as for SDF-1α treated cells, indicating that the response seen in treated cells was due to SDF-1α interaction, not time in suspension.
Figure 15. MCF-7 cells form actin pseudopodia in response to SDF-1α treatment. MCF-7 cells treated in suspension with 100 ng/ml SDF-1α and labeled with phalloidin for actin polymerization. Images show varying treatment times (a) untreated, (b) 15 s, (c) 30 s, (d) 60 s, (e) 120 s, (f) 300 s, (g) 600 s.
Figure 16. **BT-20 cells form actin pseudopodia in response to SDF-1α treatment.** BT-20 cells treated in suspension with 100 ng/ml SDF-1α and labeled with phalloidin for actin polymerization. Images show varying treatment times (a) untreated, (b) 15 s, (c) 30 s, (d) 60 s, (e) 120 s, (f) 300 s, (g) 600 s.
Figure 17. MDA-MB-231 cells do not form actin pseudopodia in response to SDF-1α treatment. MDA-MB-231 cells treated in suspension with 100 ng/ml SDF-1α and labeled with phalloidin for actin polymerization. Images show varying treatment times (a) untreated, (b) 15 s, (c) 30 s, (d) 60 s, (e) 120 s, (f) 300 s, (g) 600 s.

Figure 18. BT-20 cells do not form actin pseudopodia in the absence of SDF-1α. Control for SDF-1α specific induction of actin polymerization. BT-20 cells in suspension labeled with phalloidin. Images show varying suspension times (a) 0 s, (b) 60 s, (c) 300 s.
3.6.1. Calcium mobilization did not occur after SDF-1α stimulation

Intracellular calcium release typically occurs within a cell after stimulation with SDF-1α. Therefore, measuring calcium mobilization in response to treatment with SDF-1α is a common way to determine the reactivity of a specific cell type to this chemokine\textsuperscript{71}. To visualize whether SDF-1α interacted with breast cancer cells, calcium mobilization was observed following SDF-1α stimulation via spectrophotometer analysis of a fluorescent calcium-binding probe. Ionomycin was used as a control stimulant for intracellular calcium release. Figure 15 details the results of the experiment. Although calcium mobilization was detected after the addition of ionomycin compared to the untreated sample, no change in calcium level was visible for the cells after the addition of SDF-1α over the course of 10 minutes. This was an unexpected finding, however, literature reports that different cell types have varying responses to chemokines\textsuperscript{25,72}. The cells treated with ionomycin exhibited higher levels of calcium release in a concentration-dependent manner.
Figure 19. SDF-1α does not mobilize calcium in MCF-7 and MDA-MB-231 breast cancer cells. A calcium mobilization assay was used to determine if SDF-1α stimulates breast cancer cells causing intracellular calcium release in MCF-7 (a) and MDA-MB-231 (b) cells. Calcium levels were analyzed using Fluo-3am, which fluoresces upon binding to free calcium in the cell. Ionomycin served as a control because it is known to cause the release of intracellular calcium. A spectrophotometer detected Fluo-3 levels in untreated cells or after the addition of ionomycin or SDF-1α. Y-axis shows the units of fluorescence detected at an emission wavelength of 530 nm. Values are a result of n=1 in triplicate.

CHAPTER 4. CANCER STEM CELLS

To further investigate the role of the cancer stem cell in metastatic behavior, we sought to induce a more mesenchymal (stem-like) state in breast cancer cells.
Transforming growth factor-beta 1 (TGF-β1) has been shown to induce an epithelial mesenchymal transition (EMT) in normal mammary epithelial cells, causing a reversion to a stem cell state\textsuperscript{37}. Simply stated, the addition of TGF-β1 to cells can initiate the loss of epithelial characteristics, such as E-cadherin necessary for cell-to-cell adhesion, and gain mesenchymal cell characteristics such as vimentin\textsuperscript{73}. Therefore breast cancer cells not of the putative stem cell phenotype, BT-20 and MCF-7, were treated with TGF-β1 to induce epithelial mesenchymal transition to create cells similar to cancer stem cells.

In addition to TGF-β1 treatment to induce a stem-like state in breast cancer cells, siRNA was used to knock-down CD24 expression in CD44\textsuperscript{+}/CD24\textsuperscript{−} cell lines BT-20 and MCF-7. This technique would potentially produce breast cancer cells with the putative stem cell phenotype of CD44\textsuperscript{+}/CD24\textsuperscript{−} without directly altering other properties of the cell. Both methods provide insight into the complex nature of breast cancer metastasis.

4.1. TGF-β1 Confers Mesenchymal-like Properties to Breast Cancer Cells

Further understanding about the role of breast cancer cells in metastasis to bone marrow requires the consideration of the interaction of the cells with their microenvironment and the specific adhesion properties necessary to recruit migrating cells to endothelium. The studies in Chapter 3 observed how breast cancer cells, both of and not of the putative stem cell phenotype, responded to a chemokine in the bone marrow environment. The set of experiments to follow analyzed changes in the mobilization and adhesion behavior when epithelial-like breast cancer cells were altered to exhibit stem-like characteristics. These experiments utilized techniques to make
CD44+/CD24+ breast cancer cells more closely resemble breast cancer stem cells. Initially, TGF-β1 was used to induce an epithelial mesenchymal transition (EMT) in breast cancer cells at optimized conditions of 5 ng/ml for 48 hours. Additionally, CD24 expression was silenced using siRNA. This method would coerce a CD44+/CD24+ breast cancer cell to become CD44+/CD24-, and thereby it would phenotypically resemble a breast cancer stem cell.

