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Lung-derived selectins interact with CD44 and enhance the migration of breast cancer cells

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Graduate Program in Anatomy and Cell Biology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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Abstract

The lung is among the deadliest sites of breast cancer metastasis. Previous work from our laboratory demonstrated that aggressive CD44-expressing breast cancer cells preferentially metastasize to the lung *in vivo*, and observed the presence of multiple CD44-interacting proteins in the lung including E-, L- and P-selectin. We hypothesized that lung-derived selectins promote breast cancer migration and/or growth via interactions with CD44. Using an *ex vivo* model of the soluble lung-microenvironment, we demonstrate that lung-derived selectins enhance *in vitro* migration but not proliferation of breast cancer cells. Co-immunoprecipitation experiments reveal that CD44 expressed by breast cancer cells *in vitro* interacts with soluble selectins. The mechanism underlying the pro-migratory effect of lung-derived selectins is independent of ezrin/radixin/moesin (ERM) or CREB phosphorylation. Future studies should be aimed at elucidating mechanisms by which lung-derived selectins exert their pro-migratory function and whether these proteins can be targeted therapeutically to reduce lung metastasis of breast cancer.

Keywords: Breast cancer, organ-specific metastasis, lung microenvironment, CD44, E-selectin, L-selectin, P-selectin

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Co-Authorship Statement

Dr. Ying Xia (Research Associate, Allan Lab, London Regional Cancer Program) assisted in conducting protein phosphorylation experiments.

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Acknowledgements

I would like to begin by thanking Dr. Alison Allan for allowing me the opportunity to pursue a MSc degree. With her support and guidance, I not only developed as a researcher but as an individual overall. She taught me how to think critically, how to learn from my mistakes and what it means to take pride in one's work. Thank you, Alison, for always challenging me to further succeed and for ensuring I was prepared for any challenges that laid ahead. You have been an excellent mentor, supervisor and friend.

I would also like to thank the members of the Allan lab, especially Ying Xia, David Goodale, Ashkan Sadri, Jenna Kitz and Lori Lowes. You each contributed to a work environment, which I enjoyed coming to everyday. Thank you all for teaching me different lab techniques, for your willingness to share reagents and most of all, for your friendship. I could not have asked for a better group of people to work with and I am glad to have known each of you.

I would also like to thank my committee members, Dr. Moshmi Bhattacharya, Dr. Douglas Hamilton, Dr. Dale Laird and Dr. Paul Walton. Thank you all for donating your time, and for providing constructive feedback and invaluable advice.

Finally, I would like to thank my family, especially my parents, who always supported me and motivated me to succeed in life.

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1 INTRODUCTION

1.1 Cancer

Cancer is a family of diseases hallmarked by uncontrolled cell growth and proliferation. This dysregulation often occurs as a result of normal cells accumulating multiple genetic mutations over time[1]. Mutations relevant to cancer development often occur in either oncogenes or tumour suppressor genes. Oncogenes are genes that when activated through gain-of-function mutations will promote cancer cell growth and division[2]. In contrast, tumour suppressor genes are negative regulators of cell growth and overall malignancy; their inactivation through loss-of-function mutations leads to increased cell proliferation and survival. As cancers progress, the cells gain the characteristic abilities to self-sustain proliferative signaling, replicate infinitely, resist cell death, induce angiogenesis, activate metastasis and avoid destruction by the immune system[3]. A mass of these cancerous cells is referred to as a tumour, which can be either benign or malignant in nature.[4] Benign tumours remain confined to their original location and are usually non-life-threatening. In contrast, a malignant tumour can invade surrounding tissue and also spread to distant regions of the body; these are life-threatening.

1.2 Breast Tissue and Breast Cancer

The principal structure of the breast is the mammary gland[5]. It is composed of milk-producing cuboidal epithelial cell-lined acini organized into lobules, with each having a lactiferous duct responsible for transporting the milk to the nipple for ejection. Breast cancer is any primary tumour formation originating from the breast tissue, most commonly the milk-producing lobules and the ducts which transport it[6]. These structures are susceptible to cancerous transformation in part because of the cycling stimulation of estrogen and progesterone on the proliferation of breast epithelial cells[7]. Over time, this leads to increased cell turnover and accumulation of genetic defects[8].

There are multiple schemas by which breast cancer can be classified, one of which is based on histopathology. This takes into consideration the structure from which the tumour cells originated and whether they have remained confined to their site of origin, i.e.

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have not invaded into the surrounding tissue structures. Primary breast tumours which originate from the lobules and are confined to their site of origin are classified as lobular carcinoma *in situ*[9]. Those originating from the ductal epithelium and have not invaded into surrounding tissue are classified as ductal carcinoma *in situ*. At the *in situ* stage, the tumours are non-invasive and can be effectively treated with surgery, hormone therapy and/or radiation therapy. The five-year survival rate for affected patients is 98.6%[10]. However, once the cancer has become invasive and has begun infiltrating the surrounding tissue, the prognosis worsens. If the invasion is allowed to persist, breast cancer can spread, or metastasize, to distant organs.

An additional and more detailed system used to stratify breast cancer cases is based on molecular subtype. Cases can be categorized into one of four groups: luminal A, luminal B, HER2-enriched and basal-like (triple-negative; TN). Grouping into each group is based on the combination of their hormone receptor status (estrogen [ER] and progesterone [PR]), HER2 (human epidermal growth factor receptor 2) status and proliferation index (Ki-67). Luminal A is the most commonly diagnosed subtype of breast cancer and is characterized by $ER^+/PR^+/HER2^-$ and a low rate of proliferation[11]. Diagnosed patients show good prognosis and survival rates as they respond well to hormone therapy. Luminal B is a more aggressive phenotype than luminal A and is identified based on an ER+/PR+ phenotype with either a high proliferation rate and/or HER2+ status. Survival rates are slightly lower than those observed in luminal A breast cancer patients, but still fairly high as these patients also respond well to hormone therapy. HER2-enriched tumours are hormone receptor negative (ER-/PR-) and HER2+. Generally, these tumours are associated with a poorer prognosis and higher incidence of metastasis relative to the luminal A/B subtypes[12]. These patients are treated with HER2-targeting agents such as Herceptin (trastuzumab) that can directly interfere with HER2 receptor signaling and subsequently reduce cancer cell growth and tumour progression[13]. The only subtype for which no targeted therapies currently exist is basal-like/TN, which are characterized by an ER-/PR-/HER2- status and are highly aggressive in nature. The average expected 5-year survival rate for these patients is approximately 26%, as TN breast tumours commonly metastasize to essential physiological organs, such as the lung[10, 14].

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1.3 Metastasis

Metastasis is a complex process through which secondary tumours in distant organ sites can occur[7]. The metastatic cascade (**Figure 1**) can initiate once the primary tumour has developed its own blood supply through angiogenesis to support its metabolic demands[8]. Not only do these newly formed blood vessels increase the growth potential of the primary tumour, but they also create a means through which cancer cells can spread to other organs. The first step of the metastatic cascade is intravasation, a process by which tumour cells that have dissociated from the solid tumour will cross the endothelium and enter the blood circulatory system[15]. Tumour cells can enter the circulatory system directly or indirectly via the lymphatic system. Once in circulation, tumour cells must survive the shear stress of blood flow and evade destruction by immune cells before they can arrest in the capillary beds of distant organs. At these sites, tumour cells must extravasate in order to exit the circulatory system and enter the secondary organ. These tumour cells will only establish a metastatic tumour if they are able to survive and proliferate in the organ's microenvironment.

In the metastatic breast cancer setting, current therapies are largely ineffective and 5-year survival rates are approximately 26%[6, 7]. The majority of breast cancer-related deaths can be attributed to metastasis, as tumours in the brain, lung, liver and bone can impair essential physiological functions and eventually lead to death[16]. In comparison, primary tumours when contained within the breast are non-life threatening. For example, patients with metastatic tumours in the lung present clinically with pain, hemoptysis, pleural effusions and/or pulmonary dysfunction[17]. A pleural effusion is the build-up of fluid in the pleural space around the lungs and can negatively affect breathing and gas exchange[18, 19]. Unfortunately, pleural effusions caused by a malignancy are incurable and treatments are focused on palliative care. The median survival from clinical recognition of a malignant pleural effusion is 4 months[20]. The high mortality associated with metastasis therefore presents an important, clinically-relevant problem requiring further research to resolve.

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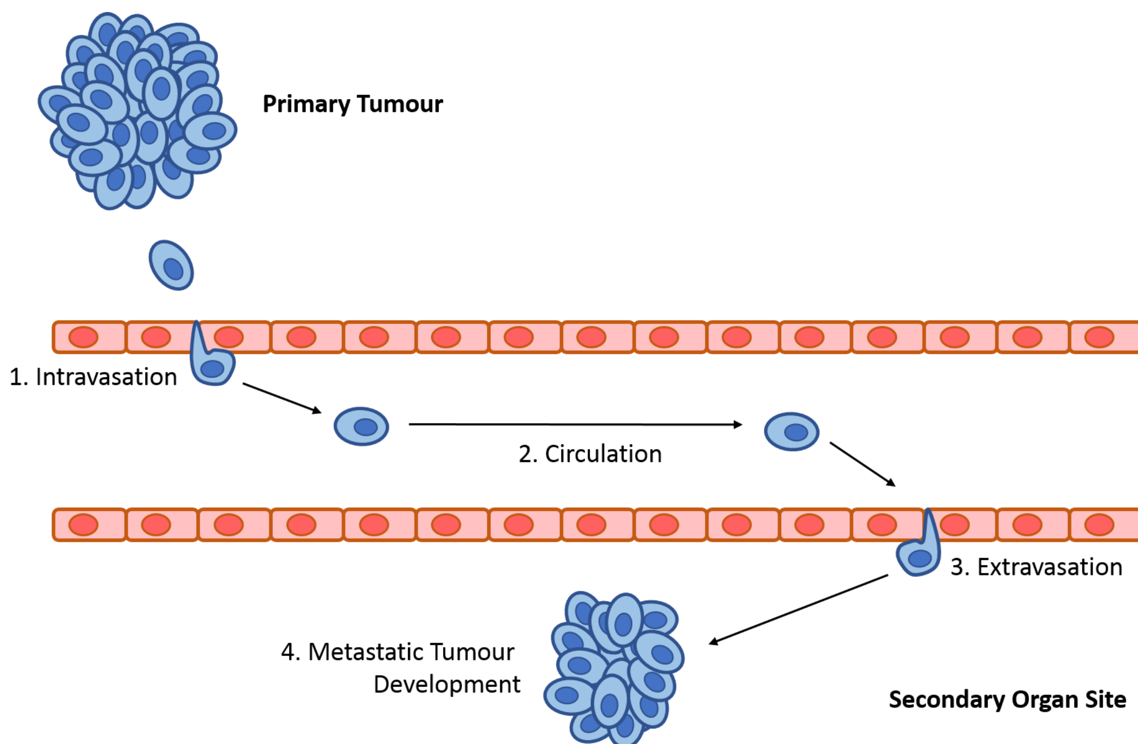

Figure 1. The metastatic cascade. Tumour cells that have dissociated from the primary tumour have the potential to spread to distant organs. This occurs through four key steps, which begins with intravasation. Intravasation is the process by which cells traverse the endothelium and enter the systemic circulation. These cells then circulate throughout the body until they reach a location where they will exit the circulation and enter the organ's parenchyma. This step is known as extravasation. If the organ microenvironment possesses ideal conditions for growth of the tumour cells, they may grow and proliferate to form secondary metastatic tumours.

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1.4 Metastatic Organ Tropism

Clinically, it has been previously observed that breast cancer metastasizes most often to the lung, bone, liver, brain and lymph nodes[21]. This phenomenon of preferential metastasis to specific organs has been observed in different types of cancer and is termed organ tropism[22]. The two main theories that have been proposed to explain organ tropism are Stephen Paget's "seed and soil" hypothesis and James Ewing's mechanical arrest theory[21]. In 1889, Paget hypothesized that organ-specific patterns of metastasis were a result of cancer cells ("seeds") requiring specific organ microenvironmental conditions at secondary sites ("soil") to be able to survive and grow[23]. This was proposed based on his observation of a non-random pattern of metastasis to specific visceral organs and bone from the autopsy records of 735 female breast cancer patients. James Ewing proposed an alternative theory forty years later, postulating that organ tropism could be accounted for by blood flow patterns[14]. Specifically, his theory emphasized that cancer cells would mechanically arrest in the first capillary bed they encountered. However, his theory could not explain why some organs that are served by only a small fraction of the blood flow (e.g. brain and bone) are disproportionately more affected by metastasis, while other organs that receive considerable blood flow (e.g. heart) are uncommonly involved in the colonization of secondary tumours[24].

It is likely that these two theories operate in concert to allow tumour cells to reach secondary organ sites and form metastases. Based on these theories, using the lung as an example, breast cancer cells that have dissociated from the primary tumour and invaded nearby capillaries would be carried within venous blood to the heart and then circulated to the lungs, where tumour cells would mechanically arrest in the first capillary bed they encounter in the lungs. Secondary tumour formation could then occur; but would be dependent on compatible characteristics of the breast cancer cells (the "seed") and supporting factors produced by the lung microenvironment (the "soil")[25].

1.4.1 The "Seed" – Breast Cancer Cells

Studies carried out by Joan Massagué's group have demonstrated the influence of breast cancer cell gene expression profiles on organ-specific metastasis. Using three

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different models of metastasis and multiple rounds of *in vivo* selection, they generated brain-seeking[26], bone-seeking[27] and lung-seeking[28] variants of the aggressive TN human breast cancer cell line MDA-MB-231. Transcriptome analysis demonstrated that each variant had a unique gene expression profile. When the data from the lung-seeking variant was compared against patient breast tumour samples and patient incidences of lung metastasis, it was found that the expression pattern of 18 univariately significant genes was enough to define a lung metastasis signature (**Table 1**)[28]. In further studies, this signature was shown to be associated with breast cancer relapse to the lung, but not to the bones, liver or lymph nodes[26, 29]. There was a weak association of the lung metastasis signature with relapse to the brain, likely because six of the 18 genes are also found in the brain metastasis signature[26]. These genes comprising the lung metastasis signature encode for a variety of different protein classes; including extracellular receptors, adhesion receptors, chemokines, secreted proteases and transcriptional regulator proteins[28]. Overall, their findings highlight the relationship between breast cancer cells, their genetic signature and the organ microenvironments to which they preferentially metastasize. However, there are other characteristics that have used to define a subset of cancer cells with an overall increased ability for metastasis.

