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Influence of the Metastatic Microenvironment on Stem-like Human Breast Cancer Cells

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Graduate Program in Anatomy and Cell Biology
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Abstract

Ninety percent of breast cancer-related mortalities result from metastasis. We have previously demonstrated that stem-like $ALDH^{hi}CD44^{+}$ breast cancer cells are critical for metastasis, and preferentially target the lung and bone marrow (BM). We hypothesize organ tropism occurs through promotion of the $ALDH^{hi}CD44^{+}$ phenotype. Using a 2D *ex vivo* model, lung and BM conditioned media (CM) were utilized to assess their influence on stem-like phenotype and behavior. Exposure of human breast cancer cells to lung-CM significantly decreased the proportion of cells with a stem-like $ALDH^{hi}CD44^{+}$ phenotype, decreased expression of cancer stem cell (CSC)-related genes, and increased gene expression related to migration ($p<0.05$). Lung-CM also induced a viable non-adherent subpopulation that expressed significantly decreased CD44 expression and was unable to form mammospheres ($p<0.05$). Analysis of lung-CM revealed presence of proteins related to migration, adhesion, and stemness. Taken together, the lung microenvironment may promote metastasis of breast cancer cells in a CSC-independent manner.

Keywords

Breast cancer, metastasis, organ tropism, cancer stem cell (CSC), aldehyde dehydrogenase (ALDH), CD44, lung microenvironment, bone marrow microenvironment, non-adherent subpopulation

Co-Authorship Statement

Proteomic analysis was carried out by Dr. Ying Xia in the Allan lab (London Health Sciences Center), in collaboration with Dr. Gilles Lajoie (Department of Biochemistry, Western University).

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I would like to take a moment and touch on something I feel is important. Upon doing research for this thesis, I have discovered a renewed sense of appreciation for those affected by breast cancer. Their resilience and strength are the reason we do this research, however incremental it may seem in the larger scheme of things. An African proverb illustrates this perfectly; "If you think you are too small to make a difference, you haven't spent the night with a mosquito". Thus, I would like to dedicate my thesis to all those individuals to whom we are working diligently to provide relief for.

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List of Abbreviations, Symbols, Nomenclature

7-AAD	7-Aminoactinomycin D
ABC	ATP-Binding Cassette
ADAM10	A Disintegrin and Metalloproteinase Domain-Containing Protein 10
ADH1	Alcohol Dehydrogenase 1
ALDH	Aldehyde Dehydrogenase
ANOVA	Analysis of Variance
ATM	Ataxia-Telangiectasia Mutated
BAA-	BODIPY-aminoacetate
BAAA	BODIPY- aminoacetaldehyde
BM	Bone Marrow
BM-CM	Bone Marrow-Conditioned Media
BMSC	Bone Marrow Stromal Cells
Ca^{+2}	Calcium Ion
Calcein-AM	Calcein-acetoxymethyl
CAM	Cell Adhesion Molecule
CAM	Chick Chorioallantoic Membrane
CD24	Cluster of Differentiation 24
CD44	Cluster of Differentiation 44
CM	Conditioned Media

CRABP2	Cytosolic Retinoic Acid Binding Protein 2
CSC	Cancer Stem Cell
CTC	Circulating Tumour Cell
CXCL8	C-X-C Motif Chemokine Ligand 8
CXCL12	C-X-C Motif Chemokine Ligand 12
CXCR4	C-X-C Motif Chemokine Receptor 4
DCIS	Ductal Carcinoma *in situ*
DEAB	Diethylaminobenzaldehyde
DMEM:F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
EMT	Epithelial-to-Mesenchymal Transition
ER	Estrogen Receptor
ETHD-1	Ethidium Homodimer-1
Ex/Em	Excitation/Emission
FABP5	Fatty Acid Binding Protein 5
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HA	Hyaluronic Acid
HEK293	Human Embryonic Human Cells 293

HER2	Human Epidermal Growth Factor Receptor 2
HSC	Hematopoietic Stem Cell
HUVEC	Human Umbilical Vein Endothelial Cells
LCIS	Lobular Carcinoma *in situ*
IDT	Integrated DNA Technologies
IE-MS	Iterative Exclusion-Mass Spectrometry
IL8	Interleukin 8
MDCK	Madin-Darby Canine Kidney
MMTV-PyMT	Mouse Mammary Tumor Virus-Polyomavirus Middle T-Antigen
MS	Mass Spectrometry
mtDNA	Mitochondrial DNA
MUC1	Mucin 1
NEK2	NIMA-related Kinase 2
OPN	Osteopontin
PBS	Phosphate-Buffered Saline
PDLSC	Periodontal Ligament Stem Cells
PE	Phycoerythrin
POSTN	Periostin
PR	Progesterone Receptor
PYLL	Potential Years of Life Lost

RA	Retinoic Acid
RBP	Retinol Binding Protein
rhPOSTN	Recombinant Human Periostin Protein
SEM	Standard Error of the Mean
siRNA	Small Interfering RNA
TGF-β	Transforming Growth Factor β
TN	Triple Negative
VCAM1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VLA-4	Very Late Antigen-4
V/W	Volume/Weight

1 INTRODUCTION

Cancer is devastating. It is the source of physical, emotional, and mental stress that not only affects the immediate individual involved, but extends further to family and friends. In 2017 alone, it is estimated that 206,200 Canadians will be diagnosed with cancer[1]. These figures have increased by nearly 10,000 new diagnoses within the past two years[2]. Unfortunately, these figures are expected to rise by an additional 20% by 2020 largely due an aging and growing population[3]. Greater emphasis on cancer prevention, adopting a healthy lifestyle, and earlier detection is necessary to counter such undesirable outcomes[3].

Breast cancer is the most common malignancy in women worldwide[4]. Despite its high prevalence, patient prognosis is strong with 5-year survival rates nearing 99% when localized to the breast[5]. Once the cancer leaves the confines of the breast, the chances of surviving this drastically decrease[5]. In fact, the dissemination of cancer from the primary affected region to distant organs accounts for 90% of all cancer related deaths[6]. Recently, it has been proposed that a rare population of tumour cells, commonly referred to as cancer stem cells, may be mediating metastasis and secondary tumour formation[7]. Therefore, mortality related to breast cancer rarely occurs as a result of the primary tumour, but rather the cascading effect of multiple organs becoming compromised, including the lung and bone [8-10].

The focus of this thesis is to investigate the importance of the native lung and bone marrow microenvironments in promoting rare, highly malignant cells to target these organs as likely sites of metastasis. Understanding the role of the microenvironment is crucial to gain a comprehensive understanding of cancer metastasis.

2 LITERATURE REVIEW

2.1 Cancer

The term "cancer" encompasses a subset of diseases that are characterized by atypical cell growth and proliferative patterns. As these aberrant cells develop, they acquire genetic disruptions that enable sustained proliferative signaling and evasion of growth suppressors[11]. Most often, the onset of this disease begins once genes involved in cell cycle regulation become mutated and dysfunctional. These genes are most often classified either as oncogenes or tumour suppressor genes. Once mutated, oncogenes become activated, giving cells the ability to induce uncontrolled cell growth[12]. In contrast, mutations in tumour suppressor genes may render normal cells incapable of DNA repair required to regulate cell growth[13]. It is likely that oncogene activation and tumour suppressor gene inactivation occur simultaneously as cancer progresses, ultimately resulting in tumour formation[14].

Tumours can be characterized by their benign or malignant nature, with the latter capable of invasive traits that represent the hallmarks of cancer. A growth that is neither invasive to surrounding or distant regions, such as a common wart, is considered benign and does not pose a significant health risk[11]. In rare cases, benign tumours may impinge on blood vessels or nerves that supply organ systems. These instances are considered higher risk and require resection, however they are not considered malignant tumours[15]. Malignant tumours tend to be life-threatening due to the capacity to leave the primary site and invade into surrounding tissues or distant secondary sites of the body via access to systemic circulation[15]. These tumours are referred to as malignant cancers and require early detection and subsequent treatment to provide the best prognosis for patients. In later stages of tumour progression, if the tumour has spread to distant sites, both primary and adjuvant treatments (e.g. tumour resection, radiation, hormonal therapy, or chemotherapy) become far less efficient at easing tumour burden[16]. Together, inefficiencies in treatment strategies and efficacy leave both patients and the healthcare system in distress.