4.1.1. E-cadherin expression is decreased after TGF-β1 treatment

There are many markers that aid in the identification of an epithelial or mesenchymal cell. Many of these molecules can be used to identify EMT that has occurred in cells. Commonly used identifiers of EMT are the loss of epithelial marker E-cadherin and gain of mesenchymal marker vimentin $^{37,42,47,74,75}$. E-cadherin expression was utilized as a primary indicator that TGF-β1 had altered breast cancer cells because it is a surface molecule and relatively easy to detect. Vimentin is an intermediate filament expressed in the cytoplasm of a cell, and was used as a secondary marker of EMT. In addition, morphology changes can be used to indicate a transition to a mesenchymal cell. Figure 20 indicates a TGF-β1 induced reduction in E-cadherin expression for BT-20 cells as indicated by a retrospective shift in the population. A similar reaction was not visualized for MCF-7 cells. The results for both BT-20 and MCF-7 cells were consistent across 3 independent experiments.
Figure 20. TGF-β1 decreases E-cadherin expression in BT-20 breast cancer cells. A decrease in E-cadherin is indicative of epithelial mesenchymal transition. The expression of E-cadherin in untreated and TGF-β1 treated BT-20 (a) and MCF-7 (b) breast cancer cells was determined by flow cytometry and immunofluorescence. Cells were incubated in normal growth medium (untreated), growth medium plus 5 μl of 4mM HCl in DPBS for a final concentration of 2 μM (control), or growth medium plus 5 μl of 10 μg/ml TGF-β1 for a final concentration of 5 ng/ml TGF-β1 (TGF-β1). Stock TGF-β1 was reconstituted in 4 mM HCl per manufacturer’s protocol, for a final concentration of 2 μM in medium on treated cells, thus a control was used to ensure that this was not altering the cells. The control treated sample is not shown because it had almost identical distribution to the untreated sample. Histograms are representative of n=3 independent experiments.

4.1.2. Breast cancer cells treated with TGF-β1 undergo a morphology change

To further characterize the changes in breast cancer cells caused by incubation with TGF-β1, cell morphologies were observed. Cells that have undergone EMT are reported as spindle-shaped as compared to the epithelial shape they had prior to the transition\cite{39,40}. The phase contrast microscopy images in Figure 21 illustrate the typical morphology of BT-20 cells untreated and treated with 5 ng/ml TGF-β1 for 48 hours, the conditions found to be optimal for a reduction in E-cadherin expression. These pictures represent the cells characterized for E-cadherin expression, as detailed above, prior to flow cytometry analysis. Similar morphological changes were observed in additional experiments with varying degrees of altered cell-shape. Although this is not conclusive
evidence for EMT, the changes seen indicate that TGF-β1 is altering the cells in some manner.

![Figure 21. TGF-β1 alters the morphology of BT-20 breast cancer cells.](image)

**Figure 21. TGF-β1 alters the morphology of BT-20 breast cancer cells.** The change in cell shape from epithelial to spindle-like implicates the occurrence of EMT. Phase microscopy was used to determine the morphological difference between untreated (a) and TGF-β1 treated BT-20 cells. Images were taken of cells from Figure 20 immediately prior to their harvest for E-cadherin detection and represent the average morphological change seen after 48 hours of TGF-β1 treatment at 5 ng/ml in normal growth medium.

### 4.1.3. Vimentin expression is increased in TGF-β1 treated breast cancer cells

In addition to E-cadherin loss and morphological changes, the expression of vimentin is also a marker of EMT. Fluorescent microscopy was able to show vimentin intracellularly and was used to analyze differences in expression for breast cancer cells when untreated or treated with TGF-β1. As shown in Figure 22, MDA-MB-231 breast cancer cells were found to always express clearly visible vimentin filaments in the cytoplasm, regardless of TGF-β1 addition. BT-20 cells showed no expression of vimentin in the cytoplasm when untreated, but when treated with 0.25 ng/ml TGF-β1, vimentin was visible although not as structurally organized as in MDA-MB-231 cells. Perhaps
longer incubations at this concentration of TGF-β1 would allow for increased formation of vimentin filaments. Analysis for EMT markers was performed with the conditions of 5 ng/ml TGF-β1 treatment for 48 hours, the same used in other experiments. However, under these treatment conditions, no changes in vimentin could be seen. Given the changes seen in E-cadherin and vimentin expression, and in morphology, it can be said that TGF-β1 is altering BT-20 breast cancer cells toward a mesenchymal state, but the transition to a mesenchymal cell may not be complete. However, it may be that EMT exists in a continuum where differing concentrations and incubation times result in different mesenchymal properties attained by the cells.
Figure 22. TGF-β1 induces vimentin expression in BT-20 cells. Vimentin in BT-20 and MDA-MB-231 breast cancer cell lines untreated or treated with TGF-β1 was determined by microscopy and immunofluorescence. Blue indicates the nucleus labeled with DAPI and vimentin is labeled in red via anti-vimentin monoclonal antibody. Cells were treated with 0.25 ng/ml TGF-β1 for 24 hours and labeled in adherent monolayers for vimentin.

4.2. CD24 Expression is Decreased in siRNA Cells

In order to make CD44+CD24+ breast cancer cells more stem-like in surface phenotype, siRNA was used to knock-down the expression of CD24. This process involved incubating breast cancer cells (MCF-7 and BT-20) with lentiviral particles containing shRNA to CD24. While complete knockdown of the glycoprotein was not achieved, Figure 23 shows the flow cytometry analysis that revealed a decrease in expression affiliated with the addition of siRNA. Therefore, these cells were used in
subsequent experiments to determine if the downregulation of CD24 played a role in the adhesion behavior of breast cancer cells.