1.4.1.1 "Stem-Like" Breast Cancer Cells

Cancer stem cells (CSCs) represent a small subpopulation of cancer cells found within solid tumours, and they have an increased ability for both primary tumour formation and metastasis[30, 31]. Their existence was first validated by John Dick and colleagues in 1997 based on findings in human acute myeloid leukemia that only a subset of cells was capable of inducing leukemia[32]. CSCs are defined as cells that have both the ability to self-renew and give rise to a wide range of differentiated cells that comprise a tumour[33]. In the context of breast cancer, CSCs were first isolated based on a $CD44^+/CD24^-$ phenotype[34]. As few as 100 $CD44^+/CD24^-$ cells when injected into the mammary fat pad of mice were able to form tumours. This contrasts with other phenotypes, where as many as 10,000 cells when injected into the mammary fat pad were unable to form tumours. Breast cancer stem cells have since then been further defined to include high activity of the aldehyde dehydrogenase 1 (ALDH1)[35]. This intracellular enzyme oxidizes aldehydes to protect stem cells and its

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Table 1. Breast cancer lung metastasis signature

Symbol	Gene Name	Also found in brain metastasis signature[26]
ANGPTL4	Angiopoietin-like 4	+
FSCN1	Fascin homologue 1, actin-bundling protein (*Strongylocentrotus purpuratus*)	+
LTBP1	Latent transforming growth factor beta binding protein 1	+
MMP1	Matrix metalloproteinase 1 (interstitial collagenase)	+
PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	+
RARRES3	Retinoic acid receptor responder (tazarotene induced) 3	+
C10orf116	Chromosome 10 open reading frame 116	–
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth-stimulating activity, alpha)	–
CXCR4	Chemokine (C-X-C motif) receptor 4	–
EREG	Epiregulin	–
ID1	Inhibitor of DNA binding 1, dominant-negative helix–loop–helix protein	–
KRTHB1	Keratin, hair, basic, 1	–
KYNU	Kynureninase (L-kynurenine hydrolase)	–
LY6E	Lymphocyte antigen 6 complex, locus E	–
MAN1A1	Mannosidase, alpha, class 1A, member 1	–
NEDD9	Neural precursor cell expressed, developmentally downregulated 9	–
TNC	Tenascin C (hexabrachion)	–
VCAM1	Vascular cell adhesion molecule 1	–

Adapted from Minn, A. J. et al, *Nature* 2005, 436 (7050), 518-24[28]

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activity is also associated with early stem cell differentiation[36, 37]. Functionally, it has been shown that if cells are isolated based on an $ALDH^{hi}/CD44^{+}/CD24^{-}$ phenotype, as few as 20 cells transplanted into a mammary fat pad could generate a tumour[35]. Previous studies by our laboratory provided the first evidence of the $ALDH^{hi}/CD44^{+}/CD24^{-}$ breast cancer cell subpopulation as metastasis-initiating cells[38]. *In vitro*, these cells demonstrated increased growth, adhesion, colony formation, migration and invasion relative to their $ALDH^{low}CD44^{low}$ counterparts. They also demonstrated that $ALDH^{hi}/CD44^{+}/CD24^{-}$ human breast cancer cells are both more tumorigenic and metastatic *in vivo* than $ALDH^{low}CD44^{-}$ cells, with a particular propensity to metastasize to the lung. Together with Massagué's observations, these studies highlight the existence of properties intrinsic to certain breast cancer cells (the "seeds") that enhance their metastatic ability to different organ sites. In particular, the role that CD44 may play in enhancing the abilities of $ALDH^{hi}CD44^{+}$ breast cancer cells to interact with their microenvironment during metastasis may relate to its function as a cell surface receptor.

1.4.1.2 Cluster of Differentiation 44 (CD44) Receptor

The cluster of differentiation 44 receptor (CD44), belongs to a family of cell adhesion molecules with well-established functions in mediating cell-cell and cell-extracellular matrix (ECM) adhesion and communication[39]. There are over 20 different isoforms of this transmembrane receptor, and they vary from 80-200 kDa in size. This variance in size is generated from differences in RNA splicing and post-translational protein modifications[40]. CD44 is encoded by a 20 exon-containing gene. Eight of the exons are constant and found in all isoforms, while others are inserted in variants. Inclusion of additional exons lengthens the amino-terminal extracellular region of the receptor and exposes new binding sites for both posttranslational modifications and ligand binding[41]. The cytoplasmic C-terminal region of the receptor does not differ between variants and plays an important role in receptor signal transduction[42, 43]. Studies have shown that CD44 is involved in the regulation of cell growth, differentiation, survival and motility[44]. Some of these processes are regulated by CD44 at the transcriptional level as the intracellular domain of CD44 can be cleaved to release a fragment capable of acting as a transcriptional factor[45]. Others such as motility have been shown to be regulated at least in part through

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interactions of CD44 with members of the Band 4.1 superfamily of proteins, including ezrin, radixin and moesin (ERM)[46]. ERM proteins allow CD44 to exert its influence on cell shape and migration by crosslinking the actin cytoskeleton to CD44. The affinity of ERM proteins for CD44 is dependent on their phosphorylation status at key tyrosine and threonine residues. The phosphorylation is mediated by either growth factor receptors such as MET[47], a receptor tyrosine kinase, or downstream proteins such as protein kinase Cα[48].

Under normal conditions, CD44 is widely distributed and its expression has been observed in the epithelial tissue of many different organs, including but not limited to lung, liver, bone marrow, mammary gland and kidneys[49]. The actions of CD44 in each tissue are likely related to the isoforms of the receptor present and ligands present, as not all variants have the same affinity for CD44 ligands. CD44 ligands that have previously been identified include hyaluronan[50], osteopontin[51], serglycin[52], E-, L- and P-selectin[53-55]. The standard isoform (CD44S) has been shown to be important in the context of breast cancer. It has specifically been shown to be involved in breast cancer cell adhesion, migration and invasion, and is the isoform utilized in isolation and functional analysis of stem-like breast cancer cells based on either the $CD44^{+}/CD24^{-}$ or $ALDH^{hi}/CD44^{+}/CD24^{-}$ phenotype[56, 57].

1.4.2 The "Soil" – Organ Microenvironments

The influence of organ microenvironments (the "soil") on tumour growth and metastasis has been previously reported based on clinical observations of cancer patients, *in vitro* experiments, and experimental murine models of cancer[58, 59]. These studies illustrate that metastasis is often largely independent of an organ's vascular anatomy, blood flow or the number of tumour cells delivered to an organ site. Instead, the potential for successful secondary tumour formation and growth is heavily dependent on the interactions between organ microenvironments and tumour cells.

The role of the soluble organ microenvironment in promoting breast cancer metastatic behavior has been previously examined by our lab[25, 60-62]. We developed an *ex vivo* model system to study the influence of organ-derived soluble factors on breast cancer cell migration and proliferation. The specific organs that were examined represented the five most common clinical sites of breast cancer metastasis, including lung, bone, liver,

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brain and lymph nodes. These different organs were aseptically harvested from female nude athymic mice and cultured to generate organ conditioned media (CM). Using this model, Chu and colleagues demonstrated that human breast cancer cells exhibit cell line-specific and organ-specific chemotactic and proliferative patterns that reflect their *in vivo* patterns of metastasis[25]. Notably, all 4 cell lines showed increased migration towards lung-conditioned media relative to basal media, and 2 of the 4 cell lines showed increased proliferation in lung-conditioned media. This suggested that specific soluble factors within the lung microenvironment may be promoting the metastatic behaviour of breast cancer cells. Using a protein array, Chu identified 70 different soluble proteins that were found in the lung-CM. Many of these factors had been previously implicated in metastasis, including 5 CD44 ligands (osteopontin, basic fibroblast factor, and E-, L-, and P-selectin) as well as proteins/pathways such as epiregulin (EREG), OPN, and urokinase-type plasminogen activator (uPA) that had been previously associated with lung metastasis of breast cancer by Massagué's group[25, 28]. The focus of this thesis is to further investigate the role of lung-derived E-, L-, and P-selectin in breast cancer metastatic behaviour.

1.5 Selectins

The selectins are a three-member family of type I cell-surface glycoproteins that function to mediate adhesions between leukocytes, platelets and the endothelium[63]. Structurally, selectins consist of a N-terminal lectin domain, followed by an epidermal growth factor domain, two to nine consensus repeats (two in L-selectin, six in E-selectin and nine in P-selectin), a transmembrane domain and a C-terminal cytoplasmic domain **(Figure 2A)**[64]. There is high sequence homology between E-, L- and P-selectin in all domains except the transmembrane and cytoplasmic domains. The lectin domain recognizes specific carbohydrates on target proteins to which selectins bind.

Selectins differ in terms of their expression. L-selectin is constitutively expressed on the surface of most leukocytes[65]. P-selectin is stored intracellularly in granules in platelets and endothelial cells until the cell is activated by pro-inflammatory stimuli, after

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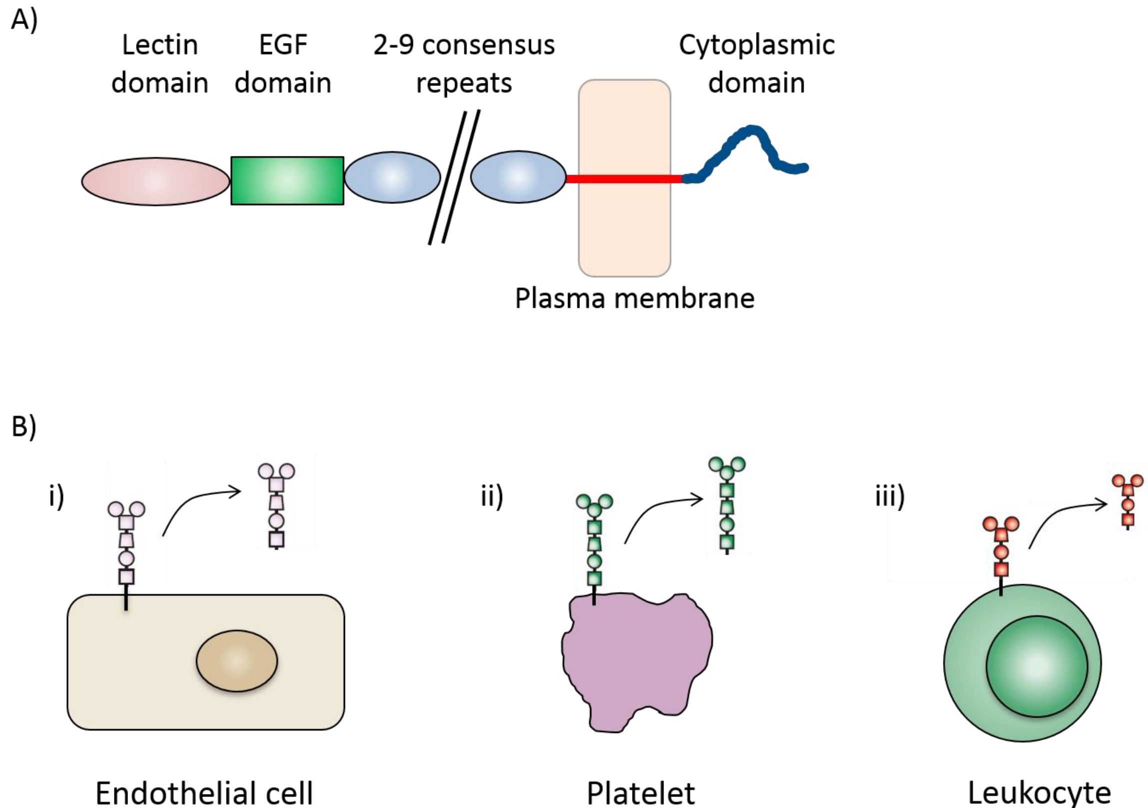

Figure 2. Membrane-bound selectins and the generation of their soluble forms. A) The general structure of selectin proteins consists of a N-terminal lectin domain, an epidermal growth factor (EGF) domain, followed by 2-9 consensus repeats, a transmembrane domain and a C-terminal cytoplasmic domain. **B)** Soluble forms of each selectin are generated through proteolytic cleavage from the cell surface. *i)* E-selectin is expressed on endothelial cells and is cleaved by an unknown mechanism into soluble E-selectin. *ii)* P-selectin is expressed on the surface of both endothelial cells and platelets. Its cleavage from the cell surface is mediated by tumor necrosis factor alpha converting enzyme. *iii)* L-selectin is expressed on the surface of leukocytes and its cleavage is mediated by matrix metalloproteinase 9.

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which P-selectin is rapidly translocated to the cell surface[66]. E-selectin is only expressed on the surface of activated endothelial cells. Its expression is increased hours after cell stimulation with pro-inflammatory mediators as E-selectin is not stored intracellularly in granules, rather it is produced *de novo*[63, 67]

Selectins exist not only as membrane bound proteins but also as cleaved soluble proteins. Cleavage is likely proximal to the membrane spanning domain as it has been noted that there is a slight difference in molecular weight between the membrane-bound and soluble forms **(Figure 2B)**[68-70]. *In vitro* studies suggest that soluble E-, L- and P-selectin remain functionally active after having been shed from the cell surface[71-73]. L-selectin and P-selectin cleavage occurs following cell activation, and the proteases responsible for generating the soluble forms include TNF-alpha converting enzyme and matrix metalloproteinase 9 (MMP-9), respectively[65, 74]. With respect to E-selectin, the protease responsible for generating the soluble form has not yet been identified. However, it is known that E-selectin is shed from the cell surface under normal conditions and that the rate of E-selectin cleavage is increased following stimulation of endothelial cells by cytokines[70].

1.5.1 Clinical Relevance of Selectins in Cancer

Of the three selectins, E-selectin is the most well-studied in the context of cancer. It has been clinically observed that the serum levels of E-selectin are significantly higher in patients with advanced breast cancer that have distant metastasis when compared to either earlier stage patients with localized primary breast tumors or those who have completed initial treatment and have no evidence of relapse at one year post-diagnosis[75-77]. Similarly, other studies have demonstrated that high E-selectin serum concentrations in patients with breast cancer are prognostic for reduced progression-free and overall survival relative to those with low serum concentrations of E-selectin[77, 78]. In addition to these prognostic studies of serum E-selectin, others have examined the effect of E-selectin polymorphisms on breast cancer prognosis. They observed that a genetic mutation resulting in the substitution of serine 128 by arginine increases the affinity of E-selectin for its binding partners[79]. Clinically, patients homozygous for this mutation have poorer survival than patients who are homozygous for the wild-type allele[80].

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For L-selectin, it has not been reported in the literature whether levels of the protein in the blood are affected by breast cancer. However, in acute myeloid leukemia, one study noted that patients with elevated plasma L-selectin levels (defined as a value three standard deviations above the average of healthy control individuals) had lower probabilities of achieving complete cancer remission and had reduced progression-free and overall survival[81]. In another study that examined patients with high-grade muscle invasive bladder cancer, higher concentrations of L-selectin were found in the serum of patients with metastatic vs. nonmetastatic stages of the disease[82]. Similarly, soluble P-selectin has been detected in increased amounts in the sera of breast cancer and colon cancer relative to healthy controls, however no significant differences were noted in P-selectin concentrations between stages of each cancer[83, 84]. Although a mechanistic understanding of selectins in the clinical setting of breast cancer is somewhat limited, studies using experimental models have provided more detailed evidence of the functional importance of selectins in cancer.

1.5.2 Experimental Evidence of the Role of Selectins in Metastasis

Animal models to represent the deficiency of one or more selectins have previously been generated through gene knockout. Phenotypically, selectin deficiencies in single selectins do not cause infertility or embryonic lethality. However, certain combinations of deficiencies can cause health issues. Mice deficient in both E- and P-selectin ($EP^{-/-}$) or E-, L- and P-selectin ($ELP^{-/-}$) were more likely to develop mucocutaneous infections that eventually lead to death. This may be caused by alterations in leukocyte homeostasis and recruitment, as supported by Robinson and colleagues. They demonstrated that peripheral blood leukocyte levels were significantly elevated in $EP^{-/-}$ (1.7 fold relative to wild-type) and $ELP^{-/-}$ mice (2.4 fold), and to a lower extent in mice deficient in P- and L-selectin ($PL^{-/-}$) (1.2 fold)[85]. This leukocytosis was likely caused by impairments of leukocyte recruitment to sites of inflammation and infection, as they also demonstrated that neutrophil influx in $EP^{-/-}$, $PL^{-/-}$ and $ELP^{-/-}$ mice to sites of chemically-induced peritonitis was less than one-third of that observed in wild-type mice.