In 2017 alone, 1 in 4 Canadians will no longer be responsive to traditional cancer treatments and will eventually succumb to the disease[1]. Further, the Canadian Cancer Society

estimates that 1 in 2 Canadians will develop cancer within their lifetime, a value that is increased from a 1 in 2.5 frequency just two years ago[1,2]. Similarly, the overall 5-year survival rates in cancer patients have also decreased from 63% in 2015, to 60% in 2017[1,2]. Despite advances in targeted treatment and enhanced screening techniques, cancer is proving to be relentless. If these patterns persist, cancer-associated deaths will account for 30% of Canadian premature deaths in 2017, [1]. Premature deaths are measured by potential years of life lost (PYLL) and account for deaths occurring at younger ages, a vital statistic when assessing economic health for any given country. Between 2010 and 2012, cancer represented the largest PYLL among Canadians, with 1.5 million years lost due to cancer[1]. Due to a loss in productivity associated with high PYLL values, not only is the growing Canadian economy impacted, but these patients also pose a significant financial burden on the healthcare system. In 2008, the Public Health Agency of Canada estimated that $3.8 billion was devoted to direct healthcare costs for cancer patients (hospitalization, treatments, etc.), while an additional $586 million was lost to indirect costs associated with decreased economic productivity[17]. Considering that cancer diagnoses have increased in recent years, the economic impact of cancer is expected to increase steadily with time.

2.2 Breast Cancer

The breasts are two prominences situated on the ventral torso of primates, morphologically identical in both male and female offspring. Once females enter puberty, secretion of sex and growth hormones (namely estrogen) promote mammary development. Subcutaneous adipose tissue within the breast supports a network of ductal and lobular tissue, that together, comprise the feeding mammary gland. The lobular epithelium of the breast serves a lactiferous function, producing and secreting milk down the ductal epithelium for expulsion out the nipple by contracting myoepithelial cells [18]. Due to hormonal cycling involved in mammary development and throughout child-bearing years, the lobular and ductal cells are most susceptible to tumour formation, and cancers that arise are termed lobular or ductal carcinomas respectively[16]. So long as the tumour is confined to primary breast tissue, the 5-year survival rate is an exceptional 98.6% among females[5]. Unfortunately, this prognosis significantly decreases to ~25% once the cancer metastasizes, spreading from the breast tissue and reaching secondary sites[5]. The most

aggressive cases of breast cancer involve secondary tumour formation at distant organs, resulting in significantly reduced organ function.

2.2.1 Histopathology and Molecular Subtypes

Recent findings regarding tumour heterogeneity suggest that each tumour is distinct and unique from patient-to-patient. This further extends into distinctions between multiple tumours identified within a single individual. Upon histopathological analyses of biopsied tumour samples from the breast, inter-tumour heterogeneity is evident[19]. Histopathology provides a rudimentary understanding of the cell subpopulations involved in tumour development, their morphology, and predicting aberrant growth patterns. The main purpose of this technique is to distinguish whether the breast tumour is originating from ductal or lobular tissue architecture[20]. At the time of detection, ductal or lobular carcinomas *in situ* (DCIS or LCIS respectively) are non-invasive as they remain confined to epithelial-lined compartments within the mammary tissue[20]. Surgical resection and localized therapy strategie*s* such as radiation are highly effective and demonstrate strong patient prognosis with a 99% survival outlook over five years[21]. Unfortunately, more than half of breast cancer incidences are invasive ductal or lobular carcinomas (IDC or ILC) upon initial diagnosis[22]. Tumours that have spread from epithelial-lined compartments of the mammary tissue into the stroma are classified as invasive carcinomas through histopathological analyses[20]. The associated treatment strategies for patients with IDC or ILC are less effective and become limited as the tumour spreads from the primary tissue. A lack of targeted therapies for invasive carcinomas is reflected in the 5-year survival rates dropping to near 25% once the tumour has acquired invasive potential[21].

In addition to histopathological distinction between breast cancer tumours, molecular classification of breast cancer cells has provided further insight regarding the cells propagating tumour growth and development. Among the invasive incidences of breast cancer, the four main molecular subtypes of interest are: Luminal A, Luminal B, HER2-enriched and basal-like (or triple-negative; TN) breast cancer[23]. The basis of this classification system is dependent on cell surface expression of hormonal receptors and intrinsic proliferation status of the cell. Luminal A breast cancer cells have a distinct phenotype expressing receptors for the hormones estrogen and progesterone (ER+ and

PR+), and lacking human epidermal growth factor receptor 2 (HER2-)[24]. Breast cancer cells with a high proliferative (Ki67) index and/or expressing HER2, in addition to both ER+/PR+, are classified as the Luminal B subtype. Both luminal A and B breast cancers have been associated with positive survival outcomes[24,25]. Because both Luminal A and B subtypes are ER+, hormone therapy is highly effective to treat patients, particularly in the adjuvant setting[19]. Despite their similarities, Luminal B breast cancer cells are genetically altered from the Luminal A subtype, resulting in poorer prognosis than those affected by Luminal A breast cancer[26]. Further, the HER2+ breast cancer subtype lacks expression of both ER and PR, rendering these cells unresponsive to targeted hormone therapy[24]. Together with increased proliferation within this subtype, HER2+ breast cancer cells tend to metastasize and spread more readily to surrounding tissues, resulting in poorer patient prognosis relative to luminal breast cancers[23]. Inhibition of HER2+ ligand-receptor interactions using HER2-targeting agents such as trastuzumab and lapatinib has improved overall patient survival and time-to-disease progression, however many patients will acquire resistance to therapy over time[27].

Perhaps the most difficult breast cancer subtype to treat is the TN breast cancer subtype which lacks the cell surface receptors found on the aforementioned breast cancer subtypes (ER-/PR-/and HER2-)[24]. Often the most aggressive subtype of breast cancer, TN breast cancer patients are highly prone to metastases which results in the poorest prognosis[28]. Despite some TN breast cancers being initially responsive to traditional chemotherapy, a hallmark of TN breast cancer is their high likelihood of distant recurrences within 3-years of initial diagnosis[29]. As endocrine treatment strategies targeting ER/PR/ or HER2 are ineffective on the TN subtype, central pathways involved in proliferation, growth and migration are being actively investigated as potential targets[30].

Although our understanding of molecular breast cancer subtypes has provided avenues for clinical intervention, it is important to consider intra-tumour heterogeneity as a factor for disease recurrence post-treatment. Cells from different regions of a solid breast tumour have shown varying levels ER, PR, and HER2 cell surface expression which is consistent with intra-tumour heterogeneity[31]. Thus, when investigating a heterogenous tumour, it is important to consider that each population of cells has a distinct composition that may

modulate tumour progression through intrinsic factors such as migration and/or invasion[19].

2.3 Epithelial-to-Mesenchymal Transition

Given the anatomical composition and functional nature of mammary tissues, primary tumours developing in the breast are epithelial-derived and termed carcinomas[32]. Understanding how healthy epithelial cells function to maintain homeostasis is necessary to predict changes in epithelial function in a diseased state. The epithelial cell utilizes networks of cell-cell and cell-extracellular matrix (ECM) interactions to provide apical-basal cell polarity. Desmosomes, adherens junctions and gap junctions are protein complexes that maintain physical association between adjacent epithelial cells (cell-cell), while cell-ECM interactions are necessitated by integrins and cell adhesion molecules (CAMs)[33,34]. In a dynamic and invasive tumour microenvironment, extracellular cues reduce characteristic epithelial cell-cell and cell-ECM interactions, and can induce a mesenchymal cell phenotype[35]. The epithelial-to-mesenchymal transition (EMT) and its reverse process, mesenchymal-to-epithelial transition (MET) describe this transformation from one phenotype to another[7]. Once in the mesenchymal state, these cells lack apical-basal polarity and possess increased migratory and invasive potential[36,37]. The role of EMT and MET were first documented in embryogenesis, but the importance of these processes further translates into cancer progression, and subsequently metastasis[7,38]. Cancer progression requires cells of the primary tumour to disassociate and invade into the surrounding stroma. This process of invasion is mediated in part by the loss of cell-cell adhesions, which enhances cellular motility, while deterioration of cell-ECM interactions allows catabolic cell secretions (e.g. matrix metalloproteinases; MMPs) to penetrate the basement membrane[34,37]. As tumourigenic cells penetrate the porous basement membrane and extend into the surrounding stroma, early stage carcinomas become invasive malignancies[7]. In order to support tumourigenic growth, invading cells secrete angiogenic factors (such as vascular endothelial growth factor [VEGF] and transforming growth factor beta [TGF-β]) to support vascular growth necessary for nutrient delivery towards the tumour[34,37]. Although recent studies document maintained tumour progression in the absence of vascular recruitment (hypoxic conditions), vascular growth is necessary for tumourigenic cells to invade systemic circulation and metastasize to distant sites[34,37,39].