![CD24 expression graphs](image)

**Figure 23.** CD24 siRNA reduces CD24 expression on the surface of BT-20 and MCF-7 breast cancer cells. CD24 expression in untreated and siRNA treated BT-20 (a) and MCF-7 (b) breast cancer cells determined by flow cytometry and immunofluorescence. Clone B CD24 siRNA produces a reduction in CD24 expression. (b) MCF-7 cells also showed a reduced level of CD24 expression with Clone B CD24 siRNA. Expression level is representative of the mean fluorescence intensity. Data are mean values +/- SEM for n=4 independent experiments.

### 4.3. EMT and siRNA Cells do not Display Differential Adhesion Interactions from Normal Cells

Earlier experiments demonstrated E-selectin as an integral part of cell adhesion to endothelium. It would therefore likely play a role in the attachment of breast cancer cells to bone marrow endothelium, and subsequent invasion of the parenchyma. In order to determine if TGF-β1 treatment or CD24 knock-down might affect the adhesion capacity of breast cancer cells, these cells were perfused over activated HUVEC and their adhesion behavior was observed.
4.3.1. Breast cancer cells treated with TGF-β1, CD24 siRNA, or untreated show similar adhesion to endothelium

After one experiment, it was found that treatment with TGF-β1 caused a decrease in E-cadherin expression but did not affect the adhesion capacity of BT-20 cells to HUVEC. Additionally, CD24 knock-down did not affect adhesion behavior of BT-20 cells. An important consideration, however, is that neither E-cadherin nor CD24 were completely removed from the cell surface indicating a stem cell state was not completely achieved. Consequently, the stem-like changes that were observed in breast cancer cells may not have been sufficient to affect adhesion capacity.

In addition to BT-20 cells, MCF-7 cells were tested for adhesion capacity after treatment with TGF-β1 or CD24 siRNA. Unexpectedly, the cells from ATCC did not show any adhesion interaction with HUVEC regardless of treatment. This result is inconsistent with data from previous experiments that indicated MCF-7 cells have a high binding capacity to HUVEC under shear flow (Figure 14, Tozeren et al, and Moss et al[11,76]). It is likely that this occurrence is due to differences in the low-passage MCF-7 cells directly purchased from ATCC used in these experiments and the original MCF-7 cells from the Sackstein laboratory used in previous flow chamber experiments. Figure 24 details these results.
Figure 24. A single experiment does not show altered adhesion behavior in untreated and TGF-β1 or CD24 siRNA treated BT-20 and MCF-7 breast cancer cells. A flow chamber adhesion assay was used to determine the effect of TGF-β1 treatment or CD24 siRNA knock-down on adhesive interactions between HUVEC and BT-20 (a) or MCF-7 (b) breast cancer cells. HUVEC were stimulated to express E-selectin 4 hours prior to use in adhesion assays. Binding interactions are the number of breast cancer cells/minute-mm² that bound to HUVEC. Data are representative of 1 independent experiment.

4.3.2. E-selectin affinity is consistent for normal, EMT, and CD24 knockdown breast cancer cells

The finding that TGF-β1 treated cells and CD24 knockdown cells did not adhere any differently to HUVEC in the flow chamber adhesion assay indicates either these changes do not affect cell adhesion or cell treatment and shear conditions are not optimized to see an effect. Therefore, to observe any differences in the affinity of these cells for E-selectin in a shear-free environment, they were incubated with an E-selectin-IgG chimera and used in flow cytometry. This method decreases the specificity of E-selectin binding for a particular physiological environment because it removes the shear force, which is an integral factor in cell adhesion to endothelium. However, it does enable a higher sensitivity of binding analysis. The data in Figures 25 and 26 shows that
E-selectin affinity is not altered due to incubation with TGF-β1 or knock-down of CD24. For this experiment, only clone B CD24 siRNA cells were used because they had shown a significant decrease in CD24 from the vector and scramble controls. However, it can also be observed from Figures 20 and 23 that neither E-cadherin nor CD24 is entirely depleted from the surface of these cells. Therefore, perhaps under ideal conditions where a complete EMT occurs and/or CD24 is not expressed resulting in a more stem-like cell, the E-selectin affinity would be altered.

**Figure 25.** TGF-β1 does not alter the E-selectin affinity of BT-20 and MCF-7 breast cancer cells. This indicates that the breast cancer stem cell phenotype does not affect cancer cell adhesion to endothelial E-selectin. Measurement of E-selectin affinity for BT-20 (a) and MCF-7 (b) breast cancer cells was determined by flow cytometry and immunofluorescence. Cells were incubated for 48 hours in normal growth medium (untreated), growth medium plus 5 μl of 4mM HCl in DPBS for a final concentration of 2 μM (control), or growth medium plus 5 μl of 10 μg/ml TGF-β1 for a final concentration of 5 ng/ml TGF-β1 (TGF-β1). Affinity was analyzed by measuring the amount of E-selectin Fc chimera that bound to breast cancer cells in suspension under static conditions. Bound E-selectin level is representative of the mean fluorescence intensity. Data are mean values +/- SEM for n=3 independent experiments.
Figure 26. Knock-down of CD24 via siRNA does not alter the E-selectin affinity of BT-20 and MCF-7 breast cancer cells. This indicates that the lack of CD24 on breast cancer stem cells does not play a role in their adhesive capacity to endothelial E-selectin. Measurement of E-selectin affinity for BT-20 (a, c) and MCF-7 (b, d) breast cancer cells was determined by flow cytometry and immunofluorescence. Affinity was analyzed by measuring the amount of E-selectin Fc chimera that bound to breast cancer cells in suspension under static conditions. Bound E-selectin level is representative of the mean fluorescence intensity. Breast cancer cells were incubated with mouse recombinant E-selectin IgG chimera (a, b), data representative of mean values of n=2 independent experiments +/- standard deviation, or human E-selectin IgG chimera (c, d), data representative of n=1 independent experiment.