While these deficiencies may negatively affect immune cell recruitment, evidence suggests that they may also be advantageous to disruption of metastasis. In an experimental

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model of spontaneous breast cancer metastasis, the number of lung metastasis foci was significantly lower in $E^{-/-}$ and $EP^{-/-}$ mice than wild-type[86]. Similar studies have been conducted to examine the influence of L- and P-selectin single or double knockout on lung metastasis in other tumour types including colon carcinoma. $P^{-/-}$ mice and $L^{-/-}$ mice demonstrated lower lung metastatic burden relative to the wild-type control[87]. Relative to the single-knockout mice, the lung metastatic burden was significantly lower in $PL^{-/-}$ mice, suggesting a synergistic effect.

Our lab has previously identified the presence of L-,E, and P-selectin in lung conditioned media. Chu and Xia et al examined the effect of depleting L-selectin from lung-conditioned media on breast cancer cell migration and proliferation. They observed that L-selectin was not involved in cell proliferation of TN breast cancer cells but did affect cell migration/ invasion[25]. However, the role of E- and P-selectin in mediating breast cancer metastatic behavior as well as the mechanisms by which this may occur require further investigation, and that is the overall focus of this thesis.

2 STUDY RATIONALE

Breast cancer is the leading cancer diagnosis and second leading cause of cancer-related death among Canadian women. Current therapies are highly effective in treating breast cancer localized to the breast. However, these therapies are largely ineffective in the metastatic setting, and metastasis accounts for the majority of breast cancer patient deaths. Previous studies from our lab and others have demonstrated that breast cancer cells which express high levels of CD44 are metastatic and preferentially spread to the lung. In accordance with Paget's "seed and soil" hypothesis, we previously sought to understand whether there were factors present in the lung that may interact with $CD44^{+}$ breast cancer cells to promote lung metastasis, and observed that E-, L- and P-selectins, known ligands of CD44, were present in lung-conditioned media. In addition, previous animal studies that involved either breast cancer or colon cancer have demonstrated that deficiency or inhibition of each selectin independently significantly reduced the extent of lung metastasis. However, it is currently unclear whether E-, L-, and/or P-selectins specifically

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derived from the lungs influence breast cancer cell metastatic behaviour, and if so, whether they do so through interactions with CD44.

3 HYPOTHESIS

Lung-derived selectins promote breast cancer migration and/or growth via interactions with CD44.

4 SPECIFIC AIMS

1. To determine the functional role of lung-derived selectins on breast cancer cell migration and/ or growth *in vitro*.
2. To investigate the mechanism(s) by which lung-derived selectins influence breast cancer cell migration and/ or growth *in vitro*.

5 MATERIALS & METHODS

5.1 Cell Culture

MDA-MB-231 (ATCC, Manassas, VA) human breast cancer cells were maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) media (Invitrogen, Burlington, ON) + 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO). At 90% confluency, cells were passaged using 1 mM ethylene diamine tetraacetic acid (EDTA; Bioshop Canada Inc., Burlington, ON). SUM149 (Asterand Inc., Detroit, MI) were maintained in HAM'S F-12 media (Invitrogen) supplemented with 5% FBS (Sigma-Aldrich), 5 μg/mL insulin (Invitrogen), 1 μg/mL hydrocortisone (Invitrogen) and 10 mM HEPES (Invitrogen). At 80% confluency, cells were passaged using 0.25% trypsin/EDTA in citrate saline (Life Technologies, Carlsbad, CA). Primary pulmonary mouse endothelial cells (generously provided by Dr. Sean Gill) were maintained in DMEM low glucose/pyruvate/L-Glutamine media (Thermo Fisher Scientific, Waltham, MA) supplemented with 18% heat-inactivated FBS (Sigma-Aldrich), penicillin-streptomycin (50 U/mL penicillin-50 μg/mL streptomycin; Invitrogen) and HEPES (Invitrogen). Cells were cultured and maintained at 37°C and 5% CO_2.

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5.2 Lung-Conditioned Media

5.2.1 Animal and Lung Harvesting

Athymic nude mice were purchased and maintained under the guidelines of the Canadian Council of Animal care as outlined by the protocol approved by the University of Western Ontario Council on Animal Care (**Appendix 1**). Healthy 5-7 week old female athymic nude mice were euthanized by CO_2 inhalation and their lungs and heart were aseptically removed *en bloc*. The heart was then carefully removed to avoid damaging the lungs and discarded, after which the lungs were placed in a pre-weighed 50 mL conical tube containing 30 mL of PBS.

5.2.2 Lung-Conditioned Media Generation

Following the harvesting of lungs (n=4 mice per session), the total weight of lungs + PBS was determined using an electronic balance. Harvested lungs were washed three times in ice cold PBS before being minced into ~1 mm^3 fragments. Lungs were weight-normalized by re-suspension in a 4:1 media to tissue (v/w) ratio in DMEM:F12 supplemented with $Mito_+$ serum extender (1X; BD Biosciences, Mississauga, ON; basal media) and penicillin-streptomycin (50 U/mL penicillin-50 μg/mL streptomycin; Invitrogen). Lung fragment/media suspensions were then incubated at 37°C and 5% CO_2 for 24 hours. Following incubation, conditioned media (CM) was further diluted four-fold in basal media, then separated from lung fragments by centrifugation at 900 *g* for 15 min at 4°C. Lung-CM was then filtered through a 0.22 μm filter (Corning Inc., Corning, NY), aliquoted and stored at -80°C until use (**Figure 3A**). The original protocol for this model was described by Chu and colleagues[21].

5.2.3 Assessment of E-selectin, L-selectin and P-selectin Concentration

To assess the presence and concentration of selectins in lung-CM, Quantikine® Mouse E-Selectin/CD26E, Quantikine® Mouse sP-selectin/CD62P, and Thermo Scientific Mouse L-selectin ELISA kits (R&D Systems, Minneapolis, MN, USA) were used. Basal media, lung-CM, and lung-CM depleted of E, L- or P-selectin (described below and in **Figure 3B**) were added to the supplied pre-coated microplates and the ELISAs were carried

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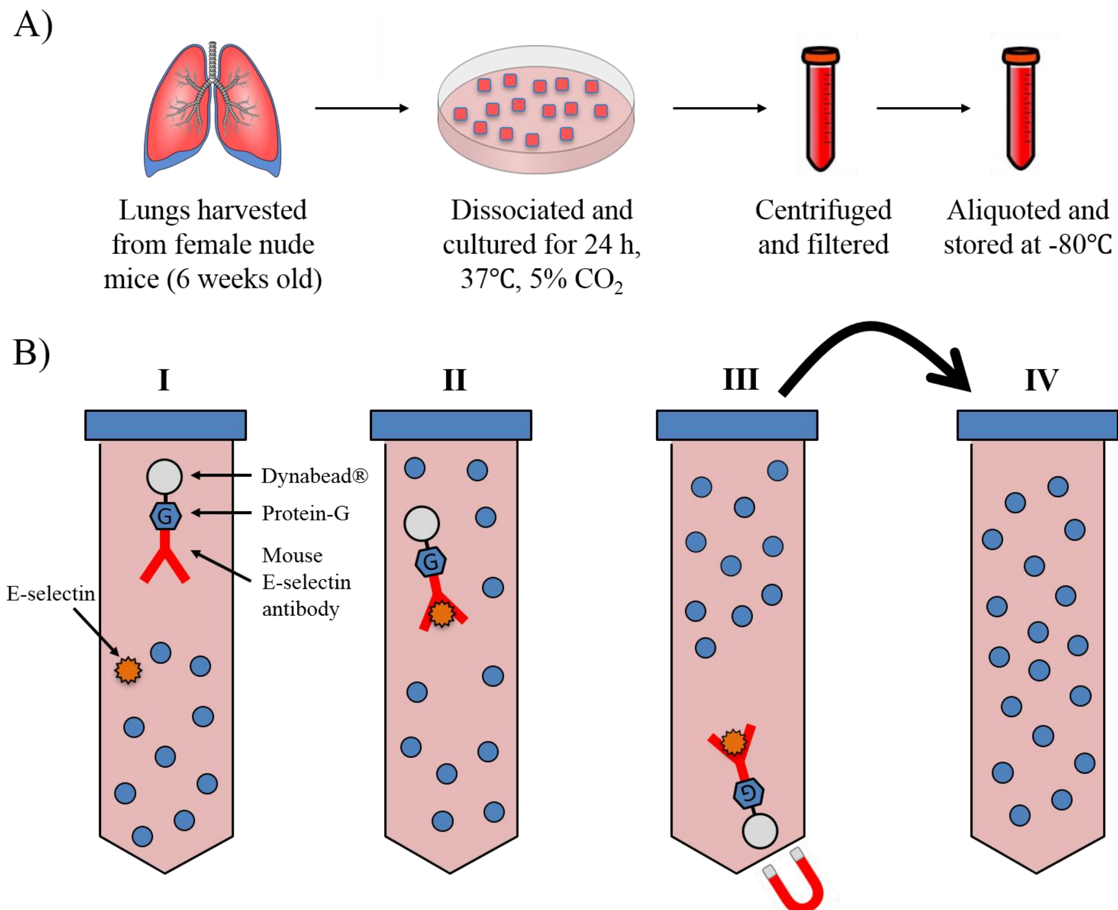

Figure 3. Lung conditioned media generation and selectin immunodepletion. (A) Healthy female nude mice were euthanized by CO_2 inhalation and their lungs removed aseptically. Harvested lungs were then washed, minced into ~1 mm^3 fragments, and resuspended in a 4:1 media to tissue (v/w) ratio for incubation at 37°C and 5% CO_2 for 24 h. Following incubation, heterogeneous solution is further diluted by three volumes of media, centrifuged and lung-CM filtered prior to use. **(B)** Immunodepletion of E-, P-, or L-selectin from lung-CM is accomplished using magnetic Protein-G Dynabeads®. *I.* An antibody specific to one type of selectin (E, P, L) bound to G-protein coupled Dynabeads® was added to lung-CM. *II.* Lung-CM/antibody-Dynabeads® mixture was mixed for 30 min to allow antibodies to bind their protein target present in lung-CM. *III.* After mixing, a magnet is used to separate lung-CM from the selectin antibody/Dynabeads® complexes. *IV.* The remaining lung-CM that is now depleted of the targeted protein is transferred to a separate tube, filtered through a 0.22 µm syringe filter and stored at -80°C until use.

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out according to the manufacturer's instructions to measure the concentration of E-, L- and P-selectin in lung-CM and the efficiency of immunodepletion. A four-parameter logistic (4-PL) standard curve was generated for each experiment using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA) and used to determine the exact protein concentration in each sample.

5.2.4 Selectin Immunodepletion from Lung-Conditioned Media

To determine the influence of lung-derived selectins on *in vitro* migration and proliferation of breast cancer cells, E-, L- and P-selectin were individually immunodepleted from lung-CM using Dynabeads® Protein G (Life Technologies). Dynabeads were suspended in 200 μL of wash buffer (PBS + 0.02% Tween-20) to equilibrate and then placed again on the magnetic rack for 2 min for separation from solution. Wash buffer was carefully removed and replaced with wash buffer + appropriate antibody.

For E-selectin depletion, 20 μL (0.6 μg) of beads and 4 μL of monoclonal E-selectin antibody (0.5 mg/mL; R&D Systems) were used per 1 mL of lung-CM. For L-selectin depletion, 10 μL (0.3 μg) of beads and 8 μL of monoclonal L-selectin (0.5 mg/mL; R&D Systems) antibody were used per 1 ml of lung-CM. For P-selectin depletion, 30 μL (0.9 μg) of beads and 50 μL of polyclonal P-selectin antibody (0.2 mg/mL; R&D Systems) were used per 1 ml of lung-CM.

The bead/antibody mixtures were first mixed by pipetting and then allowed to nutate for 20 min at RT. Following nutation, tubes were placed on the magnetic rack for 2 min to separate antibody-bound beads from solution. The supernatant was carefully removed, and the antibody-bound beads were washed three times, before the beads were exposed to 1 mL of lung-CM. The antibody-bound bead and lung-CM mixture were then nutated for 30 min at RT. Post-nutation, samples were put on the magnetic rack for 2 min to separate the selectin/antibody/bead complexes from the now depleted lung-CM. A small aliquot of depleted lung-CM was stored for quantification of protein levels by ELISA and the rest was transferred to a new tube, passed through a 0.22 μm syringe filter and stored at -80°C until use (**Figure 3B**). The selectin/antibody/bead complexes were washed in wash buffer and magnetically separated from solution three times. Selectin/antibody/bead

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complexes were then suspended in 30 μL of 1x Laemmli buffer and heated at 100℃ in a water bath for 5 min. Post-heating, samples were magnetically separated from beads, the supernatant was transferred to a clean tube and used for SDS-PAGE and immunoblotting. For rescue experiments, an amount of recombinant E-,L, or P-selectin protein that was equal to the calculated average concentration of each immunodepleted selectin was added back to depleted lung-CM (E-selectin [0.25 ng/mL], L-selectin [7.05 ng/mL]) and P-selectin [10.39 ng/mL] (R&D Systems).

5.3 Blood Collection and Lymphocyte Isolation

Healthy 5-7-week-old female athymic nude mice were euthanized by CO_2 inhalation and blood was collected by ventricular puncture into heparin-containing tubes. Blood from four mice was combined and diluted 1:1 with PBS. Blood was layered over 15 mL of Ficoll-Paque (Sigma-Aldrich) in a 50 mL conical tube and centrifuged at 500 x g for 30 min with the brake off. Immediately afterwards, a Pasteur pipette was used to carefully aspirate the mononuclear cell layer (located at the interface between the upper plasma and lower Ficoll-Paque layers) and transfer it to a new 50 mL conical tube. Ten mL of 0.8% ammonium chloride was mixed with the isolated cell layer to remove residual erythroid components. The sample was then centrifuged for 10 min at 400 x g. The supernatant was then decanted, and the cell pellet was resuspended in a small volume of cell lysis buffer (1% NP-40, 25mM Tris-HCl pH 7.5, 100 mM NaCl, 5% Glycerol, 1x Halt™ Protease Inhibitor Cocktail (Thermo Scientific)) for protein isolation.

5.4 BrdU Incorporation Assay

The bromodeoxyuridine (BrdU) incorporation assay was used to assess proliferation of MDA-MB-231 and SUM149 human breast cancer cells. Cells ($1x10^4$ cells/well) were plated on 8-well Lab-tek™ chamber slides (Thermo Scientific) and incubated for 24 h to allow adhering. Cells were then washed once gently with PBS and serum starved for 72 h. Media was then changed to either basal media, positive control media (basal media + 10% FBS), lung-CM or E-, L- or P-selectin depleted lung-CM for an additional 24 h. Following incubation in the treatment conditions, cells were exposed to BrdU (5 µg/mL; Amersham Cell Proliferation Labelling Reagent; GE Healthcare,

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Piscataway, NJ, USA) for 30 min to allow its incorporation into newly-synthesized DNA. Cells were washed with PBS and fixed with 10% neutral-buffered formalin (Fisher Scientific) for 5 min. Cell membranes were then permeabilized using 0.1% Triton X-100 (Sigma) in PBS for 10 min and subsequently treated with 2N HCl (Fisher Scientific) for another 10 min to denature DNA. To minimize non-specific binding, slides were blocked for 30 min in 5% bovine serum albumin (BSA) in 0.1% Triton X-100 in PBS. For detection, anti-BrdU primary antibody (BD Biosciences) diluted 1:75 in 5% BSA/0.1% Triton X-100 in PBS was added and slides were incubated overnight at 4°C. Unbound primary antibody was washed off with PBS and a FITC-conjugated anti-mouse IgG secondary antibody (H+L made in horse; Vector Laboratories, Burlington, ON, Canada) diluted 1:100 in 5% BSA + 0.1% Triton X-100 in PBS was added for 1 h at RT. Unbound secondary antibody was washed off using PBS and the slides were then mounted with ProLong Gold Antifade Mountant with DAPI (Invitrogen) and allowed to cure overnight in the dark at RT. Five high powered fields of view (FOV) were taken per well and nuclei enumerated using ImageJ. Results were expressed as the percentage of BrdU positive cells to total nuclei.