2.4 Metastasis

The spread of cancer from a primary tumour to a distant secondary site is referred to as metastasis, or metastatic disease. Although treatment strategies targeting the primary tumour are highly efficient, nearly 30% of women diagnosed with early-stage breast cancer will ultimately develop metastatic lesions[40-42]. For tumourigenic cells to metastasize, they must leave the primary site and enter systemic circulation by directly invading the surrounding vasculature, or indirectly through the lymphatic system[43]. As previously mentioned, the invasive behaviour of cancer cells induced by EMT and other mechanisms allows cancer cells to invade the surrounding stroma. Together with enhanced vascular recruitment (angiogenesis and vasculogenesis) surrounding the primary tumour site, cancer cells further develop means to enter into the vasculature/lymphatics and leave the primary site[43-45].

Millions of cells are capable of dissociating from the primary tumour and entering the vasculature every day. However, the relative incidence of metastatic tumour development is rare, suggesting that the metastatic process has inefficiencies[42]. As tumour cells intravasate into the circulation, experimental studies have shown that >80% of these cells can survive the shear and compressive stress associated with the circulatory phase of metastasis[41,42,46]. The majority of these circulating tumour cells (CTCs) arrest in the first capillary bed they encounter, while others remain selective for specific organ microenvironments such as the bone, lung, and brain[47-49]. Only after successful extravasation, whereby the CTCs exit the circulation and invade the distant organ, can secondary tumour formation become possible[43]. Despite the large number of cells that survive the circulatory phase of metastasis and successfully extravasate at the secondary site, the inefficiencies associated with metastasis are highlighted when assessing tumour-initiating potential at the distant tissue. Experimental studies have shown that only ~2% of cancer cells that successfully reach the secondary tissue microenvironment have the capacity to initiate a new tumour, and <0.1% of cells can persist into the successful formation of macrometastases[42]. These inefficiencies suggest that aspects of the metastatic microenvironment and/or characteristics of cancer cells can contribute to the success or failure of metastasis.

2.5 Organ Tropism of Metastasis

In the event of metastasis, patterns of cancer dissemination to secondary sites are not random, but rather coordinated[50]. Many cancers have shown preferential metastatic capacity towards particular organs, a process referred to as organ tropism. Among the various cancer subtypes, the patterns of organ tropism are variable and dependent on the cancers' origin. Some cancers predominantly metastasize to a specific organ (e.g. prostate cancer metastasizing to the bone), while other cancers follow a sequential pattern of metastasis (e.g. colorectal cancer often forms secondary metastases in the bone → lung → brain)[51]. As each organ differs in anatomical position, blood/nutrient supply, and organ microenvironment composition, invading tumour cells face different demands based on the target organ. Clinically, breast cancer metastasis has demonstrated preferential metastasis to the lung, bone, liver, brain and lymph nodes[52].

Two competing theories that attempt to elucidate mechanisms involved in organ-specific metastasis are Stephen Paget's "seed and soil" hypothesis, and Ewing's mechanical arrest theory. Initially Paget, a British surgeon, theorized that cancer cells (the "seed") require an organ microenvironment (the "soil") that can adequately support the growth of a metastatic tumour[53]. Thus, for a metastatic tumour to successfully grow, there is a requirement for favorable factors within the organ microenvironment capable of supporting tumour formation. Strengthening Paget's theory, recent findings in breast cancer research demonstrate the luminal breast cancer subtype to preferentially metastasize to the bone, while the $HER2^+$ subtype often targets the liver[54-56]. Half a century later, James Ewing proposed a novel mechanism dependent on physical characteristics of blood flow through the circulatory system that dictate eventual sites of mechanical arrest. He proposed that organs with the largest blood supply would be most prone to acquiring blood-borne metastatic cells, leading to tumour cell arrest at the first capillary bed they encounter and initiating secondary tumour formation[57]. In theory, Ewing's mechanism holds strength, however fails to fully explain clinical patterns of organ-specific metastasis. Despite receiving a similar 10%-20% of blood volume, the liver, kidney and brain tissue each show different patterns of susceptibility to metastasis development, highlighting the oversight in Ewing's theory[58].

When considering a biologically relevant theory for organ tropism, the likely mechanism is a combination of both Paget's and Ewing's hypotheses. A complex system that delivers metastatic cells to different organ tissues based on relative blood supply, and then initiation and maintenance of said tumour would be mediated by favourable interactions with the soluble and insoluble factors provided by the organ.[59,60].

2.6 The "Seeds": Stem-Like Breast Cancer Cells

Research conducted by Massagué and colleagues uncovered an association between the molecular characteristics of breast cancer cell (the "seed") and the preferential tissue to which the breast cancer cells metastasize[52]. Using *in vitro* and *in vivo* studies with the triple-negative MDA-MB-231 human breast cancer cell line, they demonstrated that specific gene expression signatures can dictate a breast cancer cell's preference to metastasize to either the lung, the bone, or the brain[47-49]. However, this work did not take into consideration the heterogeneous nature of primary metastatic breast cancer tumours. Subsequent limiting dilution analyses *in vivo* confirmed this notion by demonstrating that isolation and injection of low numbers of primary breast cancer cells into healthy immune deficient mice resulted in only a small fraction of cells harnessing the ability to initiate and produce a primary tumour[61,62]. These studies supported the idea that only a subset of cells within a primary tumor have tumour-initiating capacity, suggesting that this rare subpopulation may contain stem-like traits, often referred to as cancer stem cells (CSCs). The concept of a CSC subpopulation within tumours first originated in hematologic cancers, gaining credibility with evidence that only 1 to 4% of myeloma and leukemia cancer cells demonstrated enhanced proliferative and colony formation potential[63-65]. Although the cellular origins of cancer stem cells in some solid tumours remain controversial, recent studies conducted by Blanpain *et al* have successfully demonstrated that tumour populations in intestinal, prostate, and breast cancer can be traced back to a stem/progenitor origin, reinforcing the validity of CSC model[66].

2.6.1 Characterization of CSCs

Current methods of CSC characterization have been adapted from the pioneering field of hematopoietic stem cells (HSCs). Discovered first by James Till and Ernest McCulloch

during their investigation of hematopoietic system regeneration *in vivo*, a true HSC must fulfill two requirements: the ability to self-renew and to maintain a multipotent state[67]. Self-renewal refers to the cells' ability to produce a sister HSC without losing multipotent potential, while multipotency is the ability of a progenitor cell to differentiate into any functional cell within a given lineage[67,68]. Originating from these well-characterized HSC attributes, CSCs must be able to generate a heterogeneous tumour population (differentiation) while concomitantly maintaining their own population (self-renewal)[68]. Numerous studies have validated these stem cell characteristics to be true among a CSC subpopulation, and in addition, have demonstrated that CSCs also possess enhanced drug resistance, anchorage-independence, and increased migration relative to non-CSCs[52,61,62,69,70]. CSCs have also been associated with aggressive metastasis, and in many instances, found to express molecular markers of EMT[7]. Considering the inefficiencies previously mentioned regarding the metastatic process, it is reasonable to postulate that a rare CSC subpopulation of primary breast cancer cells may also be able to establish and drive distant secondary tumour development.

In light of their stem-like properties, CSCs can be isolated from a whole cell population using similar molecular screening techniques used with HSCs. In breast cancer, CSCs from patient tumours and various breast cancer cell lines have successfully been enriched for based on high aldehyde dehydrogenase (ALDH) enzymatic activity and the co-expression of the cluster of differentiation 44 (CD44) cell surface marker[61,62,71].

2.6.1.1 ALDH

The ALDH family is made up of 19 evolutionarily conserved isoenzymes that are localized intracellularly in the cytoplasm, mitochondria, and nucleus[72]. The main function of ALDH is to catalyze the oxidation of aldehydes into carboxylic acids, along with other functions such as ester hydrolysis and scavenging for hydroxyl radicals[72]. Of particular interest are the isoenzymes (ALDH1A1, ALDH1A2, ALDH1A3, and ALDH8A1) involved in the conversion of vitamin D to retinoic acid (RA), as they have recently been implicated in cancer cell "stemness"[73]. The lipophilic RA molecule is capable of passive diffusion in a paracrine or endocrine manner, resulting in induced transcription of biological genes related to proliferation, differentiation, cell cycle arrest, and apoptotic pathways[74]. Notably,

the human cytosolic ALDH1A subfamily (ALDH1A1 and ALDH1A3) are highly expressed in early progenitor cells and have been documented to overlap with side population cells capable of excluding Hoechst 33342 stain, another modality for identifying stem-like cells[61]. Intrinsically high ALDH (ALDH^{hi}) activity and Hoechst 33342 excluding stem-like side populations demonstrate increased expression of ABC transporters, a feature thought to provide CSCs with chemo-resistance[75]. This protective mechanism renders CSCs particularly resistant to conventional cancer therapies, permitting relapse over complete remission, and prolonging tumour longevity[7,76]. Much of the research concerning ALDH activity in cancer utilizes the metabolism of ALDEFLUOR™ substrate to isolate a subpopulation of tumourigenic cells with stem-like characteristics via flow cytometry[72]. It was initially predicted that ALDH1A1 was responsible for the majority of ALDEFLUOR™ metabolism, however recent evidence suggests ALDH1A3 is also involved[72,74]. Considering the metabolism of ALDEFLUOR™ is non-specific, it is likely that the ALDH activity detected in a cancer is due to the combined activity of two or more ALDH isoforms[72].