4.3.3. Spontaneously CD24− MCF-7 cells demonstrate similar adhesion interactions as normal MCF-7 cells

Routine characterization of breast cancer cells revealed at one point that the original MCF-7 line had spontaneously lost CD24 expression. As a result, new MCF-7
cells directly obtained from ATCC were used in the experiments that followed, including Figures 20, 21, and 23-26. Figures 4-7, and 9-16 used the original MCF-7 cell line from the laboratory of Dr. Sackstein. As the nature of this investigation was to determine how breast cancer stem cells may play a role in metastasis, this was an interesting problem in that non-stem like (MCF-7) suddenly exhibited the putative breast cancer stem cell profile. To investigate whether the loss of CD24 in the original MCF-7 cells resulted in altered adhesion behavior to endothelial cells, these cells were perfused over activated HUVEC in a flow chamber adhesion assay. The result was a level of binding interactions similar to that seen for the previously tested CD24 positive original MCF-7 cells. Specifically, for the CD24 negative cells, 53.1 cells/min-mm² interacted with HUVEC as compared to the average of normal, CD24 positive cells of 46.1 cells/min-mm² (as seen in the control for flow chamber assays with SDF-1α incubation). Therefore, the spontaneous disappearance of CD24 on the surface of CD24 cells did not appear to have an effect in the adhesion properties of MCF-7 cells.

4.4. Summary of Treatments and Responses

The following chart, Table 2, details the different treatments applied to breast cancer cells in the previous experiments. The formerly described issue with MCF-7 cells spontaneously losing CD24 expression resulted in the use of two different lines of MCF-7 cells throughout this study. The MCF-7 cells labeled “Sackstein” are the original cell line obtained from the Sackstein laboratory. The MCF-7 cells labeled “ATCC” were purchased directly from ATCC.
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<th>CXCR 4</th>
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CHAPTER 5. DISCUSSION & CONCLUSIONS

5.1. Discussion

The study above investigated how two very different cytokines, SDF-1α and TGF-β1, affect changes in breast cancer cells. Stromal cell-derived factor-1α has been shown to play a crucial role in the ability of migrating leukocytes and cancer cells to adhere to endothelium and subsequently move into the tissue parenchyma\textsuperscript{22,77}. This is particularly true for bone marrow endothelium, as it constitutively expresses an SDF-1α gradient into the surrounding milieu\textsuperscript{21}. Hematopoietic stem cells are known to utilize the CXCR4 expressed on their surface to interact with an SDF-1α gradient in order to home and adhere to bone marrow\textsuperscript{21}. It is therefore logical that breast cancer cells expressing CXCR4 would take advantage of this process and use it to facilitate bone marrow metastasis. Given this information, we embarked on a journey to discover specifically if SDF-1α affected the ability of breast cancer cells to adhere to endothelium.

Initially, it was crucial to characterize the expression of CXCR4 on the surface of breast cancer cells. While the expression of CXCR4 on some of the breast cancer cell lines analyzed has been shown in literature, analysis of all seven cell lines has not previously been performed. Without the expression of this chemokine receptor, the migrating cells would not be able to interact with SDF-1α. While many of the breast cancer cells analyzed expressed CXCR4, MCF-7 and MDA-MB-231 expressed it at the highest levels. In addition, these cells are characterized as CD44\textsuperscript{+}/CD24\textsuperscript{+} or non-stem like, and CD44\textsuperscript{+}/CD24\textsuperscript{−} or stem-like, respectively. This profile of surface marker
expression is important because the discovery of breast cancer stem cells by Al Hajj et al.\textsuperscript{27} has indicated that there may be a specific type of cancer cell responsible for metastasis, and that the stemness of a cell may determine its metastatic inclination\textsuperscript{35,42,78}. Interestingly, the specific role of the cancer stem cell in adhesion to endothelium is a yet unstudied, but crucial part of metastasis. Experiments in this study aimed to identify differences in the cancer stem cell and non-stem cell in terms of adhesive interactions to endothelium and responsiveness to adhesion related molecules at the potential metastatic site. In the experiments that observed the effects of SDF-1α, MCF-7 and MDA-MB-231 and BT-20 cells were used to understand if the cancer stem cell-like nature of MDA-MB-231 cells alters its reaction to SDF-1α compared to non-stem like breast cancer cells.

Breast cancer cells may respond to SDF-1α in a similar manner as leukocytes by calcium mobilization and actin polymerization\textsuperscript{14,25}, therefore these responses were studied. For MCF-7 and MDA-MB-231 breast cancer cells, calcium mobilization in response to SDF-1α treatment was not observed. While this result was surprising, literature reports that different cell types exhibit varied responses to chemokines\textsuperscript{25,72}. Actin polymerization, however, was observed in some types of breast cancer cells in the form of pseudopodia at the edge of the cell. This same behavior can be seen for breast cancer cells upon exposure to SDF-1α in previous experiments by Müller et al as they demonstrated active CXCR4 on the surface of MDA-MB-231 cells\textsuperscript{6}. Actin polymerization of suspended BT-20 cells in response to SDF-1α has not been shown before. These
experiments were performed with cells in suspension to better mimic the *in vivo* scenario where cells would be exposed to SDF-1α. Non cancer stem cell-like MCF-7 and BT-20 cells, both CD44+/CD24+, showed actin polymerization and the formation of pseudopodia in response to SDF-1α treatment. Interestingly, cancer stem cell-like MDA-MB-231 cells did not appear to react to SDF-1α in the milieu. Another study observed actin polymerization in MDA-MB-231 cells in response to incubation with SDF-1α, so our result was unexpected⁶. However, in that experiment, a higher concentration of SDF-1α and a longer incubation time were necessary to see an effect.