5.5 Transwell Migration Assay

The migration of MDA-MB-231 and SUM149 human breast cancer cells towards lung-CM was assessed using transwell migration assays. Prior to the start of the assay, Fluoroblok™ transwell inserts (24-well, 6.4 mm polyethylene terephthalate membrane, 8 μm pore size; Corning Inc., Corning, NY, USA) were coated with 100 μL of gelatin (6 μg/well; Bioshop) and allowed to dry overnight in a sterile environment at RT. Immediately prior to the assay, the gelatin was reconstituted with 100 μL of control media (DMEM:F12 + Mito$^+$ + 0.1% BSA; Bioshop) and agitated for 90 min at RT. During the reconstitution incubation, MDA-MB-231 or SUM149 cells were harvested, washed twice with PBS and suspended at a concentration of 5 x 10^5 cells/mL. After reconstitution, excess media from the top of the transwells was carefully removed and 100 μL of cell suspension was added in replacement. In the lower compartment of the transwell chamber, 600 μL of either negative basal media, 1:2 diluted lung-CM or 1:2 diluted lung-CM depleted of E-, L- or P-selectin was added. Transwells were then incubated for 18 hours at 37°C and 5% CO_2 to allow migration. After 18 hours, transwell chambers were removed, fixed with 1%

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glutaraldehyde in PBS (Fisher Scientific) for 20 min. Transwell membranes were then washed once with distilled water and a cotton swab was used to remove the non-migrated cells from the inner portion of the membrane. Membranes were then carefully cut out, placed on microscope slides and mounted using ProLong® Gold Antifade Mountant with DAPI (Life Technologies). Five high powered FOV were captured for each membrane, and a mean number of migrated cells/FOV was calculated using ImageJ software (NIH, Bethesda, MD, USA).

5.6 Co-immunoprecipitation

To assess potential interactions between selectins and CD44, co-immunoprecipitation was performed. Confluent 100 mm dishes of MDA-MB-231 cells were washed three times with PBS and lysed with 500 μL of lysis buffer (1% NP-40, 25mM Tris-HCl pH 7.5, 100 mM NaCl, 5% Glycerol, 1x Halt™ Protease Inhibitor Cocktail [Thermo Scientific]). Lysates were collected using cell scrapers and transferred to a 1.5 mL microfuge tube that was then placed on ice for 1 h. Following the incubation period, lysates were centrifuged for 10 min at 13,000 *g* and 4°C. During this period, 20 μL (0.6 μg) of Dynabeads were equilibrated in 200 μL of wash buffer (25mM Tris-HCl pH 7.5, 100 mM NaCl, 5% Glycerol) and placed on a magnetic rack for 2 min to separate from solution. Wash buffer was carefully removed and replaced with 500 μL of MDA-MB-231 cell lysate supernatant. The remaining pellet from the cell lysates was discarded.

Two micrograms of recombinant mouse E-selectin Fc chimera protein, recombinant mouse L-selectin Fc chimera protein or recombinant mouse P-selectin Fc chimera protein (R&D Systems) was added to each tube containing cell lysate/beads. Cell lysate/beads without recombinant protein was used as a negative control. Lysate/bead/recombinant protein mixtures were placed on a rotator overnight at 4°C. The following morning, tubes were placed on a magnetic rack to separate beads/bound proteins from solution. The supernatant was then carefully removed, and remaining beads were resuspended in wash buffer, washed and magnetically separated. The wash and separation steps were repeated twice, beads were suspended in 30 μL of Laemmli buffer, and boiled for 5 min in a 100 °C water bath. After boiling, samples were placed on the magnetic rack

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for 2 min and the supernatant was transferred to a clean 1.5 ml microfuge tube for storage at -20℃ until SDS-PAGE and immunoblotting were performed.

5.7 Immunoblotting

Cell lysates were quantified using the DC Protein Assay (Biorad, Hercules, CA). Thirty micrograms of cell lysates or 30 μL of immunoprecipitated samples were subjected to separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (PVDF; Millipore, Billerica, MA). Membranes were blocked using 5% skim milk in Tris-buffered saline + 0.1% Tween-20 (TBS-T; Sigma) for all western blots, unless otherwise indicated. Primary and secondary antibody concentrations used are described in **Table 2**. Protein expression was visualized using Clarity Max™ Western ECL Substrate (Bio-Rad) and normalized by immunostaining for beta-actin as a loading control.

5.8 Phosphokinase Array

MDA-MB-231 human breast cancer cells were serum starved for 24 h and then exposed to one of three conditions for 15 min: basal media, lung-CM or lung-CM depleted of L-selectin. Immediately after treatment, cells were gently washed three times with cold 1x PBS and lysed with lysis buffer. Lysates protein concentrations were measured using the BioRad DC protein assay (BioRad). The phospho-kinase array (Proteome Profiler™ Human Phospho-Kinase Array; R&D Systems) was carried out using 200 μg of each lysate as per the manufacturer's guidelines. Using the kit-provided wash buffer, unbound proteins were removed from membranes, and a cocktail of detection antibodies was applied. To detect the amount of phosphorylated protein bound to each capture spot, streptavidin-HRP and chemiluminescent agents were used to produce a signal. The signals were then visualized using a ChemiDoc System and analyzed for densitometry using Image Lab™ Software (BioRad) (n=3 for each treatment condition). To validate the accuracy of the phospho-kinase array and expand the number of treatment conditions being examined with respect to identified proteins of interest, immunoblotting was performed. MDA-MB-231 cells were exposed to basal media, lung-CM (native or depleted of individual selectins) or

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Table 2. Details of primary and secondary antibodies used for immunoblotting

1° or 2° antibody	Target Species	Target Protein	Clone	Commercial Source	Antibody Host	Antibody Conditions
1°	Human	β-actin	Polyclonal	Sigma	Rabbit	1:5000 O/N at 4°C
1°	Human	CD44S	156-3C11	Invitrogen	Mouse	1:100 O/N at 4°C
1°	Mouse	E-Selectin	96403	R&D Systems	Rat	1:500 O/N at 4°C
1°	Mouse	L-Selectin	95205	R&D Systems	Rat	1:1000 O/N at 4°C
1°	Mouse	P-Selectin	Polyclonal	R&D Systems	Goat	1:1000 O/N at 4°C
1°	Human	Ezrin/ Radixin/ Moesin	Polyclonal	Cell Signaling Technology	Rabbit	1:500 O/N at 4°C
1°	Human	Phospho-Ezrin (Thr567/ Radixin(Thr564)/ Moesin (Thr558)	Polyclonal	Cell Signaling Technology	Rabbit	1:500 O/N at 4°C
1°	Human	CREB	Monoclonal	Cell Signaling Technology	Rabbit	1:1000 O/N at 4°C
1°	Human	Phospho-CREB (Ser133)	Monoclonal	Cell Signaling Technology	Rabbit	1:1000 O/N at 4°C
2°	Goat	Goat IgG	Polyclonal	Calbiochem	Rabbit	1:2000 (1 h @ RT)
2°	Mouse	Mouse IgG	Polyclonal	Calbiochem	Goat	1:2000 (1 h @ RT)
2°	Rabbit	Rabbit IgG	Polyclonal	Calbiochem	Goat	1:2000 (1 h @ RT)
2°	Rat	Rat IgG	Polyclonal	Calbiochem	Goat	1:2000 (1 h @ RT)

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recombinant protein (individual selectins diluted in basal media) conditions for 15 min as described earlier and analyzed by immunoblotting for specific proteins of interest.

5.9 Statistical Analysis

A minimum of three biological replicates were performed for each experiment, with three technical replicates within each experiment for transwell migration and BrdU incorporation assays. All statistical analyses were performed using GraphPad Prism 6.0 (San Diego, CA, USA), and data are presented as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was used to compare means between treatment groups, followed by a Tukey's post-hoc test to confirm significance. In some cases, a Students t-test was used for pair-wise comparisons. P values less than 0.05 were deemed statistically significant.

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6 RESULTS

6.1 E-, L-, and P-selectin are present in lung-CM

We have previously demonstrated that lung-CM generated from the lungs of healthy female mice can be used as a model of the soluble lung microenvironment. Using protein array analysis, we identified over 70 lung-derived proteins in lung-CM that are not present in basal media[25]. In particular, E-, L- and P selectin were identified as proteins for further investigation based on their previous association with metastasis and the fact that they were known CD44 ligands[88-90]. Using ELISA, the presence and concentration of each selectin protein was quantified in lung-CM relative to basal media. We observed that E-selectin (**Figure 4A**), L-selectin (**Figure 4B)** and P-selectin (**Figure 4C)** are present in lung-CM at significantly higher concentrations than in basal media ($p<0.05$). Each selectin could be successfully individually immunodepleted, resulting in lung-CM that had significantly reduced levels of E-, L, or P-selectin compared to non-depleted lung-CM ($p<0.05$). In the absence of a specific antibody, Dynabeads® Protein G alone had no significant effect on immunodepletion of selectins. Analysis of positive controls (activated endothelial cells) demonstrated the specificity of the ELISA for the selectins of interest.

We were also interested in trying to determine which selectin forms (cleaved/soluble versus full-length) were present in lung-CM and attempted to characterize this by immunoblot analysis of the immunoprecipitated fractions (**Appendix 2**). However, lack of optimal antibodies for immunoblotting and the small difference in molecular weight and antibody specificity made it very difficult to resolve two close bands in the case where both forms might be potentially present, or to identify a band as one form with certainty (in humans, it has been reported that the soluble forms of each selectin are 3-6 kDa smaller than their full-length counterparts, as they are cleaved at a membrane proximal region such that the transmembrane and cytoplasmic regions are removed[68-70]).

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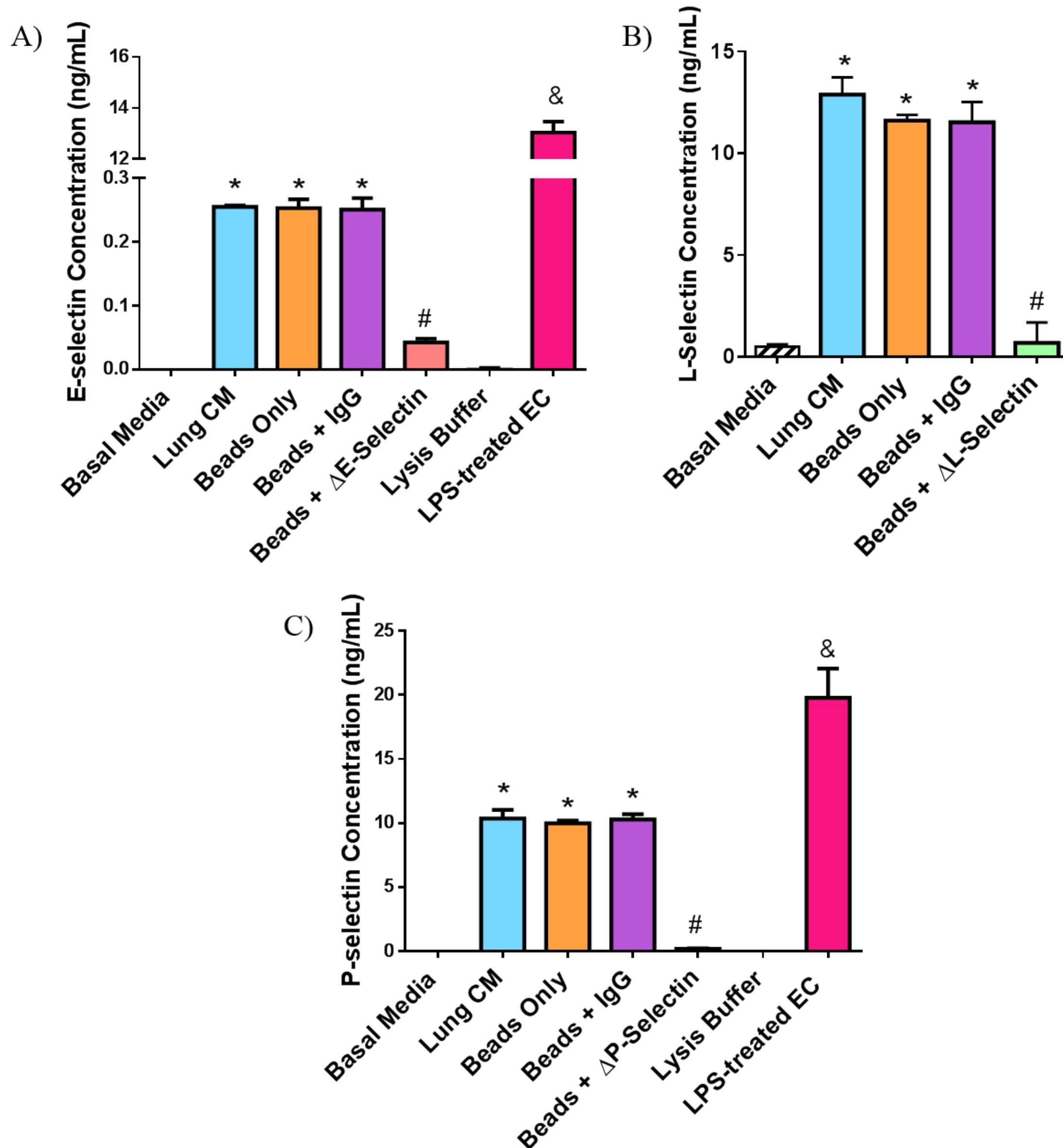

Figure 4. E-, L-, and P-selectin are present in lung-conditioned media (CM) and can be immunodepleted. Lung-CM generated from the lungs of healthy female nude mice was assessed for the presence and concentration of (A) E-selectin, (B) L-selectin, and (C) P-selectin protein by ELISA, and immunodepletion (denoted by Δ symbol) was carried out using specific respective antibodies coupled to Dynabeads® Protein G. Immunodepletion controls included basal media, lung-CM exposed to Dynabeads Protein G only, and lung-CM exposed to Dynabeads plus a nonspecific anti-mouse IgG antibody. Lysates of lipopolysaccharide-stimulated mouse endothelial cells (EC) were used as an additional positive control for E- and P-selectin. Data is presented as mean protein concentration +/- SEM. (n=3). Analysis was carried out using a one-way ANOVA and Tukey's post hoc test. * = significantly different than basal media (p<0.05); # = significantly different than respective non-depleted media (p<0.05); & = significantly different than lysis buffer (p<0.05).

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6.2 Lung-derived selectins enhance breast cancer cell migration

Recent findings from our lab have demonstrated that cell migration of several breast cancer cell lines including MDA-MB-231 and SUM149 is increased in response to lung-CM relative to basal media[25]. To determine whether this effect is due in part to the presence of E-selectin, L-selectin and/or P-selectin in lung-CM, a transwell migration assay was used. MDA-MB-231 (**Figure 5A**) or SUM149 (**Figure 5B**) human breast cancer cells were exposed to one of the following conditions for 18 hours: basal media, lung-CM, or lung-CM depleted of E-selectin, L-selectin or P-selectin. Relative to the negative control basal media, MDA-MB-231 and SUM149 human breast cancer cells demonstrated significantly higher migration towards lung-CM ($p<0.05$). This increased migration towards lung-CM could be abrogated in both cell lines when each selectin was individually immunodepleted ($p<0.05$). Addition of specific recombinant mouse selectins to depleted lung-CM at a level that restored selectin protein levels to those detected in the naive lung-CM rescued the pro-migratory effect of lung-CM to levels consistent with the non-depleted lung-CM ($p>0.05$). Taken together, these results indicate that lung-derived E-, L-, and P-selectins can enhance breast cancer cell migration.