Analyses of intracellular ALDH activity in liver, lung, esophageal, and breast cancer cells has been a useful tool to estimate how these tumourigenic cells may behave *in vivo*[73]. In breast cancer, cells with elevated ALDH activity have demonstrated increased migratory capacity and the ability to form mammospheres *in vitro*[70,77]. The importance of ALDH activity *in vivo* was emphasized by Ginestier *et al.* after transplantation of 50,000 ALDH^{lo} human breast cancer cells into the mammary fat pad of immunocompromised mice was insufficient for tumour formation, but transplantation as few as 500 ALDH^{hi} cells resulted in tumour formation within 40 days[61,78]. Both ALDH1A1 and ALDH1A3 have been implicated in driving tumourigenesis after breast cancer cell transplantation in xenograft models[79,80]. Moreover, Marcato *et al* demonstrated ALDH1A3 overexpression in human breast cancer cells is case specific, as ALDH1A3 overexpression was tumour-promoting in MDA-MB-435 and MDA-MB-231 cell lines, while tumour-suppressive in MDA-MB-468 cells[79]. Clinically, Marselos *et al* identified ALDH^{hi} activity to have a strong correlation with metastatic lesions among patients with colon cancer, relative to healthy adjacent tissues[81]. More recently, a study of 87 female patients diagnosed with metastatic breast cancer found the incidence of ALDH1 expression significantly increased in the

metastatic site (43.7%) compared to the primary tumour (28.7%), suggesting the importance of ALDH1 in metastatic disease and secondary tumour formation[82]. With accumulating evidence supporting the tumorigenic role of ALDH in patients with metastatic breast cancer, a meta-analysis assessing 921 patients for elevated ALDH1A1 expression in breast cancer tumours concluded that ALDH1A1^{+} can be used as a biomarker for the prediction of tumour progression and poor patient outcome[83].

2.6.1.2 CD44

Membrane-spanning CD44 is a glycosylated cell surface receptor that has well-defined roles in cell-cell and cytoskeletal cell-ECM interactions (via Rho GTPase signaling), promotion of cell survival and invasion (via PI3/Akt and MAPK-Ras pathways). Importantly, CD44 has a strong association with cell migration through interactions with matrix metalloproteases (MMPs) and other matrix remodeling enzymes which together coordinate cellular locomotion[62,84]. The principal ligand for CD44 is hyaluronic acid (HA), a major component of extracellular matrices, which has also been reported to maintain long term self-renewal[85]. Functional CD44 protein is encoded by a single gene with 20 exons, where exons 1-5 and 16-20 comprise the standard isoform (CD44s), while exons 6-15 are alternatively spliced to produce CD44 variants (CD44v)[86]. Although CD44s has been implicated repeatedly in a variety of cancers, recent investigations have begun to examine specific splice variants and their association in cancer progression[87]. CD44v4 in human breast cancer cells was found to preferentially interact with E-selectin expressed human umbilical vein endothelial cells (HUVECs) and promoted trans-migration[88]. Moreover, a clinicopathologic study investigating the role of CD44v6 in 85 untreated primary breast cancer patients reported that a decrease in CD44v6 mRNA correlated with poor survival[89]. Due to conflicting data suggesting upregulation and/or downregulation of certain CD44v isoforms implicated in cancer development, the CD44s isoform is most consistently used for CD44 assessment[90]. Nevertheless, CD44 remains an important marker for identification of tumourigenic cancer cell populations, both *in vitro* and *in vivo*. Research conducted by Al-Hajj et al successfully identified a CD44^{+}/CD24^{-} subpopulation of breast cancer cells with heightened CSCs characteristics. *In vivo* studies demonstrate that as few as 100 CD44^{+}/CD24^{-} cells were capable of forming tumours in mice, while the CD44^{-}/CD24^{+}

subpopulation was unable to form tumours, even after injection of 500,000 cells[62]. These findings suggest CD44 expression has an important role in tumour development.

2.6.2 Stem-Like Cancer Cells and Metastasis

Metastasis has been correlated with poor overall survival and mortality in several types of cancers, a major obstacle in cancer treatment. The involvement of CSCs in metastatic disease has received particular attention because they have been implicated in the initiation of the metastatic cascade through EMT processes[91]. Interestingly, both ALDH and/or CD44 have been used as markers for the identification of metastasis-prone cancer cell subpopulations. Previous investigation conducted in the Allan lab by Croker *et al* found breast cancer cells expressing the stem-like $ALDH^{hi}CD44^{+}$ phenotype demonstrated enhanced metastatic behavior *in vitro*, as their ability to migrate and invade was significantly increased relative to the non-CSC $ALDH^{low}CD44^{-}$ subpopulation[70]. Moreover, these stem-like breast cancer cells exhibited increased ability to form anchorage-independent colonies *in vitro* when compared to non-CSCs, suggesting these cells could potentially colonize in a distant organ microenvironment after detachment and dissemination[70]. Subsequent *in vivo* analyses confirmed the metastatic potential of stem-like breast cancer cells after orthotopic injection into the mammary fat pad of NOD/SCID-IL2Rγ mice led to increased spontaneous metastases to the liver, spleen, and most notably the lung[70]. Not only did stem-like cells preferentially metastasize to these organs relative to their non-CSC counterpart, the mean tumour volume and metastatic burden to the lung was significantly increased in mice injected with the stem-like breast cancer cell subpopulation[70]. Surprisingly, investigation of ALDH1A1 and ALDH1A3 isoenzymes demonstrated differential roles related to their involvement in metastasis[92]. Using a knockdown model, human breast cancer cells devoid of ALDH1A1 demonstrated a significant reduction in their ability to migrate, and were less adherent *in vitro*[92]. In contrast, knockdown of ALDH1A3 resulted in increased cell migration and adhesion *in vitro*[92]. Knockdown of either ALDH1A1 or ALDH1A3 significantly decreased the number of tumoursphere colonies formed *in vitro*[92]. Further, in a chick chorioallantoic membrane (CAM) assay, ALDH1A1 knockdown resulted in reduced ability to of breast cancer cells to extravasate from the vasculature, as well as reduced number of micrometastatic tumours

with either knockout of ALDH1A1 or ALDH1A3[92]. Collectively, ALDH activity among CSC subpopulations has been demonstrated to be a critical factor in cancer dissemination through coordinated activity of ALDH1 isoenzymes.

The concerns revolving around CSCs and metastasis extend past their ability to disseminate and colonize distant organs. Tumour cells expressing stem-like characteristics present a significant barrier between effective cancer therapy and improved patient prognosis[91]. As CSC are postulated to have a slow rate of division and efficient efflux pumps capable of removing toxic agents, traditional chemo- and radiation therapies become inadequate in targeting CSC without off-target effects on healthy tissues[91]. Despite these limitations, conventional therapy remains the first line of treatment and often results in cancer recurrence due to a residual CSC subpopulation[91]. A subsequent study conducted by Croker *et al* investigated the role of stem-like $ALDH^{hi}CD44^{+}$ breast cancer in therapy resistance. By inhibiting ALDH enzymatic activity, CSCs became transiently sensitized to chemotherapy (doxorubicin/paclitaxel) or radiotherapy measured by decreased cell viability and colony formation *in vitro*[93]. Moreover, therapy resistance was attributed to ALDH activity in part by the ALDH1A1 isoenzyme, and not ALDH1A3, demonstrated by siRNA knockdown *in vitro*[92]. These findings suggest ALDH activity is an important mediator of CSC therapy resistance, and has since been supported by several studies reporting similar sensitization through ALDH inhibition[94,95]. Interestingly, a recent study by Yang *et al* demonstrated that ALDH1A1 overexpression directly correlated with increased activity of multidrug efflux pumps through phosphorylation by NIMA-related kinase 2 (NEK2)[96]. An increase in efflux pump activity could support CSCs with high ALDH activity to remove therapeutic toxins and allow the tumourigenic cell to continue through to metastasis. Considering the importance of ALDH to drug resistance, without ALDH inhibition in CSC subpopulations, tumorigenic cells remain resistant to therapy and could be detrimental to patients as metastasis persists.

Taken together, the importance of CSC throughout metastatic progression is well-supported. ALDH activity appears to provide CSCs with the capacity to support individual steps of the metastatic cascade with regards to extravasation, migration, invasion, and colony formation. Moreover, the function of ALDH in therapy resistance also promotes

metastasis as CSCs become less likely to undergo apoptosis in response to therapy. Despite their supportive role in the cancer progression, transient activation and inactivation of cellular processes by CSCs is required to complete the metastatic cascade, suggesting CSC plasticity as a key contributor to metastasis.