Adhesion assays to analyze the binding behavior of breast cancer cells in the presence or absence of SDF-1α, showed that MCF-7 cells exhibit a consistent, but non-statistically significant increase in adhesive interactions when they were incubated with SDF-1α as compared to assays where neither cancer cells nor HUVEC were treated or only endothelial cells were treated. The consistent trend of heightened binding interaction when breast cancer cells are treated with SDF-1α indicates that these results may become statistically significant if this experiment is repeated. This observation is not likely due to the transcription of CXCR4 because treatments were only 2 minutes. Therefore, SDF-1α treatment may activate the expression of other adhesion molecules such as VLA-4 that enhance cell interactions with endothelium¹⁶. Further study is necessary to identify the mechanism by which SDF-1α increases adhesion between migrating cells and endothelium.
Intriguingly, the stem-like MDA-MB-231 cells did not form appreciable adhesion interactions with endothelial cells under any circumstances. The adhesion of MDA-MB-231 cells to endothelial cells *in vitro* is a controversial subject in the literature. Some researchers indicate MDA-MB-231 cells do adhere to endothelial cells *in vitro*\(^\text{79}\), and other studies show this cell line as being able to extravasate and develop metastases *in vivo*\(^\text{24,80}\). Therefore, the experimental assay may not capture the conditions necessary for MDA-MB-231 cell adhesion to endothelium, and cannot observe their response to SDF-1α for the same reason. Perhaps also, stimulation with SDF-1α alone is not enough to induce changes in some breast cancer cells. Other cytokines, also present in the bone marrow microenvironment such as TGF-β1, may be obligatory to visualize cellular response to adhesion-regulating chemokines. In addition, it may be that properties of non stem-like breast cancer cells are necessary for adhesion to endothelium and the cancer stem cell is necessary for former or subsequent stages of metastasis.

One of the advantages for cancer cells in becoming stem-like cells is that they are able to self-renew and differentiate – two processes that aid in the formation of a secondary tumor. TGF-β1 contributes to this process by decreasing E-cadherin and increasing vimentin expression via EMT\(^\text{42}\). Further, enhanced invasivity and metastasis correlate with vimentin expression for breast cancer\(^\text{81}\), and the loss of E-cadherin disrupts intercellular contacts contributing to metastasis\(^\text{82}\). Therefore, the phenomena of EMT enables cancer cells to more aggressively pursue metastatic sites\(^\text{42}\). In the experiments we performed using TGF-β1, it was not possible to confirm EMT had
completely occurred, but incubation with the cytokine did alter the cells to become more mesenchymal (Figures 20-22). There are many reasons why the transition may not have been complete; among them is the finding that EMT is a difficult process to induce in vitro\textsuperscript{47}. Additionally, epithelial mesenchymal transition does not necessarily confer a complete loss of epithelial characteristics, even in vivo\textsuperscript{83}. It is also possible that EMT exists in a continuum, at least for cancer cells. At different concentrations of TGF-\(\beta\)1, different effects are seen in cells. For example, a less TGF-\(\beta\)1 treatment (0.25 ng/ml for 24 hours) showed a change in vimentin expression for BT-20 cells, but no change in E-cadherin expression (not shown). Cell incubation with 5 ng/ml TGF-\(\beta\)1 for 48 hours resulted in a decrease of E-cadherin expression, but no visible change in vimentin levels. The varying results seen for EMT experiments illustrate the inherent complexity of this process and its analysis.

Considering that TGF-\(\beta\)1 was able to alter BT-20 breast cancer cells, even though EMT may not have been complete, experiments were performed to understand the meaning of cellular transformation in the context of metastasis. MCF-7 and BT-20 cells were chosen because they were CD44\(^+\)/CD24\(^-\) cell lines and presumed to be amenable to transition into the CD44\(^+\)/CD24\(^-\) putative breast cancer stem cell phenotype.