6.3 Breast cancer cell proliferation is independent of lung-derived selectins

Our group has also previously observed that lung-CM can not only increase migration, but also influences the proliferation of human breast cancer cells[25]. To determine whether selectins are contributing to the observed proliferative effect of lung-CM, BrdU incorporation assays were used. MDA-MB-231 and SUM149 human breast cancer cells were exposed to basal media, lung-CM, or lung-CM depleted of E-selectin, L-selectin or P-selectin for 24 h. Cells were then transiently exposed to BrdU and incorporation was detected by immunofluorescence. Consistent with our previous study[25], we observed that a greater proportion of MDA-MB-231 (**Figure 6A,B**) and SUM149 cells (**Figure 6C,D**) were BrdU-positive following exposure to lung-CM relative to basal media ($p<0.05$). However, the proportion of BrdU-positive cells was unaffected by the individual

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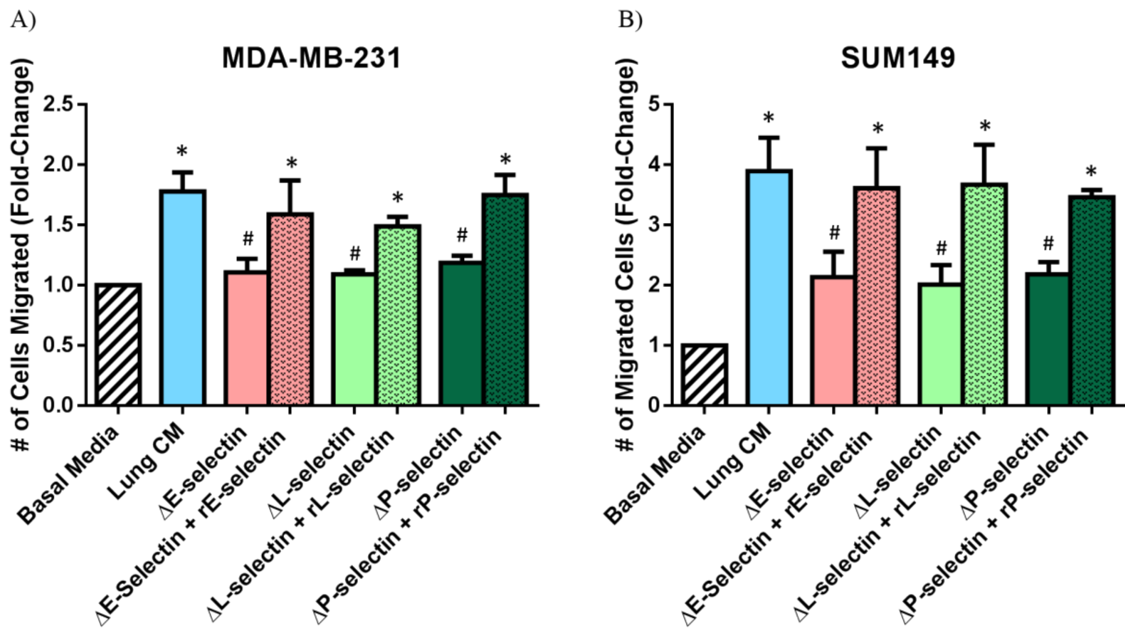

Figure 5. Lung-derived selectins enhance breast cancer cell migration. (A) MDA-MB-231 or (B) SUM149 human breast cancer cells (5 x 10^4 per well) were plated in triplicate (n= 3) on top of gelatin-coated Corning® Fluroblok™ transwells (8-μm pore size) before exposure to one of the following conditions: basal media; lung-CM; lung-CM depleted of E-selectin; L-selectin or P-selectin (denoted by Δ symbol); or depleted lung-CM + recombinant E-selectin, L-selectin or P-selectin (added at the concentration needed to restore protein levels to that observed in native lung-CM). Cells were allowed to migrate for 18 hours at 37°C and 5% CO_2. Transwells were fixed, stained with DAPI, and five high-powered images were captured per transwell. Migrated cells were analyzed using ImageJ software (NIH). Data is presented as mean fold-change in number of migrated cells relative to basal media +/- SEM (n=3). Analysis was done with one-way ANOVA and Tukey's post hoc test. * = significantly different from basal media (p<0.05); # = significantly different from non-depleted lung-CM (P<0.05).

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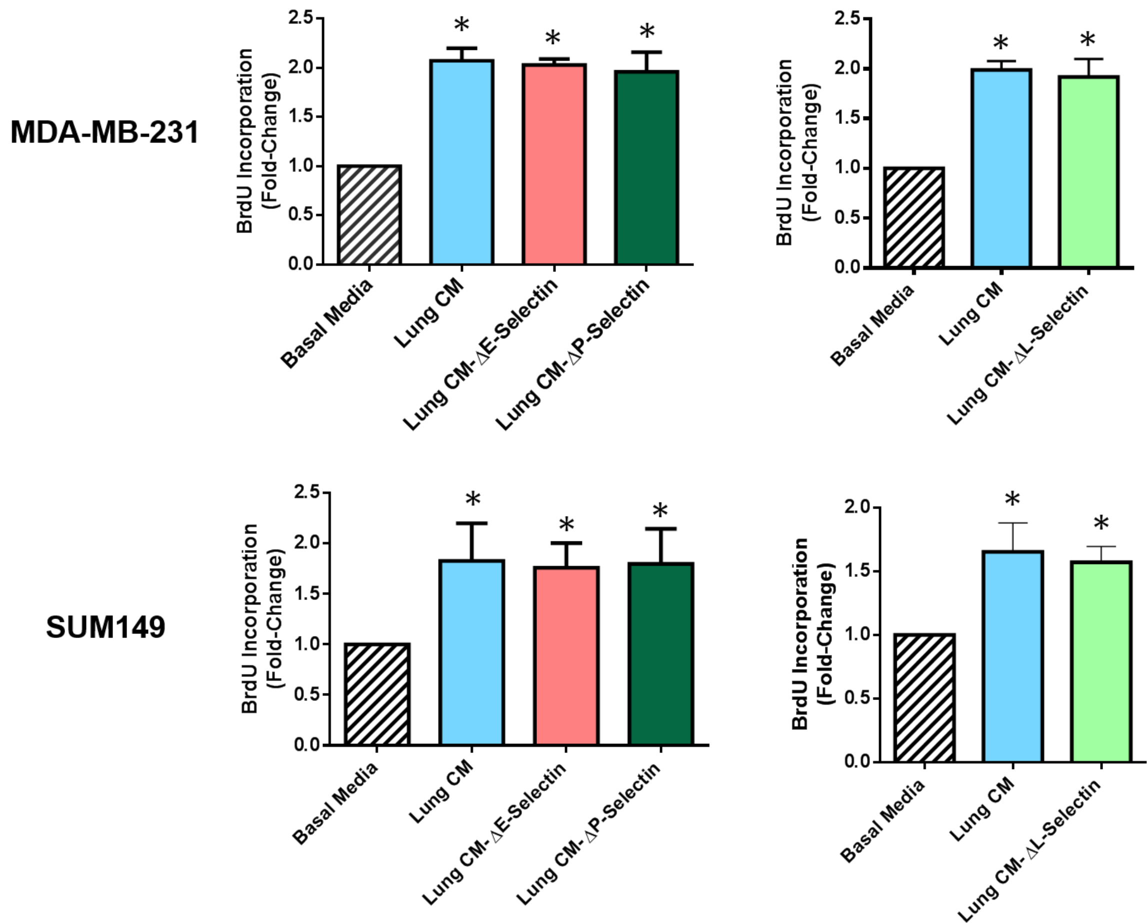

Figure 6. Breast cancer cell proliferation is independent of lung-derived selectins. (A,B) MDA-MB-231 and (C,D) SUM149 human breast cancer cells were plated at a density of $1x10^4$ cells/well on 8-well chamber slides and serum starved for 72 hrs. Cells were then exposed to basal media, lung-CM, and lung-CM depleted of E-selectin, P-selectin or L-selectin (denoted by Δ symbol) for an additional 24 h before assessing proliferation based BrdU incorporation. Five high-powered images per well were captured and used to enumerate the percentage of BrdU-positive cells using ImageJ software (NIH). Data is presented as mean fold-change in BrdU relative to basal media +/- SEM (n=3). *= significantly different from basal media (p<0.05).

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depletion of E-selectin, L-selectin and P-selectin from lung-CM ($p>0.05$), suggesting that breast cancer cell proliferation is independent of lung-derived selectins.

6.4 Mouse selectins interact with human CD44 expressed by MDA-MB-231 cells

To assess whether CD44S expressed by human breast cancer cells could interact with murine selectins, we performed co-immunoprecipitation. The three selectin proteins used were the soluble forms of mouse E-selectin-human IgG_1 chimeric protein, mouse L-selectin-human IgG_1 chimeric protein and mouse P-selectin-human IgG_1 chimeric proteins (R&D Systems). Since all three proteins contained a fused IgG region, it was theoretically possible for them to interact directly with Dynabead® Protein G. We confirmed this by incubating Dynabead® Protein G alone or in combination with one of the selectin proteins overnight at 4℃. Samples were immunoprecipitated and analyzed by immunoblotting (**Figure 7**) for the respective selectin protein using antibodies and conditions described in **Table 2**.

A single band was observed in each lane containing recombinant protein that immunoprecipitated with the Dynabead® Protein G (**Figure 7A**). This suggested that the recombinant chimeric selectins could interact directly with the Dynabead® Protein G without additional antibodies. We then went on to test whether CD44S interacted with the murine selectin proteins. Cell lysates from MDA-MB-231 human breast cancer cells were incubated overnight at 4℃ with Dynabead® Protein G alone or in combination with one of the murine selectin-human IgG_1 chimeric proteins. Immunoprecipitates were then assessed by immunoblotting for human CD44S using antibodies and conditions described in **Table 2.** Bands corresponding to proteins of approximately 90 kDa in weight reacted with the CD44S antibody and were observed in each of E-, L- and P-selectin immunoprecipitates (**Figure 7B**). The protein size detected was consistent with the band observed from the MDA-MB- 231 cell lysate input control. This suggests that murine E-, L-, and P-selectin can interact with CD44S expressed by human breast cancer cells.

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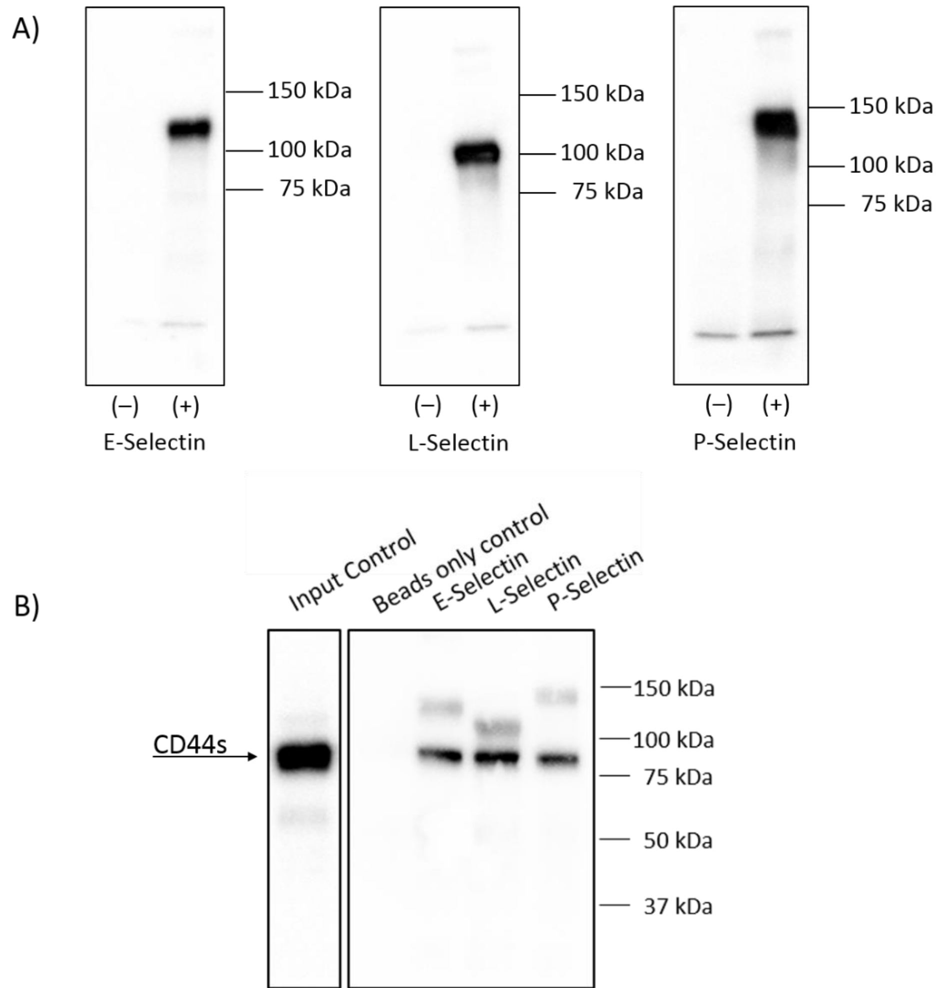

Figure 7. Mouse selectins interact with human CD44S expressed by MDA-MB-231 cells. A) Dynabead® Protein G were incubated alone or with a murine E-, L- or P-selectin-human IgG_1 chimeric protein (R&D Systems) overnight at 4°C. Proteins potentially bound to Dynabead® Protein G were isolated and analyzed by immunoblotting. Membranes were blocked with 5% BSA in TTBS for 1 h at room temperature before overnight incubation at 4°C with an anti-E-selectin (1:500), anti-L-selectin (1:1000) or anti-P-selectin (1:1000) antibody diluted in 5% BSA in TTBS. Membranes were then washed with PBS and incubated at room temperature for 1 h with an anti-rat IgG, anti-rat IgG or anti-goat IgG secondary antibody (all diluted 1:2000 in 5% BSA in TTBS) for E-, L- and P-selectin, respectively. Membranes were visualized by chemiluminescence and captured on a ChemiDoc™ Imaging Systems. (n=3). B) MDA-MB-231 cell lysates were incubated overnight at 4°C with Dynabeads® Protein G alone or with 2μg of murine E-, L- or P-selectin-human IgG_1 chimeric protein. Immunoprecipitates were isolated and immunoblotted. Membranes were blocked with 5% BSA in TTBS for 1 h at room temperature, then incubated overnight at 4°C with an anti-CD44S antibody (1:100 diluted in 5% BSA in TTBS) and incubated at room temperature for 1 h with an anti-mouse IgG secondary antibody (1:2000 dilution in 5% BSA in TTBS) the following morning. Membranes were then visualized by chemiluminescence and captured on a ChemiDoc™ Imaging Systems. (n=3).