2.6.3 Plasticity of Stem-Like Cancer Cells

It is generally believed that normal development is largely unidirectional, where slow-proliferating stem cell populations gives rise to highly proliferative progenitor cells, ultimately producing terminally differentiated mature cell types that regulate organ function[97]. The unidirectional nature of the cellular maturation process allows distinct cell types with varying specialties to be present within a single organ system and maintain functional homeostasis throughout the organisms lifespan[97]. This is especially clear in organs such as the heart, where the annual cardiac myocyte turnover rate is ~1% per year at age 20, drastically decreasing to ~0.4% after the age of 75[98]. That is not to say every post-mitotic mature cell is incapable of proliferation. Within the pancreas, mature pancreatic β-cells have been reported to expand their population through self-duplication as opposed to stem-cell differentiation[99]. Instances such as self-duplication suggest mature cells may have alternative options related to cell fate, despite having undergone terminal differentiation. In particular, the concept of cell plasticity has received much attention recently as it supports the notion that a cell can alter its phenotype or behaviour in response to environmental queues[100]. The metabolic and epigenetic mechanisms required to induce phenotypic plasticity were first documented during early embryogenesis, but have been shown to re-activate during normal tissue regeneration, inflammation, and notably during tumour development[101].

The re-activation of cellular plasticity in tumour cells has been associated with acquisition of a CSC state capable of therapeutic evasion, increased motility, and survival under hypoxic conditions[101]. A prime example of tumour cell plasticity involves the aforementioned EMT process. As cells transition from an epithelial state towards a mesenchymal phenotype, intrinsic alterations are activated to induce changes in cell phenotype and behaviour. In a study conducted by Liu *et al*, stem-like populations of breast cancer cells expressing either a $CD44^{+}CD24^{-}$ or $ALDH^{hi}$ phenotype were found to originate

from one patient sample, yet represent two distinct subpopulations of breast CSCs expressing mesenchymal-like (EMT) and epithelial-like (MET) phenotypes respectively[102]. Interestingly, the EMT-CSC population was associated with a quiescent state and preferentially localized to the invading tumour front, while the MET-CSC phenotype was more proliferative and centrally localized[102]. During tumour development, it was initially thought that EMT-CSC mediate invasion into surrounding tissues, while the MET-CSCs drive tumour growth internally. As the tumour progresses, CSCs change states in order to maintain invasion and proliferation accordingly. These findings suggest that plasticity between an epithelial or mesenchymal state in breast CSCs is a transient behaviour, rather than a fixed state. Thus, the role of CSC plasticity during tumour propagation, invasion, and metastasis is an important consideration in patient treatment.

Furthermore, plasticity among CSC populations is also evident during therapy. Initially, it was postulated that conventional therapeutic agents target and deplete non-CSCs, while CSCs evade treatment and expand their population to form a more aggressive tumour upon recurrence[103]. In a recent study conducted by Goldman *et al*, therapeutic treatment of taxanes or anthracyclines on human breast cancer cells *in vitro* and *in vivo* not only induced apoptosis in the majority of breast cancer cells, but also promoted the transition of non-CSCs towards a CSC state[104]. The therapy-resistant cells demonstrated increased expression of breast CSC markers ($CD44^{+}CD24^{-}$) and augmented tumour growth, while decreasing survival using patient derived xenograft models in mice. Indeed, these effects were not due to an enrichment for the CSC population but rather a transition from non-CSC to CSC state, demonstrated by the dose-dependent increase of the CSC population after acute low dose treatment[104]. Importantly, the plastic nature of CSCs was demonstrated as removal of the chemotherapeutic agent reverted newly generated CSCs back to a non-CSC state[104]. CSC plasticity is not specific to breast cancer as similar findings have been reported in prostate and ovarian cancer as well[103]. Nonetheless, the plasticity of the CSC state highlights several complexities with regards to the identification of tumourigenic cells and their subsequent response to conventional therapies.

Overall, plasticity among CSC populations plays a significant role in tumour progression, metastasis, and therapeutic resistance. Despite the validation of tumourigenic CSCs in

several studies, the scientific community has faced difficulties in identifying a universal method for identification of elusive CSCs[105]. This is likely attributed to the search for a specific CSC phenotype, rather than a highly plastic subpopulation of tumourigenic cells capable of transitioning from one state to another.

2.7 The “Soil”: Organ Microenvironments

Cancer metastasis follows an organ-specific pattern of dissemination[53]. If the metastatic site is compatible with the disseminated cancer cells, interactions between these cells and the microenvironment will likely promote colonization and secondary tumour formation[106]. Both clinical observation and experimental murine models suggest that organ-specific metastasis occurs independent of anatomical position, rate of blood flow, and number of cancer cells reaching the organ[106]. Using radiolabeled melanoma cells, cancer cell progression through systemic circulation and successful delivery to key organs was monitored after intravenous injection into murine models. Interestingly, radiolabeled melanoma cells were selective in colonizing specific organs, demonstrating that although tumour cells were capable of reaching the secondary organ, they required a congenial microenvironment to support extravasation and tumour development[107]. More recently, high expression of very late antigen 4 (VLA-4) on the endothelial cells surrounding the lung, bone, and brain tissue have been demonstrated to support homing of circulating breast cancer cells toward these organs through binding of their natural receptor, vascular cell adhesion molecule-1 (VCAM-1), aberrantly expressed on the surface of breast CTCs[108].

In our lab, research conducted by Chu et al has demonstrated the role of soluble organ-derived factors in promoting metastatic behaviour of breast cancer cells using a novel *ex vivo* model system[52]. Clinically relevant organs representing common sites of breast cancer metastasis (lung, bone marrow, liver, brain, LN) were harvested from female nude mice and cultured to produce organ-specific conditioned media (CM) for use in functional assays. The findings suggested that native soluble factors within organ-CM induced chemotactic and proliferative functions among the MDA-MB-231, MDA-MB-468, SUM149, and SUM159 human breast cancer cell lines analyzed[52,109]. Interestingly, these patterns of migration and proliferation occurred in a manner that reflected *in vivo* patterns

of metastasis[52].

2.7.1 The Lung Microenvironment and Lung Metastasis

The lungs are an indispensable organ involved in respiratory function, mediating the intake of oxygen and disposal of carbon dioxide during normal physiological conditions[110]. The functional anatomy of the lungs is divided into two zones. First air enters the conduction zone in the upper respiratory tract, travelling down the trachea and directed into each lung via the bronchi and terminal bronchioles[110]. The lower respiratory tract represents the respiratory zone, where air is shuttled past the terminal bronchioles and into the respiratory bronchioles, eventually reaching the alveolar ducts where individual alveoli necessitate gas exchange through an expansive network of capillaries[110]. Interestingly, the lungs are often implicated in various cancers, both as a direct cause from external factors (e.g. chemical pollutants) and/or dysregulation of normal physiology[111]. In particular, the lungs are a major site for tumour metastasis of breast and other cancers. A recent investigation of 1,088 medical records from non-metastatic breast cancer patients between 2004 and 2012 demonstrated that metastases to the lungs developed in 35% of patients, after a median follow-up time of 6.9 years[112]. Although incidence of breast cancer metastases are higher in bone marrow than in lung tissue, tumour formation in respiratory organs severely impact the quality of life and are the leading cause of breast cancer related deaths[113]. Often, the more aggressive subtypes such as HER2+ and TN breast cancers metastasize to the lung where they largely contribute to impaired respiratory function, leading to intense pain, laboured breathing, and often hemoptysis[114-117].

Behind the endothelial-lined capillary networks that supply the lung tissue with nutrients and gas exchange, a porous alveolar microenvironment exists that is often targeted by CTCs[118]. Once tumour cells have infiltrated the lung parenchyma, interactions between invading tumour cells and the rich stromal microenvironment promote survival and tumorigenic behaviour in the cancer cells[51]. Using a mouse mammary tumor virus promoter-polyomavirus middle T-antigen (MMTV-PyMT) breast cancer model, secretion of transforming growth factor-β3 (TGF-β3) by stem-like breast cancer cells demonstrated direct stimulation of pulmonary fibroblasts to secrete extracellular matrix protein, periostin

(POSTN), into the tumour-stroma microenvironment[119,120]. POSTN is a nonstructural soluble protein that is present at low levels in healthy adults, but becomes significantly overexpressed at sites of inflammation and within the tumour stroma[121]. Findings by Malanchi et al demonstrate the necessity for POSTN in secondary tumour development as knockout of POSTN in PyMT mice had no effect on primary breast tumour size and volume, but significantly reduced incidence of pulmonary metastases[120]. More recently, treatment of human periodontal ligament mesenchymal stem cells (PDLSCs) with recombinant human periostin protein (rhPOSTN) accelerated migratory and proliferative capacity among treated hPDLSCs[122].