The first experiments used TGF-\(\beta\)1 to induce EMT in BT-20 and MCF-7 cells. The optimization of TGF-\(\beta\)1 concentration and incubation time was an arduous task. The few reports of EMT occurring in vitro used many different concentrations and treatment times to achieve results. In addition, it was prior to this analysis that our MCF-7 cells
were found to have lost CD24 expression, and new cells had to be obtained. Interestingly, only BT-20 cells responded to TGF-β1 treatment at any concentration so they, along with MCF-7 cells as a control, were analyzed in subsequent experiments. Although a decrease in E-cadherin levels was achieved through incubation of breast cancer cells with TGF-β1, this reduction did not affect adhesion interactions nor did it alter the E-selectin affinity of the cells. This is the first time that the effect of TGF-β1 treatment on the adhesion behavior of breast cancer cells has been observed. Surprisingly, the MCF-7 cells obtained from ATCC though CD44+/CD24+, exhibited dramatically reduced interaction with endothelial cells and E-selectin. The reason for this is unknown, but requires further study. The MCF-7 cells from ATCC also expressed higher levels of CD24 than the cells previously analyzed during characterization experiments (data not shown). The importance of this finding remains elusive because a link between cancer stem cell properties and adhesion capacity has yet to be established. Further, the relationship between stem cell phenotype and endothelial adhesion is relatively unstudied even in normal stem cells, making a correlation between normal stem cells and cancer stem cells in adhesion a new and important area of study. It is therefore necessary to consider that E-selectin may be under the control of factors independent of the stem cell phenotype in a cancer cell. However, there is still a possibility that adhesion and stemness are coordinated through similar mechanisms and further investigation is essential to illuminate this relationship.
Aigner et al demonstrated that P-selectin serves as a binding partner for CD24 for some breast cancer cells\textsuperscript{30}. However other than P-selectin endothelial binding partners of CD24 are unknown. This study showed for the first time that CD24 is not an E-selectin ligand for BT-20, MDA-MB-468, and ZR-75-1 breast cancer cells. As a consequence, if CD24 does affect breast cancer cell adhesion to endothelium, it is not through direct binding to E-selectin. It is possible that CD24 has a minor role in the adhesion of breast cancer cells to endothelium that is not identifiable in the above experiment which only assessed E-selectin binding. To further understand if CD24 may bind currently unknown molecules on endothelium to facilitate adhesion, a comparison of cells with and without CD24 expression is necessary. siRNA was used to knock-down the expression of CD24 to determine if CD24 loss alters adhesion interactions. The procedure did in fact decrease the expression of CD24 on the cell surface for both cell lines; however it did not reduce it to isotype control levels. This is the first study to observe the effects of CD24 knock-down in breast cancer cells on adhesion behavior. The lack of difference in E-selectin affinity and adhesion may be due to the fact that some CD24 still existed on the surface of these cells. However, the results from an adhesion assay using the MCF-7 cells that spontaneously lost CD24 showed no difference in binding interactions regardless if CD24 was expressed. Therefore, a possible mechanism for CD24 involvement in adhesion of breast cancer cells to endothelium remains unknown.
Overall, this study demonstrated that stromal cell-derived factor-1α and transforming growth factor-β1 are able to enact changes in breast cancer cells that would potentially enable the cells to better metastasize. On the one hand, SDF-1α induces structural changes that enhance cell mobilization and surface alterations that allow better interaction with endothelial cells. The adhesion assay results in Figure 14 demonstrate for the first time that breast cancer cells incubated with SDF-1α consistently show increased adhesive interactions with endothelial cells, but the trend of enhanced adhesion was not statistically significant. On the other hand, TGF-β1 makes cells more stem-like, potentially enabling them to form a secondary colony at a metastatic site. Treatment of BT-20 cells with TGF-β1 led to the novel observation that these cells can be made stem-like. Both of these processes are believed necessary for successful metastasis to occur. However, further study is necessary to clearly elucidate the role of SDF-1α and TGF-β1 in breast cancer progression.

An additional study to determine the utility of hBMEC in adhesion studies investigated the use of human bone marrow endothelial cells as a replacement for HUVEC in adhesion assays. hBMEC would provide a model of adhesion in breast cancer metastasis to bone marrow that resembles physiology more accurately and therefore would produce more relevant data. It has already been determined that breast cancer cell binding to HUVEC is E-selectin dependent. Additionally, it has been shown that untreated breast cancer cells do not utilize VLA-4, a binding partner for endothelial VCAM-1, to facilitate the adhesion of migrating cells to endothelium. Interestingly,
SDF-1α has been shown to induce the expression of active VLA-4 in multiple myeloma cells and CD34+ leukocytes\textsuperscript{16,19}. Physiologically, SDF-1α resident in the bone marrow microenvironment may activate VLA-4 on migrating breast cancer cells, enhancing their adhesion to VCAM-1 on the endothelium. This phenomenon has yet to be investigated and merits further study. Therefore, because untreated cancer cells were used in hBMEC experiments, hBMEC were characterized based on the role of E-selectin in adhesion. This analysis showed that MCF-7 breast cancer cell binding is indeed dependent on E-selectin, but that MDA-MB-231 cells do not adhere any better to hBMEC than to HUVEC. Future experiments investigating breast cancer cell adhesion to bone marrow should utilize hBMEC as a representative endothelial wall in addition to HUVEC to obtain a more complete understanding of the adhesion interactions observed.

Figure 27 briefly illustrates the results discussed in this section.
Figure 27. Summary of Results discussed in this section. It is known that SDF-1α shows a trend of enhancing the adhesion of certain breast cancer cells to HUVEC, but the exact mechanism is unknown. Also, TGF-β1 induces an epithelial mesenchymal transition in BT-20 cells, but the effect of that change on breast cancer cell adhesion to endothelium is not conclusive. CD24 siRNA can be used to induce the breast cancer stem cell phenotype of CD44+/CD24- in cells positive for both glycoproteins. However, the effect of this alteration on adhesion to endothelium has not yet been definitively shown.
5.2. Conclusions

This study observed changes in the adhesion behavior of various breast cancer cell lines treated with SDF-1α or TGF-β1 that implicate a role of these cytokines in breast cancer metastasis. For the first time, it was shown that short-term exposure of breast cancer cells to SDF-1α consistently increases breast cancer cell adhesion to HUVEC, although at n=3 this result was not statistically significant. This has important implications in breast cancer progression, as the bone marrow microenvironment is rich in SDF-1α that can interact with a migrating breast cancer cell and enhance adhesion. Additionally, it was determined that MCF-7 breast cancer cell binding to human bone marrow endothelial cells is E-selectin dependent. The finding that SDF-1α and E-selectin affect breast cancer cells in a manner promoting adhesion to endothelium in this study signifies their role in breast cancer metastasis. The novel finding that the adhesion cascade for hBMEC is similar to that of HUVEC in terms of E-selectin binding demonstrates that it is possible to use both cell types in adhesion assays to observe the unique properties of hBMEC that may potentiate breast cancer metastasis to bone marrow.