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6.5 Effect of lung-derived selectins and CD44 on migration-related signaling pathways

To determine whether the interaction between selectins and CD44S contributes to the pro-migratory effect of selectins that we observed in our earlier experiments, immunoblotting was used to investigate the effect of lung-derived selectins and/or CD44 on migration-related signaling pathways. The highly related ERM (ezrin, radixin, moesin) proteins were of interest in examining because when phosphorylated they have been shown to serve as linkers between CD44 receptors and the cytoskeleton[91, 92]. Through this role, ERM proteins may facilitate pro-migratory signals[93]. Serum-starved MDA-MB-231 cells were exposed for 15 min to native lung-CM or lung-CM that was depleted of E-, L- or P-selectin. Cellular response to lung-CM in the presence of a functional blocking antibody against CD44S was also assessed. Cell lysates were collected and assessed by immunoblotting for total ERM and phosphorylated ERM proteins (**Figure 8**). No statistically significant changes in the ratio of ERM phosphorylation were observed among treatments when examined together by one-way ANOVA. However, a direct comparison of basal media and lung-CM treatments by t-test demonstrated that phosphorylation was significantly increased in response to lung-CM ($p<0.05$). Taken together, this data suggests that ERM phosphorylation is increased by proteins found in lung-CM but the effect is independent of lung-derived selectins.

Given the complexity of intracellular signalling pathways and how they may mediate migration and/ or proliferation, we used the Proteome Profiler™ Human Phospho-Kinase Array (R&D Systems) to determine what additional proteins may be involved. This array simultaneously profiles changes in the phosphorylation of 43 protein kinases and two related total proteins. Lysates examined were from MDA-MB-231 cells that were treated for 15 min with either basal media, lung-CM or lung-CM depleted of L-selectin. Relative to the basal media treatment, the phosphorylation of a few proteins was significantly increased by lung-CM treatment (**Figure 9**) ($p<0.05$). These proteins included extracellular signal-regulated kinase 1 and 2 (ERK1/2), mitogen- and stress-activated protein kinases 1 and 2 (MSK1/2), cAMP response element binding protein (CREB) and tyrosine-protein kinase (LYN). A significant effect of L-selectin immunodepletion from lung-CM relative

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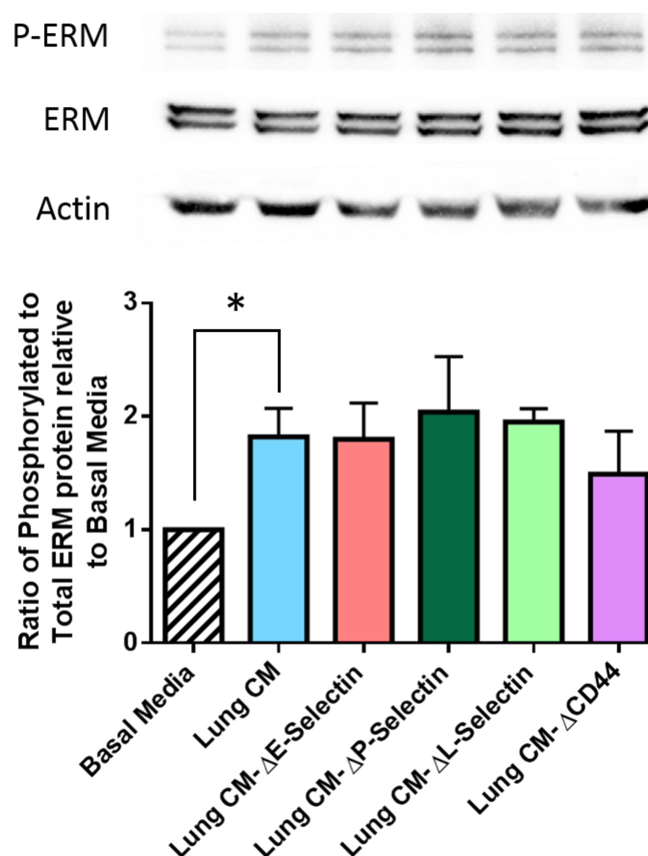

Figure 8. **Effect of lung-conditioned media on ERM protein phosphorylation in human breast cancer cells.** MDA-MB-231 cells were cultured to 90% confluence and then serum starved for 24 h. Cells were then exposed for 15 min to one of the following conditions: basal media, lung CM, or lung CM depleted of E-selectin, P-selectin, L-selectin (denoted by Δ symbol). Cellular response to lung-CM in the presence of a functional blocking antibody against CD44S (CD44Δ) was also assessed. Cells were lysed, protein was quantified by Lowry assay and analyzed by immunoblotting. Membranes were blocked with 5% BSA in TTBS for 1 h at room temperature before immunostaining overnight at 4℃ with the primary antibody for ERM (1:1000 diluted in 5% BSA in TTBS) or phosphorylated ERM (1:1000 diluted in 5% BSA in TTBS). The region of the membrane at the molecular weight of actin was cut and probed with an anti-actin antibody (1:5000 diluted in 5% BSA in TTBS.) The following day, membranes were washed with PBS and incubated with anti-rabbit secondary antibody (1:2000) for 1 hour at room temperature. Bands were visualized using enhanced chemiluminence (ECL) and captured on a ChemiDoc™ Imaging Systems. Quantification was carried out by densitometry using ImageLab software. Data is presented as fold-change in ratio of total:phosphorylated ERM protein relative to basal media condition. (n=3). *= significantly different from basal media ($p<0.05$; t-test).

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Figure 9. **Effect of lung conditioned media on protein phosphorylation in breast cancer cells.** MDA-MB-231 human breast cancer cells were incubated in basal media, lung-CM or lung-CM depleted of L-selectin (Δ) for 15 minutes. Cells were then collected, lysed and incubated with the Human Phospho-Kinase Array membrane (R&D systems) as per the manufacturer's instructions. The presence of phosphorylated proteins was detected using biotinylated antibodies and visualized by chemiluminescence. A) Representative arrays of the basal media, lung-CM and lung-CM depleted of L-selectin are presented. Boxes outlined with dashed lines indicate internal positive controls. Boxes outlined with a solid line are proteins of interest. Extracellular signal-regulated kinase 1 and 2 (ERK1/2), mitogen- and stress-activated protein kinase 1 and 2 (MSK1/2), cAMP response element-binding protein (CREB) and tyrosine-protein kinase Lyn are numbered 1 to 4, respectively. B) Densitometry analysis was performed using Image Lab software (Bio-Rad) and one-way ANOVA with Tukey's post-hoc statistical analysis was performed using GraphPad Prism software. * = significantly different from basal media ($p<0.05$); # = significantly different from lung-CM ($p<0.05$). Only kinases/proteins with significantly different phosphorylation or expression between at least two treatments are shown.

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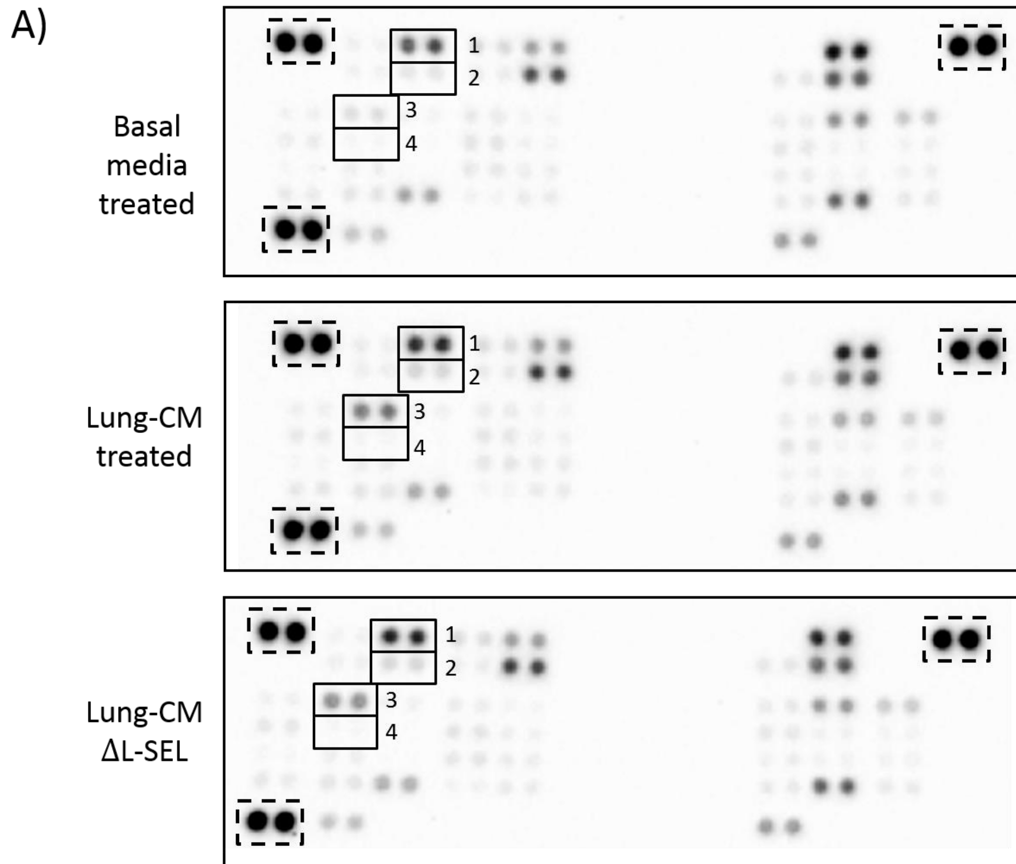

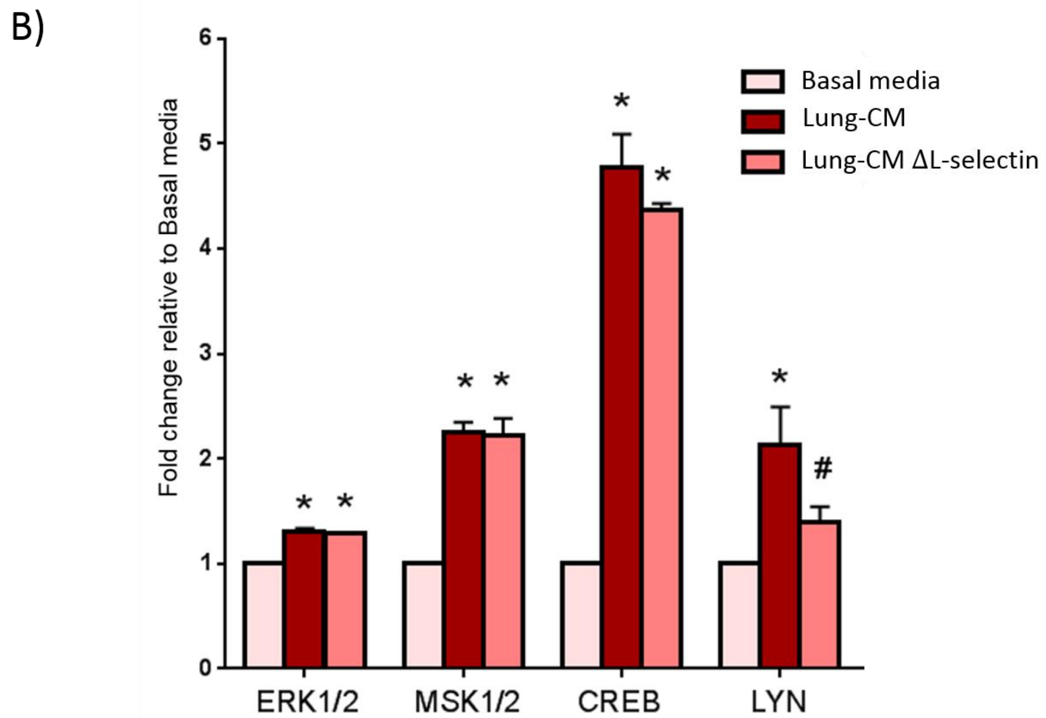

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to non-depleted lung-CM (*p*<0.05) was only observed for the kinase LYN, although a non-significant trend (*p*>0.05) was also observed for CREB. Although the LYN effect could not be validated by immunoblotting (data not shown), CREB was analyzed in greater detail to determine whether lung-derived selectins might signal through this pathway. MDA-MB-231 cells were exposed to non-depleted lung-CM or that which had been depleted of E-, L-, or P-selectin. Cellular response to lung-CM in the presence of a functional blocking antibody against CD44S was also assessed. Cell lysates were analyzed by immunoblotting for total and phosphorylated CREB. We observed that while lung-CM treatment significantly increased CREB protein phosphorylation relative to basal media (*p*<0.05) (consistent with phosphokinase array results), immunodepletion of single selectins or blocking with CD44 did not significantly affect CREB phosphorylation (*p*>0.05), although a non-significant trend could be seen whereby CREB phosphorylation appears to be lower in cells exposed to selectin-depleted lung-CM compared to native lung-CM, or cells blocked by CD44 (**Figure 10A**). In an alternative approach, when MDA-MB-231 cells were treated with recombinant E-, L- and P-selectin at concentrations 10x higher than those observed in lung-CM, a slight but non-significant increase in CREB phosphorylation relative to the basal media was observed only in the presence of E-selectin (*p*>0.05) (**Figure 10B**).

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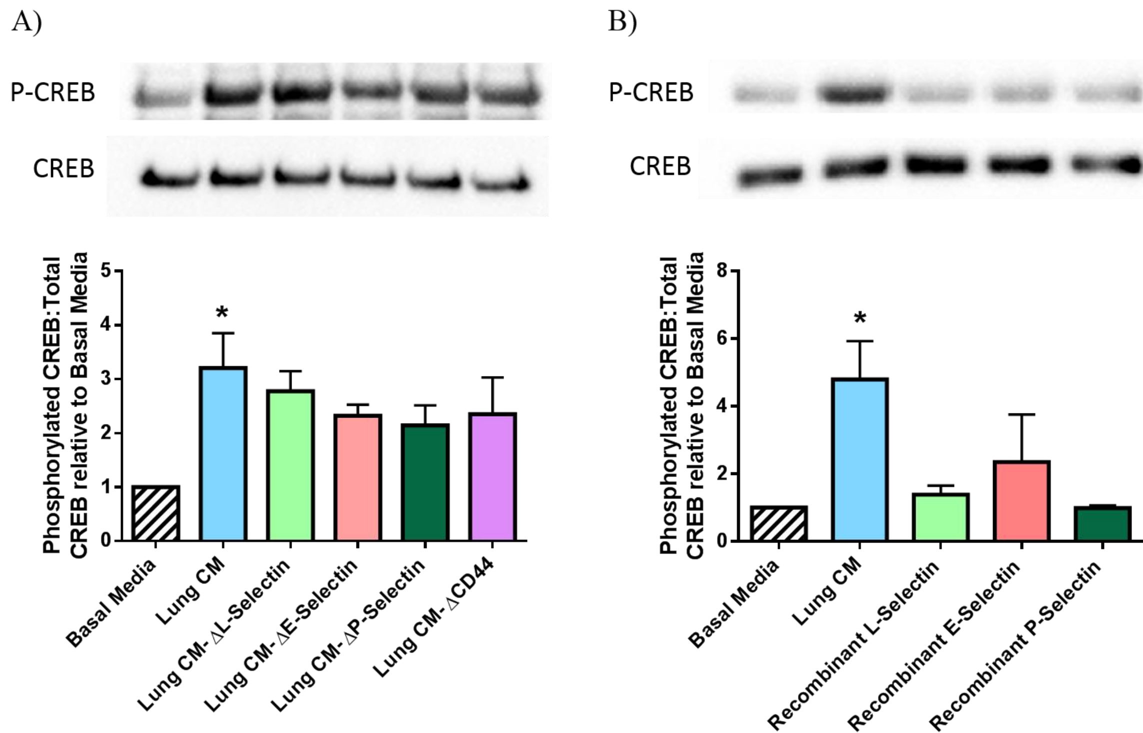

Figure 10. Lung-conditioned media enhances CREB phosphorylation. MDA-MB-231 cells were cultured to confluence and subjected to serum starvation for 24 hours. Following this period, cells were exposed to treatment conditions for 15 min and then harvested, lysed and their protein concentrations quantified by BioRad DC Protein Assay. 30 ug of protein from each lysate was analyzed by immunoblotting. Membranes were blocked for 1 h at room temperature with 5% milk in TTBS, followed by incubation with either P-CREB (1:1000 dilution in 5% milk in TTBS) or CREB (1:1000 in 5% milk in TTBS) primary antibody overnight at 4℃. The following morning, membranes were washed with TTBS and then incubated with an anti-rabbit IgG secondary antibody for 1 hour before visualization by chemiluminescence. Images were captured on a BioRad ChemiDoc system and quantified by densitometry using ImageLab® software. One-way ANOVA and Tukey's post-hoc test were performed using GraphPad Prism software, and results are presented as mean ± SEM (n=3) (A) Cells were treated with basal media, lung-conditioned media (lung-CM) or lung-CM depleted of L-selectin, E-selectin, P-selectin. Cellular response to lung-CM in the presence of a functional blocking antibody against CD44S (CD44Δ) was also assessed. (B) Cells were treated with basal media, lung-CM or recombinant protein in basal media (mouse E-, L- or P-selectin) at concentrations ten-fold higher than those detected in lung-CM. * = significantly different from basal media (p<0.05)

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7 DISCUSSION

Breast cancer is the most commonly diagnosed cancer (with the exception of non-melanoma skin cancers) and second leading cause of cancer-related deaths among Canadian women[94]. Since the 1970s, considerable progress has been made in the clinical management of the disease, with five-year survival rates having risen from 53% in the 1970s to almost 87% for women in 2016[95]. This improvement can be primarily attributed to improved early detection and the development of therapies that target the estrogen receptor (e.g. tamoxifen) and the HER2 receptor (trastuzumab)[96-98]. Unfortunately, triple-negative breast cancer, the most aggressive subtype, does not express ER or HER2 receptors and no other effective targeted therapy exists[99]. In the metastatic setting, breast cancer currently remains incurable.