Further investigation of the lung microenvironment and its relation to tumour formation has been modeled *in vivo,* however *in vitro* techniques for more detailed molecular characterization of the lung microenvironment are limited as it is difficult to adequately represent the complexities of native lung tissue in culture[123]. Utilizing the aforementioned *ex vivo* model of organ-conditioned media, exposure to lung-CM has been shown to induce migration of human breast cancer cell lines, as well as increase proliferative capacity in two cell lines, MDA-MB-231 and MDA-MB-468[52]. In addition, the stem-like $ALDH^{hi}CD44^{+}$ breast cancer subpopulation was exposed to organ-specific CM (bone marrow, lymph, liver, lung, and brain) to assess how this subset responded to soluble organ-derived factors. Interestingly, $ALDH^{hi}CD44^{+}$ breast cancer cells were found to preferentially migrate towards lung-CM over all other organ conditions *in vitro*[52]. These findings support observations by Croker et al, where $ALDH^{hi}CD44^{+}$ breast cancer cells were observed to preferentially metastasize to the lung *in vivo*[70].

To better understand the specific soluble factors within the lung-CM that promote migration and growth of breast cancer cells, Chu et al carried out protein array analyses[52]. They observed that lung-CM contained ~70 proteins that were absent in the basal media, many of which have previously been shown to have specific roles in metastasis and migration [48,49,52]. Among the identified proteins, five ligands of CD44 (osteopontin [OPN], basic fibroblast growth factor, and E-, L-, P-selectins) were identified to mediate growth and metastasis of $CD44^{+}$ breast cancer cells including stem-like $ALDH^{hi}CD44^{+}$ cells. These findings provided insight to the lung microenvironment and its potential role in

recruiting metastasis-initiating cells to the lung. However, little is known regarding the processes leading to organ-specific metastasis in the context of the CSC model.

2.7.2 The Bone Microenvironment and Bone Metastasis

In addition to the lungs, bones of the axial skeleton are another major tissue susceptible to metastasis in breast cancer patients. Approximately 60-85% of breast cancer patients develop bone metastases which significantly affect the integrity and resilience of the bone, resulting in chronic pain, bone resorption and pathological fractures in affected patients[124]. Often metastatic colonies form in regions of the skeleton that are heavily vascularized such as the pelvis, sternum, ribs, and particularly the marrow of long bones[125]. Structurally, the framework of cancellous bone is organized in a three-dimensional lattice structure, akin to lung tissue in that both are porous and supplied by a rich source of nutrients[126]. What sets apart the bone marrow as a metastatic target from other tissues is its ability to support the hematopoietic system and related stem cell niche. Within long bones (namely the femur), osteoclasts, osteoblasts and bone marrow stromal cells are involved in HSC regulation. Osteoblasts are specialized cells involved in the secretion of matrix proteins, and function in a coordinated manner with bone-resorbing osteoclasts to maintain physiological homeostasis. Interestingly, both cell types have been associated with supporting the HSC niche within the marrow. Although somewhat controversial, osteoblasts have been reported to interact directly with N-cadherin expressed on HSC to maintain quiescence and HSC activity during serial BM transplantation[127]. Further, activated osteoblasts have been demonstrated to secrete OPN, angiopoietin-1, and thrombopoietin, which have been implicated in limiting HSC expansion and maintaining quiescence[128-130]. The active resorption of bone by osteoclasts directly releases calcium into the bone marrow where calcium receptors on HSCs bind and promote HSC localization through engraftment to the endosteal surface of bone[131]. In addition, resorption of bone causes several chemokines secreted by bone marrow stromal cells, such as C-X-C motif chemokine ligand 12 (CXCL12) which is also involved in HSC homing and mobilization, to be released into the marrow[132]. Conveniently, CSCs from various cancers have been reported to express elevated levels of chemokine C-X-C motif receptor 4 (CXCR4), which through interaction with its ligand CXCL12, has been implicated in modulating the tumour microenvironment

to support a CSC niche[133]. In a glioblastoma model, inhibition of CXCR4/CXCL12 interactions led to decreased self-renewal and survival among CSC populations, emphasizing the importance of the bone marrow niche in supporting CSC populations[134]. It would be beneficial to achieve a stronger understanding related to the interactions that occur between HSC and the native bone marrow microenvironment as these interactions may be translatable to CSCs.

As is the case with the lungs, the complexities of the bone marrow microenvironment make it very difficult to accurately investigate its role in cancer metastasis and CSC maintenance *in vitro*, resulting in the majority of research being performed in animal models[135]. Our preliminary findings utilizing the *ex vivo* organ-CM model demonstrate MDA-MB-231 and SUM159 breast cancer cell lines exposed to bone marrow-conditioned media (BM-CM) exhibit enhanced migratory potential in both stem-like $ALDH^{hi}CD44^{+}$ and whole cell populations[136]. Similar to the analysis of lung-CM protein content mentioned previously, protein array analysis of BM-CM highlighted potential mediators of metastasis, including the CD44 ligand OPN[136]. Notably, exposure of MDA-MB-231 breast cancer cells to BM-CM induced stem-like behavior including tumorsphere formation and colony-forming ability, mediated at least in part by OPN[136]. Although present in CM generated from bone marrow stromal cells, the bone matrix and cancer cells themselves are also capable of producing soluble OPN[137]. The relevance of OPN to bone metastatic capacity is emphasized by experiments utilizing murine models deficient in, or overexpressing OPN, where there was a strong correlation between OPN and the likelihood of skeletal metastasis[137].

Taken together, our previous findings from both the lung-CM and BM-CM protein arrays combined with data gathered from functional assays suggests that proteins produced by the lung and bone marrow support the growth and migration of stem-like $ALDH^{hi}CD44^{+}$ breast cancer cells that facilitates their metastatic capacity towards these organs. However, the role of these organ microenvironments not only supporting but promoting a stem-like breast cancer phenotype requires further investigation, and this is the topic of this thesis.

2.8 Study Rationale

Ninety percent of breast cancer-related mortalities result from metastasis, a process whereby the primary tumour disseminates and targets distant secondary organs. Interestingly, events leading up to secondary tumour formation have marked inefficiencies, with only a very small proportion of primary tumour cells able to reach, persist and grow into a secondary tumour[138]. We believe this rare subset of cells may be stem-like cancer cells. CSCs possess unique capabilities of self-renewal and differentiation, and help to potentiate the development of secondary tumours. Breast CSCs from patient tumours and cell lines have been successfully isolated based on high ALDH enzymatic activity and co-expression of the cell-surface glycoprotein CD44. These two CSC markers actively provide the cell with protective detoxifying mechanisms as well as enhanced metastatic capacity, respectively[52,76,84]. We have previously observed that $ALDH^{hi}CD44^{+}$ cells preferentially migrate and/or metastasize to the lung and bone marrow microenvironments, where secondary tumours severely impact organ function. In addition, previous work in our lab has demonstrated that bone marrow-conditioned media can enhance the stem-like behavior of breast cancer cells. However, the specific role of the lung and bone microenvironments promoting metastasis of stem-like $ALDH^{hi}CD44^{+}$ cells remain poorly understood.

Our preliminary studies have shown that the lung and bone marrow microenvironments provide necessary factors to ***support*** the stem-like $ALDH^{hi}CD44^{+}$ breast cancer subpopulation, following a hierarchical model (**Figure 1**). In this thesis, we propose that the lung and/or bone microenvironments may induce cellular plasticity in breast cancer cells to ***promote*** $ALDH^{hi}CD44^{+}$ phenotype and subsequent acquisition of metastasis-initiating capacity (**Figure 1**). Understanding whether organ microenvironments promote stem-like phenotype and function could provide further insight into the mechanisms underlying organ-specific breast cancer metastasis.