The MDA-MB-231 cells exhibited the putative phenotype of breast cancer stem cells. These cells expressed high levels of cytoplasmic vimentin, a marker of mesenchymal cells. The vastly decreased binding interactions MDA-MB-231 cells have with endothelial cells compared to non stem-like MCF-7 cells may be attributed to the stem cell properties of the former cell line. It is plausible then, that a non-stem cancer
cell is required to interact with E-selectin for adhesion to bone marrow endothelium, and upon infiltration into the bone marrow parenchyma, it reverts to a stem cell state enabling the development of a secondary colony. A novel finding of this study is that, treatment of non stem-like BT-20 breast cancer cells with TGF-β1 causes them to become more mesenchymal in phenotype through the loss of E-cadherin and gain of cytoplasmic vimentin. TGF-β1 is expressed in the bone marrow microenvironment and would be able to interact with nearby disseminated breast cancer cells. These findings indicate a unique role for the breast cancer stem cell in metastasis in that it cannot attach to E-selectin at a potential metastatic site as shown in flow adhesion assays. Instead the cancer cell may revert to a stem-like state once inside the bone marrow via TGF-B1 to enable later stages of the metastatic cascade, including tumorigenesis.

In conclusion, molecules present in the bone marrow microenvironment enact changes in breast cancer cells that have the capacity to enhance the progression of disease. A main player in the development metastases is the capacity of cells to adhere to endothelium at potential metastatic sites. However, the specific mechanism through which these changes operate is unknown and further experimentation is necessary to fully elucidate the phenomenon of metastasis. The progress made in this study will contribute positively to future research and investigation in the area of breast cancer metastasis.

The results of this set of experiments combined with future studies will lead to further clarification of the molecular pathways used by breast cancer cells to
metastasize. An understanding of the involvement of cytokine-receptor interactions in metastasis will provide a foundation for novel therapies targeting the inhibition of this deadly occurrence. By blocking these interactions, it may be possible to prevent metastasis and thereby the main cause of all cancer death. The illumination of metastatic mechanisms will provide a rational basis for the development of diagnostic and therapeutic strategies directed against breast cancer metastasis.
CHAPTER 6. FUTURE STUDIES

6.1. Determine how SDF-1α Enhances Adhesion of Breast Cancer Cells to HUVEC

Experiments described previously demonstrated a trend that short-term incubation of breast cancer cells with SDF-1α increased their adhesion to endothelial cells. The treatment period was not sufficiently long to allow novel transcription and expression of CXCR4 at the cellular surface. Therefore, SDF-1α must stimulate the breast cancer cells in another manner conducive to adhesion interactions. It is known that SDF-1α can cause the activation of integrins, such as VLA-4, on hematopoietic stem cells\(^\text{16}\). These integrins facilitate adhesion interactions of HSC with bone marrow endothelium. SDF-1α may be inducing a similar response in breast cancer cells that leads to increase binding interaction with bone marrow endothelium. However, the specific alteration that occurs in breast cancer cells treated upon short incubations with SDF-1α requires further examination to uncover.

In another scenario, cancer cells may be exposed to SDF-1α over long periods of time while in the bone marrow microenvironment prior to tissue infiltration. Flow adhesion and actin polymerization assays from the study in Chapter 3 have demonstrated that breast cancer cells respond to exposure to SDF-1α in the environment in a manner that may increase adhesion capacity. Previous studies also indicate that breast cancer cells with a propensity for bone metastasis express comparatively heightened CXCR4 levels\(^\text{80}\). Increased expression of CXCR4 implicates an enhanced ability to respond to SDF-1α in the surrounding milieu subsequently causing
firm adhesion of migrating cells to the endothelium. Therefore, one way that SDF-1α could be affecting the breast cancer cells is by stimulating increased expression of CXCR4 on the cell surface. An important consideration for these experiments is the finding that CXCR4 is continuously internalized and cycles back to the cell membrane over time\textsuperscript{84}. This process may result in varying levels of CXCR4 expression independent of SDF-1α. Investigation into the effect of SDF-1α on CXCR4 expression may shed light on how the chemokine contributes to bone marrow metastasis.

### 6.2. Analyze Breast Cancer Cells for ALDH1 and HER2

To characterize breast cancer stem cells, including cell line MDA-MB-231 as well as cells made stem-like through \textit{in vitro} techniques, it is important to use multiple markers. CD44\textsuperscript{+}/CD24\textsuperscript{−} is recognized as the putative breast cancer stem cell profile, but is not sufficient for specific identification\textsuperscript{33}. In addition to CD44 expression and lack of CD24 expression, ALDH1 is now identified as a candidate marker of breast cancer stem cells\textsuperscript{85}. Interestingly, ALDH1 is a protective mechanism in hematopoietic stem cells and may enable chemotherapeutic resistance in cancer stem cells\textsuperscript{86}. Therefore in addition to being a marker of the breast cancer stem cell phenotype, ALDH1 may also contribute to the persistence of cancer stem cells following treatment.