The "seed and soil" hypothesis was originally proposed by Stephen Paget in 1889 and postulated that the formation of metastatic tumours at distant sites was related to not only the intrinsic properties of the breast cancer cell (the “seed”) but also to factors found in the organ microenvironment (the “soil”) that would allow the tumour cell to survive and grow[24]. For three of the most common sites of breast cancer metastasis (lung, brain and bone), studies conducted by Joan Massagué and colleagues elegantly demonstrated how the genetic signature of breast cancer cells can contribute to determining organ-specific metastasis[26-28].

To study the influence of organ microenvironments common to breast cancer metastasis, our group previously developed an *ex vivo* model that allowed the soluble factors produced by a given tissue to be identified and their influence examined[25]. With respect to the lung, we found that at least 70 unique soluble proteins are present, and that these proteins include chemokines, cytokines, growth factors and extracellular matrix components. Five of the identified proteins are known interactors of CD44, an extracellular receptor that is a marker for aggressive breast cancer cells[34]. These proteins include basic fibroblast growth factor[100], osteopontin[51], E-[53], L-[54] and P-selectin[88]. Our group has demonstrated that basic fibroblast growth factor promotes the proliferation of breast cancer

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cells[101], L-selectin promotes the migration of breast cancer cells and OPN enhances both metastatic behaviours *in vitro*[25].

This thesis focused on investigating the role and potential mechanisms of lung-derived E-, L, and P-selectin in breast cancer metastatic behavior. We hypothesized that lung-derived selectins promote breast cancer migration and/or growth via interactions with CD44.

7.1 Summary of Experimental Findings

1. E-selectin, L-selectin and P-selectin are present in significant concentrations in lung-CM and can be immunodepleted.
2. Each of the three selectin proteins promotes the *in vitro* migration of MDA-MB-231 and SUM-149 human breast cancer cells towards lung-CM.
3. Lung-CM promotes the *in vitro* proliferation of MDA-MB-231 and SUM-149 breast cancer cells but its effect is independent of selectins.
4. CD44S expressed by MDA-MB-231 human breast cancer cells *in vitro* interacts with soluble mouse E-, L- and P-selectin.
5. The phosphorylation of ezrin/radixin/moesin (ERM) proteins in MDA-MB-231 breast cancer cells is enhanced by exposure to lung-CM but is not significantly affected by depletion of individual selectins.
6. Phosphorylation of CREB in MDA-MB-231 cells is significantly increased following exposure to lung-CM relative to a basal media control but is not significantly influenced by selectin depletion or addition.

7.2 Implications of Experimental Findings

The findings of this project support the hypothesis that lung-derived selectins have a pro-metastatic role in breast cancer, specifically through migration. Our group was the first to examine the response of breast cancer cells to selectins in the context of the lung microenvironment.

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7.2.1 Forms of Selectins Found in Lung-Conditioned Media

In the literature, the *in vivo* presence and expression of selectins in the lung has been well-established[25, 102, 103]. Using ELISA, we quantified the amount of each selectin in lung-conditioned media. While we cannot confirm whether the concentrations observed are equal to those observed in the native lung microenvironment, we can at least appreciate their presence, the relative amounts of each selectin family member, and begin to understand the role they may have in breast cancer metastatic behavior.

Due to the nature of our lung-CM model and the need for mechanical dissociation of mouse lungs, the possibility exists that some of each selectin detected by ELISA was of the full-length form. Full-length forms of the protein could have been released from the cell membrane if mechanical dissociation of the lungs damaged the cells. We attempted to determine the presence and relative amount of full-length versus soluble forms of each selectin by immunoprecipitation and subsequent immunoblotting. In humans, the soluble form of each selectin is approximately 3-6 kDa smaller that its full-length counterpart, and the soluble forms of the protein are generated by cleavage at a membrane proximal site such that the transmembrane and cytoplasmic regions are removed[68-70]. Unfortunately, a lack of optimal antibodies and the small difference in molecular weights between the two forms of each selectin made it difficult to resolve and identify which species were present in our lung-CM. However, studies suggest that both soluble and full-length forms of each protein (E-selectin[104, 105], L-selectin[73] and P-selectin[106]) interact with the same receptors/binding partners. Therefore, both forms should theoretically exert the same functional influence on breast cancer cells in our *ex vivo* model.

7.2.2 Lung-derived Selectins are Important for Breast Cancer Migration but not Growth

Next we assessed the functional influence of lung-derived selectins on breast cancer cells and whether it occurs through CD44S, a cell surface receptor that has a well-characterized role in regulating the proliferation, migration and invasion of breast cancer cells[107]. Selectins present in lung-CM were observed to play significant roles in migration of breast cancer cells but did not influence proliferation, as assessed by transwell migration

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and BrdU incorporation assays, respectively. A study conducted by Kumar and colleagues was among the first to demonstrate that soluble E-selectin had a chemotactic effect on immune cells[72]. This was followed by a recently published study in which soluble E-selectin was shown to promote the migration of MDA-MB-231 human breast cancer cells potentially through interactions with CD44[108], which supports our findings. With respect to proliferation, there is no direct evidence implicating E-selectin in the proliferation of cancer cells *in vitro*. However, soluble E-selectin has previously been shown to mediate angiogenesis *in vitro* and in the context of breast cancer, E-selectin deficiency has reduced the number of lung metastatic foci[86]. It is difficult to extrapolate whether changes in the number of metastatic foci observed were related to migration and/or proliferation, as no comparison in the size of metastatic foci was made between wild-type and E-selectin deficient mice.

In comparison to E-selectin, relatively little is known about L-selectin and its influence in breast cancer. In the context of breast cancer, previous work by our laboratory demonstrated that the migration of MDA-MB-231 human breast cancer cells towards lung-CM was mediated in part by L-selectin[25]. The work of this thesis added to our previous findings by demonstrating that the migration of MDA-MB-231 breast cancer cells abrogated by depletion of L-selectin from lung-CM could be rescued through addition of recombinant L-selectin, and by replicating these experiments with all three selectins and in a second breast cancer cell line, SUM149.

There are many studies that have used animal models of cancer to examine the role of P-selectin in metastasis. One of these used a spontaneous model of breast cancer metastasis and interestingly observed that although P-selectin deficiency in mice did not decrease the incidence of lung metastasis, the overall metastatic burden in the lung was significantly higher than that of their wild-type counterparts[86]. They suggested an impaired ability of P-selectin deficient mice in recruiting neutrophils and monocytes was driving this observation[109]. However, in other cancers, such as melanoma and colon carcinoma, opposing results were observed. In an experimental model of melanoma metastasis in which cancer cells were intravenously administered into mice, the number of lung metastases in P-selectin deficient mice was less than half of that observed in wild-type

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mice[110]. Similarly, in a spontaneous metastasis model of colon carcinoma, only 11% of P-selectin deficient mice had any histological signs of tumour cells or microscopic nodules in the lungs. In comparison, signs of metastasis were observed in 89% of wild-type mice injected with tumour cells[103].

At the cellular level, published evidence has suggested a role for P-selectin in cancer cell proliferation, with subcutaneously implanted colon carcinoma cells having significantly lower primary tumour growth in P-selectin deficient mice versus in wild-type[103]. To the best of our knowledge, no previous studies have investigated the influence of P-selectin (lung-derived or otherwise) on migration or proliferation in the context of breast cancer, although a previous study by Morbidelli and colleagues demonstrated that soluble P-selectin could promote endothelial cell migration *in vitro*[111].

While we did not explore the effects of depleting multiple selectins from lung-CM, we did observe that depletion of each selectin individually significantly affected migration of breast cancer cells. It is possible that the selectins may either function through interactions with different receptors at the cell surface or by activating different intracellular mechanisms. This idea is supported by a study on lung metastasis of adenocarcinoma in mice. Borsig and colleagues observed that mice deficient in P-selectin and L-selectin had a significantly lower burden of lung metastasis than wild-type mice or those deficient in only one of the selectins (L- or P-selectin)[87]. How selectins function in the context of metastasis, whether it is through a single receptor or mutual downstream mechanisms, has not been well examined. Furthermore, it is still not clear whether each selectin functions differently/synergistically in migration of breast cancer cells, and/or whether E-,L- and P-selectin may be redundant/compensatory for each other.

7.2.3 Interaction of Lung-Derived Selectins with CD44

The receptor that we were most interested in examining was CD44, as previous evidence has not only supported a role for the receptor in cell migration/invasion[57], but also demonstrated that it can interact with all three selectins[53-55]. An interaction of E-selectin with CD44 expressed by LS174T colon carcinoma cells was originally hypothesized based on CD44 immunoreactivity with the HECA-452 antibody, which detects sialofucosylated

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oligosaccharides (a group that E-selectin binds to)[112]. For P- and L-selectin, studies demonstrated an interaction with CD44 by blot rolling assays[88]. However, detection of a direct interaction by co-immunoprecipitation of selectins with CD44 expressed by breast cancer cells has only been reported for E-selectin[113]. We therefore investigated this further using recombinant mouse soluble selectin-human IgG chimera proteins, which are capable of directly interacting with Dynabead® Protein G. This allowed us to avoid potential issues of immunoprecipitating CD44 or selectins using antibodies that target the ectodomains through which they may interact, since the IgG region that interacts with the Dynabead® Protein G is located near the C-terminus of the chimera, far from the ectodomains. We tested this using soluble mouse selectins, as these were the forms that we hypothesized were found in our lung-CM model.

The interaction of CD44 with mouse selectins that we observed was facilitated by the high degree of homology shared between human and mouse selectins. There is on average, 72% homology in the lectin region of selectins between mouse and human[63]. Whether and/or how this interaction might activate CD44-associated pro-migratory signaling pathways was examined in further studies.

7.2.4 Potential Mechanisms Underlying the Pro-Migratory Effect of Lung-Derived Selectins

Previous studies have suggested ERM (ezrin/radixin/moesin) proteins are involved in promoting migration, potentially through their roles as crosslinkers between CD44 and the underlying actin cytoskeleton[114, 115]. For example, the N-terminal domain of phosphorylated ezrin binds to the cytoplasmic tail of CD44 and the C-terminal domain of ezrin binds to actin. This function is only possible when the ERM proteins are phosphorylated at key threonine residues[91]. In their respective unphosphorylated states, the N- and C-terminal domains of ERM proteins self-associate and make the protein conformationally inaccessible to binding partners[116]. Based on these previous studies, we therefore examined whether lung-derived selectins affected the phosphorylation of the ERM proteins.

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In our results, we observed that exposure of human breast cancer cells to lung-CM increased ERM phosphorylation relative to the basal media negative control. However, no significant differences in phosphorylation were observed between control, lung-CM, lung-CM depleted of a single selectin type or when cells were pre-treated with a neutralizing antibody for CD44. This suggested that the effect of selectins on CD44-associated migration may not be through induced changes in ERM phosphorylation. Rather, selectin interactions with CD44 may affect CD44-pERM interaction. A recent study by Donatello and colleagues observed that there were no differences in the ratio of phosphorylated to total ERM proteins between migrating and non-migrating breast cancer cells[117]. However, by co-immunoprecipitation they did observe greater association of CD44 with pERM in migrating cells than non-migrating cells. This is something that should be further explored in future studies.

In addition to our investigation of ERM phosphorylation, we next sought to identify alternative intracellular mechanisms by which lung-derived selectins may affect breast cancer cell migration. This was done using a human phospho-kinase array. When comparing the effects of basal media, lung-CM and lung-CM depleted of L-selectin on human breast cancer cells, we identified CREB as a potential protein of interest because it had the greatest fold change vs. control in its phosphorylation response to lung-CM. Upon further analysis, CREB phosphorylation was validated by immunoblotting to be significantly elevated in breast cancer cells treated with lung-CM and slightly but non-significantly reduced upon depletion of a single selectin from lung-CM. Similarly, treatment of these cells with recombinant selectin proteins led to a small but non-significant increase in CREB phosphorylation relative to the basal media control, leading to the conclusion that we were unable to demonstrate a role for selectins in CREB phosphorylation. Although previous studies have associated CREB with migration of breast cancer cells and hepatocellular carcinoma cells, the observed effect may be through other soluble proteins found in lung-CM [118, 119].

In our phospho-kinase array, another kinase, tyrosine kinase Lyn, was observed to have increased phosphorylation in breast cancer cells exposed to lung-CM relative to those exposed to basal media. Interestingly, depletion of L-selectin from lung-CM significantly

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reduced phosphorylation of Lyn. In the context of human colon cancer, Lyn has been implicated as a mediator of cell migration downstream of CD44. Researchers observed that CD44 interacted with Lyn and that siRNA knockdown of CD44 in colon cancer cells was associated with lower cell migration and Lyn expression relative to the parental cell line[120]. To our knowledge, the role of Lyn kinase downstream of CD44 in breast cancer has not been investigated and it is an avenue for our group to investigate further in the future.

8 Limitations of the Study

A considerable portion of our study involved an *ex vivo* model system of the soluble lung microenvironment. While it allowed us to easily explore the effects of lung-derived selectins on breast cancer cells, there are limitations that should be acknowledged. The first is that by examining the effects of soluble lung-derived factors on breast cancer cells in isolation, we are ignoring the important contribution that insoluble lung ECM factors and other cells of the lung may have on the cancer cells. Studies of small cell lung cancer have shown that interaction of the cells with ECM proteins such as fibronectin, laminin and collagen IV can both enhance tumorigenicity and confer resistance to chemotherapeutic agents[121]. With respect to the cell-cell interactions, a 2011 study by Qian and colleagues demonstrated that the interactions between monocytes and breast cancer cells were involved in lung metastasis. They noted that breast cancer cells secreted a chemokine capable of activating monocytes that would go on to support cancer cell transmigration[122].