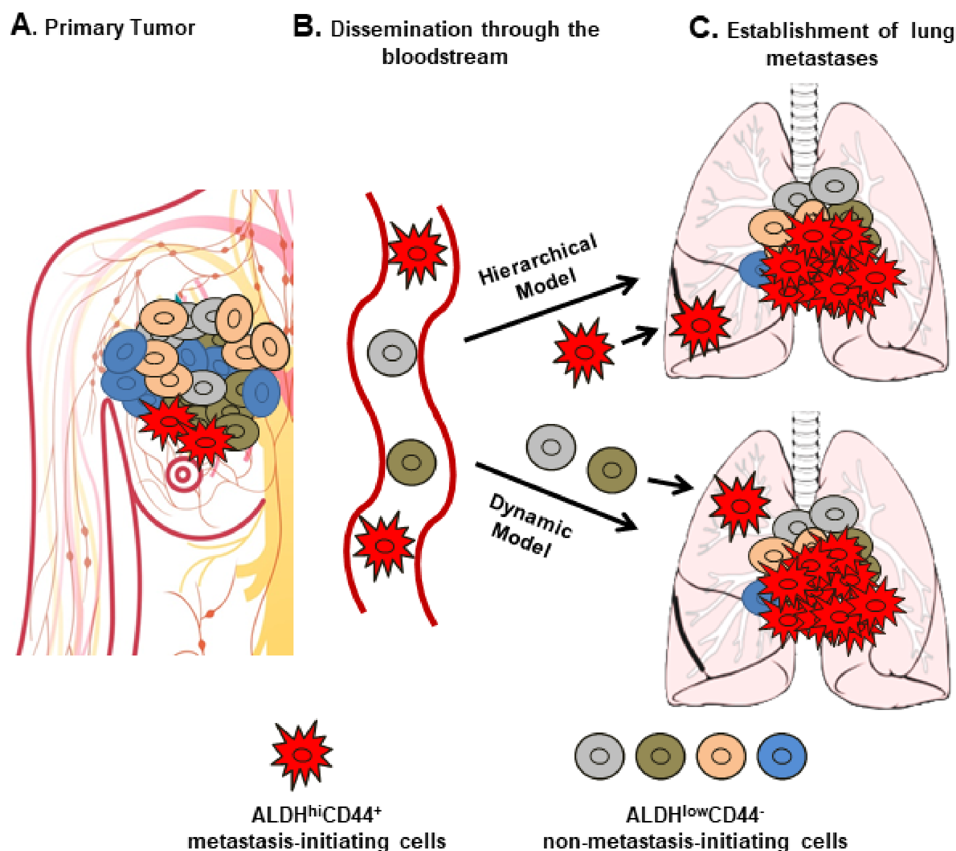


Figure 1. The ALDH^{hi}CD44^{+} phenotype in breast cancer cells enhances metastasis-initiating capacity. *(A)* In the primary breast tumor, ALDH^{hi}CD44^{+} cells comprise a subpopulation of the total tumor; the percentage of which may be higher in aggressive tumors (i.e. triple-negative breast cancer). ***(B)*** Early dissemination steps in metastasis are very efficient and may include both ALDH^{hi}CD44^{+} and ALDH^{low}CD44^{-} cells. However, only breast cancer cells with an ALDH^{hi}CD44^{+} phenotype can initiate and maintain metastasis. We hypothesize that this may occur either via a hierarchical model, in which ALDH^{hi}CD44^{+} cells are pre-existing before entering the secondary site (i.e. lung) and microenvironmental factors support their ability to initiate metastases; and/or via a dynamic model, in which the influence of the lung microenvironment may facilitate cellular plasticity to promote the development of an ALDH^{hi}CD44^{+} phenotype and acquisition of metastasis-initiating capacity. In both cases the population of ALDH^{hi}CD44^{+} cells are enriched in the metastatic site and can generate a heterogeneous tumor.

3 HYPOTHESIS AND OBJECTIVES

3.1 Hypothesis

The lung and bone microenvironments promote stem-like and metastatic behavior of human breast cancer cells.

3.2 Objectives

To determine the role of lung and bone microenvironments in promoting ***(1)*** stem-like phenotype, and ***(2)*** stem-like functional behavior of human breast cancer cells *in vitro.*

4 MATERIALS and METHODS

4.1 Cell Culture and Reagents

Several genetically unique immortalized human breast cancer cell lines were used in this study. The cell lines MDA-MB-468 (TN subtype), SUM159 (TN subtype), MDA-MB-231 (TN subtype) and MCF-7 (Luminal A subtype) are epithelial in origin and have adherent culture properties. The metastatic capacity of these cell lines *in vivo* (from greatest to least) are SUM159 > MDA-MB-231 > MDA-MB-468 > MCF-7[47,52,139]. Human breast cancer culturing conditions are described in **Table 1**. Media was purchased from Invitrogen (Burlington, ON, Canada). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO, USA) and VWR (Mississauga, ON, Canada). Tissue culture plastic was purchased from Nunc™ (Fisher Scientific, Ottawa, ON, Canada). Trypsin was purchased from Invitrogen and used at a concentration of 0.25% in citrate saline. Ethylene diamine tetraacetic acid (EDTA) was purchased from Bioshop Canada Inc. (Burlington, ON, Canada) and used at a concentration of 2 mM in deionized water. Cells were cultured at low passage numbers (<10) for all experiments and maintained under normal culture conditions of 37°C and 5% CO_2.

4.2 Lung and Bone Marrow *Ex Vivo* Model Systems

Using a novel technique adapted by Chu *et al*, lung-CM and BM-CM were generated as described below and used to investigate the significance of organ-derived soluble factors and their influence on stem-like breast cancer phenotype and function [52,136].

4.2.1 Organ Conditioned Media Generation

Healthy 5-7 week old female athymic nude mice (Hsd: Athymic Nude-*Foxn1*nu; Envigo, Indianapolis, IN) were purchased and monitored under the guidelines of the Canadian Council of Animal care as outlined by the protocol approved by the University of Western Ontario Council of Animal care (protocol #2009-064; **Appendix 1**). Mice were euthanized by CO_2 inhalation and lungs, or tibia and femur were aseptically removed and

Table 1. Human Breast Cancer Cell Lines and Culturing Conditions

Cell Line	Culturing Conditions	Source
MDA-MB-468	αMEM + 10% FBS	MD Anderson Cancer Center
MDA-MB-231	DMEM:F12 + 10% FBS	MD Anderson Cancer Center
MCF7	DMEM + 10% FBS	Koropatrick Lab
SUM159	HAM F:12 + 5% FBS, 0.5% insulin, 0.1% Hydrocortisone, 1% HEPES	Asterand

placed into pre-weighed 50-mL conical tubes with 30mL sterile phosphate-buffered saline (PBS).

4.2.1.1 Lung Conditioned Media (Lung-CM)

Harvested lungs were washed three times in ice cold PBS before being dissociated into ~1 mm^3 fragments. Lung tissues were weight-normalized by resuspension in a 4:1 media to tissue (v/w) ratio in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM:F12) supplemented with $Mito^+$ serum extender (1X, BD Biosciences, Mississauga, Canada) and penicillin-streptomycin. Lung fragments and media were cultured at 37°C and 5% CO_2 for 24 hours. Following culturing, conditioned media (CM) was harvested, diluted by three volumes of media and centrifuged at 900g for 15 minutes at 4°C to remove residual cell debris. Lung-CM was passed through a 0.22µm syringe filter (Corning, Germany), aliquoted and stored at -80°C until use. To account for mouse-to-mouse variability, lung-CM from multiple mice was pooled prior to use in experimental studies **(Figure 2A)**.

4.2.1.2 Bone Marrow Conditioned Media (BM-CM)

Isolated tibia and femurs from mice were trimmed clean of excess muscle tissue and epiphyses removed. Since the cellular content in bone marrow (BM) is lower than in other organs, a different approach was used to generate BM conditioned media (BM-CM). Using a 27-gauge x ½ inch needle, PBS was flushed through the shaft of each long bone. The collected BM cells which had been previously characterized by Chu et al to be bone marrow stromal cells (BMSC), were then centrifuged for 5 minutes at 1000g, resuspended in DMEM + 10% FBS + pen/strep and incubated at 37°C + 5% CO_2. BMSCs were seeded at a concentration of ~1 x 10^7 cells/flask in T-75 flasks and cultured at 37°C and 5% CO_2 for 2-3 passages. The BMSC monolayer was washed and finally exposed to DMEM/F12 + $Mito^+$ + pen/strep for 72 hours, after which BM-CM was collected by centrifugation at 900g for 15 minutes at 4°C, passed through a 0.22µm syringe filter (Corning, Germany) and stored at -80°C until use. To account for mouse-to-mouse variability, BM-CM from multiple mice was pooled prior to use in experimental studies **(Figure 2B)**.