In addition to ALDH1, the expression of HER2 on breast cancer cells has been indicated as a factor to enhance the progression of disease\textsuperscript{36}. HER2 over-expression is correlated with enhanced invasion and metastatic capacity in breast cancer cells, and is shown to further develop the cancer stem cell population in a tumor. However, the
same phenomenon is not observed in more differentiated cancer cells, as determined by ALDH activity\textsuperscript{87}. Therefore, examination of the HER2 status and ALDH1 expression in breast cancer cells will provide more, although still tentative, indicators for the stemness of breast cancer cells used in experimentation. Further scrutiny of the stem-like nature of breast cancer cells would inevitably lead to more accurate discrimination between non-stem versus stem cell activities in cancer metastasis.

6.3. Determine if Breast Cancer Cells Secrete TGF-\(\beta\)1 and have TGF-\(\beta\)1 Receptors

Experiments described in this study have demonstrated that BT-20 breast cancer cells respond to treatment with TGF-\(\beta\)1, but MCF-7 cells do not. One factor that may contribute to this discrepancy is the expression, or lack of expression, of TGF-\(\beta\)1 receptors on the surface of breast cancer cells. Without the receptors, the cells cannot react to TGF-\(\beta\)1, and detection of TGF-\(\beta\)1 receptors on cancer cells may determine which breast cancer cells are capable of EMT via treatment with TGF-\(\beta\)1.

A number of cells in the body including macrophages, lymphocytes, endothelial cells, and chondrocytes, secrete TGF-\(\beta\)1\textsuperscript{88}. TGF-\(\beta\)1 present in the tumor microenvironment has been shown to contribute to the aberrant signaling seen in cancer cells\textsuperscript{89}. Surprisingly, TGF-\(\beta\)1 is capable of dual activity by affecting cancer cells in opposite ways: it can limit cell proliferation behaving as a tumor suppressor, or it can be oncogenic through the induction of EMT\textsuperscript{90}. Characterization of the ability of breast cancer cells to secrete TGF-\(\beta\)1 would provide more information on the nature of cellular responses to the cytokine. If a particular phenotype of breast cancer cell does not
respond to external TGF-β1 treatment, it may be that the cell produces TGF-β1 itself and is constantly stimulated in an autocrine manner or that the pathway has been mutated and cannot respond.

6.4. Optimize EMT in Breast Cancer Cells

The studies described above demonstrated that TGF-β1 produces changes in BT-20 cells reminiscent of epithelial mesenchymal transition. However, a complete EMT was not confirmed. It is possible that the TGF-β1 treatment conditions were not optimized, preventing the completion of EMT. Therefore a future study should look into optimizing EMT and validating a complete transition through RT-PCR analysis in addition to flow cytometry. Using RT-PCR would allow more sensitive analysis of changes in the expression of epithelial and mesenchymal markers, especially over short time periods.

6.5. Optimize CD24 Knock-down via siRNA in Breast Cancer Cells

Although it was shown that CD24 expression could be decreased through the methods used for siRNA knock-down, CD24 was still expressed on the surface of the breast cancer cells. In order to fully observe the contribution that CD24 plays in breast cancer cell adhesion to endothelium, a complete knock-down of the glycoprotein should be achieved. Therefore, the siRNA knock-down of CD24 in breast cancer cells needs to be optimized. It may be necessary to purchase siRNA from a different company, or it may be as simple as increasing multiplicity of infection (MOI), but it is a necessary step for future experiments involving CD24 analysis.
6.6. Treat CD24 Knockdown Breast Cancer Cells with TGF-β1

Once CD24 knock-down of breast cancer cells has been obtained, it would be interesting to observe how the lack of CD24 affects the expression of other cellular molecules. For example, does the removal of CD24 affect E-cadherin expression or increase levels of mesenchymal cell markers? To further that question, an exciting project would be to observe how CD24 knockdown cells react to treatment with SDF-1α or TGF-β1. Differences in responses between knockdown and normal cells would shed light on the role of CD24 in breast cancer progression and would also open up many new avenues of research.

6.7. Observe CD24 siRNA Cells and EMT Cells in a Mouse Model for Bone Marrow Metastasis

To finalize the observations made on CD24 knockdown and EMT cells, it would be ideal to analyze cellular behavior in vivo. In particular, the actions of these cells in a mouse model for bone metastasis would further illuminate the role they play in cancer metastasis. Kuperwasser et al, has described a murine model of human breast cancer metastasis to bone. Through implantation of a functional section of human bone in a mouse dorsal flank, this model was able to demonstrate metastatic behavior physiologically similar to human skeletal metastasis. The use of a mouse model to analyze the effects of CD24 knock-down in migration to bone marrow endothelium would provide valuable insight into the unique properties of cancer stem cells in metastasis. Additionally, it would be beneficial to observe how cells that have
undergone EMT interact with the bone marrow microenvironment to facilitate metastasis in a physiological setting.

6.8. Examine Relationship between CD24 and CXCR4 Expression

A novel study by Schabath et al demonstrated that the expression of CD24 on breast cancer cells negatively influences the capacity of those cells to respond to SDF-1α. By comparing the tumorigenicity of normal MDA-MB-231 breast cancer cells to that of CD24 overexpressing MDA-MB-231 cells, it was also determined that cells with low CD24 expression more readily formed tumors. Importantly, this finding indicates that breast cancer cells of the putative stem cell phenotype, CD44+/CD24−, may respond better to bone marrow resident SDF-1α and further promote metastasis as compared to non stem-like breast cancer cells. Given this information, observation of the effect CD24 has on CXCR4 expression and responsiveness in breast cancer cell lines would be an important part of understanding the mechanism of breast cancer metastasis. Indeed, it might explain why some breast cancer cells respond better to SDF-1α than others.
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