The second limitation of our lung-CM model is that it involves lungs harvested from athymic nude mice. Athymic nude mice lack T-cells and lack cell-mediated immunity. This impairs their ability to mount robust immune responses, something that is increasingly recognized as an important factor in cancer development and progression. For example, the number of $CD8^+$ T-cells present in primary breast tumours has been associated with better patient survival[123]. Similarly, a study on non-small-cell lung cancer demonstrated that increased infiltration of $CD4^+$ and $CD8^+$ T-cells in lung tumours was associated with better patient prognosis as well[124]. In future investigations, models that take into consideration the potential influence of the immune system, cell-ECM and cell-cell interactions should be used.

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The third limitation of the study is the assumption that human breast cancer cells can interact with murine lung soluble factors. Generally, it should not be a concern because the protein-coding regions of the mouse and human genomes are 85% identical on average[125]. A previous study has also shown that of 4,000 genes examined between humans and mice, there are less than ten gene products which exist in one species, but no closely-related form exists in the other. Nonetheless, we must express caution in our interpretation of relevance to human disease, as exceptions have been shown in the literature where human proteins cannot interact with the mouse equivalents of their known binding partners[126].

It is also important to be cognizant of our use of immortalized human breast cancer cell lines rather than primary human breast cancer cells. While the cell lines we used are well characterized in the literature, they may not fully recapitulate the inherent heterogeneity that is seen in the breast cancer patient populations. Researchers previously observed differences in the expression of genes related to metabolism and cell communication between primary metastatic breast cancer cells and established breast cancer cell lines[127]. To try to minimize concerns of discrepancies, our cells are maintained at low passage numbers and routinely authenticated.

Finally, protein phosphorylation, especially that of ERM, is a dose- and time-dependent event[128]. While we were unable to observe a significant change in ERM phosphorylation following cell treatment with different depleted lung-CM, the possibility exists that a noticeable effect may occur, but outside of the time window we had examined.

9 Future Directions

While this study contributes to our understanding of selectins and the role they may play in breast cancer metastasis to the lung, some questions remain unanswered, in particular related to the potential intracellular mechanisms by which selectins exert their influence on breast cancer cells.

As mentioned above, binding of CD44 by selectins may not necessarily increase ERM protein phosphorylation but could alternatively affect the interaction between p-ERM and CD44. By immunofluorescence, we should examine whether ERM proteins and CD44

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colocalize after cell stimulation with recombinant selectin proteins. To determine if any colocalization observed is a result of direct protein interactions, co-immunoprecipitation of phosphorylated ERM proteins with CD44 could be examined. These findings could allow for more definitive conclusions to be made regarding whether the pro-migratory roles of selectins occur via CD44 and ERM.

Next, we should examine other proteins of interest identified by the phospho-kinase array, and in particular, tyrosine kinase Lyn, which has previously been shown to function downstream of CD44[120]. In our experiments with the phospho-kinase array, we observed greater phosphorylation of Lyn in response to lung-CM treatment relative to control, and the extent of phosphorylation to lung-CM was partially abrogated by L-selectin depletion. In future experiments, improved Lyn antibodies for immunoblotting should be used to examine the effects of depleting each selectin from lung-CM or blocking CD44 on Lyn phosphorylation in breast cancer cells.

Another idea to explore is whether there is synergy between selectins when influencing breast cancer cells. While we have shown the effects of removing selectins individually from lung-CM on breast cancer cells, it is unknown as to whether the influence on migration would be strengthened further if two or more selectins were depleted. *In vivo* evidence from experimental models of lung metastasis of colon adenocarcinoma suggests that L- and P-selectin can function synergistically to enhance metastasis[87]. In addition to studies that examine the effect of depleting lung-CM of multiple selectins, we should explore effects of inhibiting selectins with small molecules. Rivipansel (GMI-1070) is a pan-selectin inhibitor that is currently in a phase 3 clinical trial for the treatment of vaso-occlusive crises secondary to sickle cell disease[129]. Although it is not yet commercially available, this drug has been shown to have excellent safety and tolerability in humans[130]. Testing of this drug in our *ex vivo* model of the lung microenvironment could provide valuable preclinical data for Rivipansel's therapeutic value in the treatment of breast cancer metastasis to the lung.

Although the lung-CM model can be used to provide valuable insight about lung-derived soluble factors, it does not consider cell to cell and cell to insoluble ECM protein

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interactions. This can be addressed using a 3D *ex vivo* model of the lung microenvironment. The pulmonary metastasis assay (PuMA) is an *ex vivo* system in which mouse lungs are cut into thin slices (1-2 mm thick) and maintained in culture[62, 101, 131]. Our lab has previously demonstrated that if animals are intravenously injected with breast cancer cells 15 min prior to euthanasia, this is enough time for tumour cells to be seeded in the lung[101]. By then examining lung slices at different time-points, we can see cancer progression from the single cell to micrometastatic to macrometastatic stages. Using the PuMA, we could compare tumour progression between breast cancer cells that do or do not express CD44 in conjunction with comparisons of this in wild-type and selectin-knockout (single or multiple selectin deficiency) mice. These experiments could further our understanding of the importance of CD44 interactions with lung-derived selectins in breast cancer metastasis.

Finally, since we were unable to identify which forms of each selectin (soluble and/or full-length) were present in our lung-CM, we should further examine this using alternative approaches. Two different primary antibodies (from different host species) for each selectin should be used, one for immunoprecipitation and the other for immunoblotting. By using a different primary antibody from different host species such that the secondary antibody only interacts with the antibody used for immunoblotting and not co-immunoprecipitation, we may avoid the interference on our immunoblots that residual co-immunoprecipitation antibody left in our samples may cause.

For E-selectin and P-selectin, the molecular weight at which selectins in lung-CM are detected should be compared to samples from LPS-activated mouse endothelial cell supernatant (for the soluble form) and their cell lysates (membrane bound form), as they are appropriate positive controls. For L-selectin isolated from lung-CM, the molecular weight observed should be compared with the supernatant of LPS-stimulated neutrophils and lysates of untreated neutrophils isolated from mice[132]. L-selectin is constitutively expressed by neutrophils but LPS-stimulation causes L-selectin cleavage from the cell surface into its soluble form[133].

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10 Final Conclusions

It has long been known that breast cancer displays a pattern of preferential metastasis, and one common site to which it spreads is the lung. Since breast cancer metastasis to the lung is a major contributor to the disease-related mortality, it is important that we elucidate why cancer cells preferentially metastasize to the lungs and how we can prevent/treat this cancer progression. Using lung-CM as an *ex vivo* model system, we tested the hypothesis that lung-derived selectins promote breast cancer migration and/or growth via interactions with CD44. Using our model in conjunction with *in vitro* transwell migration and BrdU incorporation assays, we observed that all three lung-derived selectins promoted the migration of breast cancer cells but were not involved in breast cancer cell proliferation. By immunoprecipitation, we also demonstrated that CD44 expressed by MDA-MB-231 human breast cancer cells interacts with selectins and that these interactions may mediate the pro-migratory effects observed. Although we were unable to define a pathway by which the CD44-selectin interactions may be propagating the signal intracellularly, we did identify potential targets of interest and they remain under investigation. Nonetheless, the findings of this thesis support the idea of the lung microenvironment playing a role in breast cancer metastasis. In accordance with the "seed and soil" theory, targeting the lung microenvironment and interfering with factors that would otherwise enhance the metastatic behaviour of breast cancer cells could be a potential therapeutic strategy. This approach could be feasible for treating lung metastases of breast cancer, as it should be easier to target the constant composition of the lung between patients than the highly heterogeneous population of cells that make up breast cancer.

In conclusion, our investigation has provided the foundation for the involvement of selectins in lung metastasis of breast cancer. Taken together with further studies, this could contribute to improved treatment of breast cancer in the metastatic setting and help reduce the burden of this disease on humanity.

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Appendices

Appendix 1: Approved Animal Use Protocol

AUP Number: 2017-095
PI Name: Allan, Alison
AUP Title: Detection and Analysis of Rare Metastatic Events in Mouse Models of Metastatic Breast Cancer
Approval Date: 09/01/2017

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2017-095:1: entitled " Detection and Analysis of Rare Metastatic Events in Mouse Models of Metastatic Breast Cancer"
has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

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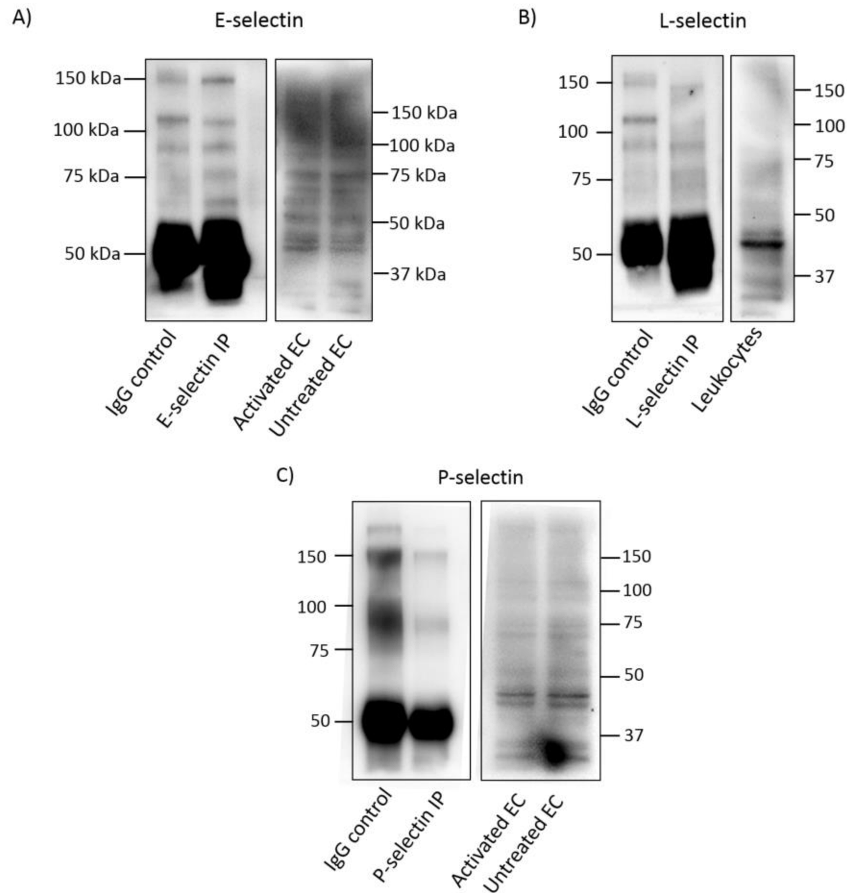

Appendix 2: Immunoblotting analysis of immunoprecipitated selectins from lung-CM. Lysates from normal and activated mouse pulmonary vein endothelial cells (EC) were used as positive controls for E-selectin and P-selectin. Lysates from primary mouse leukocytes were used as a positive control for L-selectin. (A) Immunoblot analysis of E-selectin immunoprecipitated samples from lung-CM revealed 2 bands: one at 75 kDa and another at approximately 65 kDa. These two bands were not observed in the IgG control. For the positive control of mouse pulmonary vein endothelial cells (MPVEC), both the untreated and those activated by lipopolysaccharide (LPS) treatment displayed numerous bands when subject to immunoblotting. A specific molecular size for E-selectin could therefore not be inferred from the MPVEC. (B) Immunoblot analysis of L-selectin immunoprecipitated from lung-CM revealed non-specific binding and no differences in banding pattern relative to the IgG control. A positive control of leukocytes isolated from the blood of female nude athymic mice revealed a band at approximately 45 kDa in size. This region is at the same molecular size as IgG heavy chains (~50 kDa) that were otherwise strongly stained for in immunoprecipitated samples, suggesting a masking of this band in the immunoprecipitated samples. (C) Similarly, immunoblot analysis of P-selectin immunoprecipitated from lung-CM revealed non-specific binding and no differences in banding pattern relative to the IgG control. However, a set of two bands at approximately 45 kDa were observed in immunoblots of untreated and activated endothelial cell lysates.

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Curriculum Vitae

Sami Unnabi Khan

EDUCATION:

University of Toronto – *Doctor of Pharmacy* (In Progress) **Sept. 2017–Present**

Western University – *Master of Science* **Sept. 2015–Dec. 2018**
- Department of Anatomy and Cell Biology
- Supervisor: Dr. Alison Allan
  (Leave of Absence January-April 2018)

Western University - *Honours Bachelor of Medical Sciences* **Sept. 2011-Apr. 2015**
- Honours Specialization in Physiology and Pharmacology

RESEARCH AND TEACHING EXPERIENCE:

Graduate Research Assistant with Dr. Alison Allan **Sept. 2015–Present**
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Honours Thesis Student with Dr. Frank Beier **Sept. 2014–Aug. 2015**
Department of Physiology & Pharmacology
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Research Assistant with Dr. Dale W. Laird **Sept. 2013–Aug. 2014**
Western University, London, Ontario

ACADEMIC AWARDS AND SCHOLARSHIPS:

Colonel F.A. Tilston Admission Scholarship **Sept. 2017**
University of Toronto, School of Pharmacy Award Amount: $1,000

Translational Breast Cancer Studentship **Sept. 2015–Dec. 2017**
Breast Cancer Society of Canada Total Funding: $40,000

Western Graduate Research Scholarship **Sept. 2015–Present**
Western University Total Funding: $9,000

CIHR Summer Undergraduate Student Research Award **May 2015–Aug. 2015**
CIHR Institute of Musculoskeletal Health and Arthritis (IMHA) Total Funding: $5,000

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Cancer Research and Technology Transfer Summer Studentship — **May 2014–Aug. 2014**
CIHR, Western University and London Regional Cancer Program — Total Funding: $3,575

Continuing Admission Scholarship — **Sept. 2011–Apr. 2015**
Western University — Award Amount: $10,000

Dean's Honor List — **2011–2015**
Faculty of Science at Western University

PUBLICATIONS:

Moon PM, Penuela S, Barr K, **Khan S**, Pin CL, Welch I, Attur M, Abramson SB, Laird DW, Beier F. (2015) Deletion of *Panx3* prevents the development of surgically induced osteoarthritis. J Mol Med (Berl) 93(8):845-856.

PRESENTATIONS:

Khan SU, Chu JE, Xia Y and Allan AL. Soluble lung-derived selectins promote migration. Poster presentation, AACR Annual Meeting, Washington, D.C. April 2017

Khan SU, Chu JE, Xia Y and Allan AL. Soluble lung-derived selectins promote migration. Poster presentation, London Health Research Day, London, ON. March 2017

Khan SU and Allan AL. The role of lung-derived selectins in breast cancer growth and migration. Oral presentation, Anatomy & Cell Biology Research Day, London, ON. October 2016

Khan SU, Xia Y and Allan AL. Role of lung-derived selectins in promoting metastatic behavior of breast cancer cells. Poster presentation, Oncology Research & Education Day, London, ON. June 2016

Khan SU and Allan AL. Breast cancer and its attraction to the lung. Oral presentation, Retiring With Strong Minds Presentation Series. London, ON. December 2015.

Khan SU, Moon PM and Beier F. Effects of global Pannexin 3 deletion on the development of surgically induced osteoarthritis in mice. Poster presentation, Physiology and Pharmacology Undergraduate Research Day, London, ON. March 2015.

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