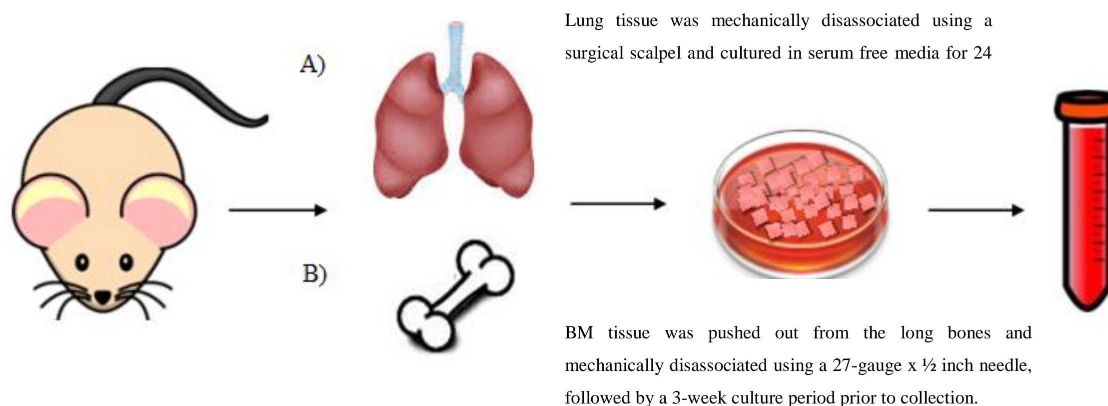


Figure 2. Generation of organ-conditioned media. Healthy female nude mice were euthanized by CO_2 inhalation and organs were removed aseptically. ***(A)*** Harvested lungs were washed, minced into ~1 mm^3 fragments, and resuspended in a 4:1 media to tissue (v/w) ratio for culturing at 37°C and 5% CO_2 for 24 h. Following culture, lung-CM media is collected and further diluted by three volumes of basal media. ***(B)*** Femurs and tibias were excised from female nude mice and subject to bone marrow extraction using a 27gauge x ½ inch needle to flush out the BM contents. Collected cellular bone marrow stromal cells (BMSC) were seeded and cultured for 2-3 passages before collection of BM-CM. All organ-CM is centrifuged to remove cellular debris and subjected to sterile filtration prior to use in experimental studies.

4.3 Flow Cytometry Analysis

Flow cytometry was used to identify the frequency of $ALDH^{hi}$, $CD44^{+}$, and $ALDH^{hi}CD44^{+}$cells from both the MDA-MB-468 and SUM159 breast cancer cell lines. Initial seeding densities were determined based on 60% tissue culture confluency at a 48-hour timepoint. MDA-MB-468 (4×10^5 cells) and SUM159 (1.5×10^5 cells) were seeded and grown on 60 mm tissue culture dishes in regular growth media for 48 hours. Cells were then washed with PBS and exposed to three different treatments: BM-CM, lung-CM, or basal media as negative control. Cultured breast cancer cells were harvested using trypsin (1x) after 24, 48, and 72 hours and labeled as described below.

The Aldefluor™ assay kit (StemCell Technologies, Vancouver, BC, Canada) was used to assess ALDH activity. The Aldefluor™ kit uses an uncharged fluorescent ALDH substrate [BODIPY-aminoacetaldehyde (BAAA)] that passively diffuses into cells. Cellular ALDH activity converts uncharged BAAA molecules to negatively charged BODIPY-aminoacetate (BAA-) molecules, preventing diffusion out of the cell. Trapped BAA-molecules increase green fluorescence in $ALDH^{hi}$ cells. Cells were kept on ice prior to sorting to prevent the efflux of BAA- from labeled cells by ABC-transporters. In addition, the Aldefluor™ buffer contains a pharmacological inhibitor of ABC-transporters. Approximately 2×10^6 cells were harvested, washed in PBS, centrifuged at 1000g for 5 min, resuspended in Aldefluor™ buffer and incubated with Aldefluor™ substrate (10 µL BAAA/10^6 cells). A control sample was also prepared in which 5 µL the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB; 1.5 mM) was co-incubated with Aldefluor™. DEAB inhibits ALDH enzyme activity and allows BAAA to remain in its uncharged form and passively diffuse out of the cell. Following a 45-minute incubation at 37°C, samples were centrifuged (1000g for 5 min), washed with PBS/centrifuged (1000g for 5 min), and resuspended with Aldefluor™ assay buffer. Subsequent labelling with 10 µL CD44-phycoerythrin (PE; BD Biosciences) antibody was performed at 4°C for 30 min. Cells were again washed with PBS/centrifuged (1000g for 5 min) and resuspended in assay buffer. Following resuspension, 5 µL of 7-aminoactinomycin D (7-AAD; BD Biosciences) was added to samples to monitor cell viability. Samples were stored on ice and analyzed by

flow cytometry for intrinsic ALDH activity and/or CD44 cell surface expression using the gating strategy illustrated in **Figures 3, 4 and 5** (SUM159 cells; adherent MDA-MB-468 cells; and non-adherent MDA-MB-468 cells, respectively). Analysis was performed using a Beckman Coulter FC500 flow cytometer, with acquisition analyses carried out using Kaluza 1.5 software (Beckman Coulter, USA).

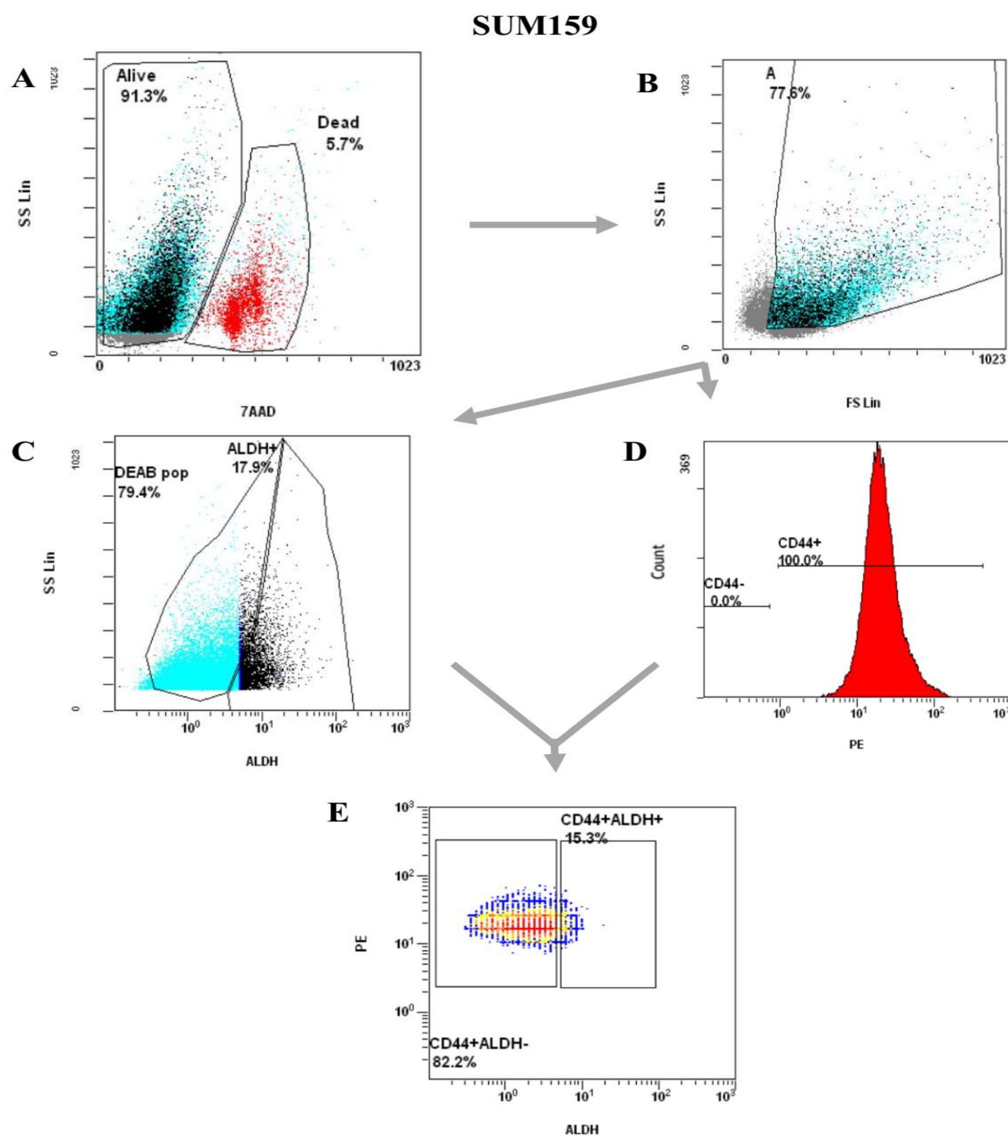


Figure 3. Representative flow cytometry gating strategy for analysis of ALDH activity and/or CD44 cell surface expression in SUM159 human breast cancer cells. Whole cell populations of SUM159 breast cancer cells were harvested and labelled with 7-AAD, CD44-PE, and the Aldefluor™ assay kit. Analysis was performed using a three-colour multi-parameter gating strategy on a Beckman Coulter EPICS XL-MCL flow cytometer. **(A)** Viable cells were identified based on 7-AAD exclusion, and **(B)** gated based on forward scatter. Cells satisfying viability criteria were then assessed for **(C)** ALDH activity relative to a DEAB control, and **(D)** CD44 expression relative to a cells only control. **(E)** Breast cancer cells expressing both high ALDH activity and CD44 expression were used to identify the $ALDH^{hi}CD44^{+}$ phenotype. Analysis performed using 50,000 events.

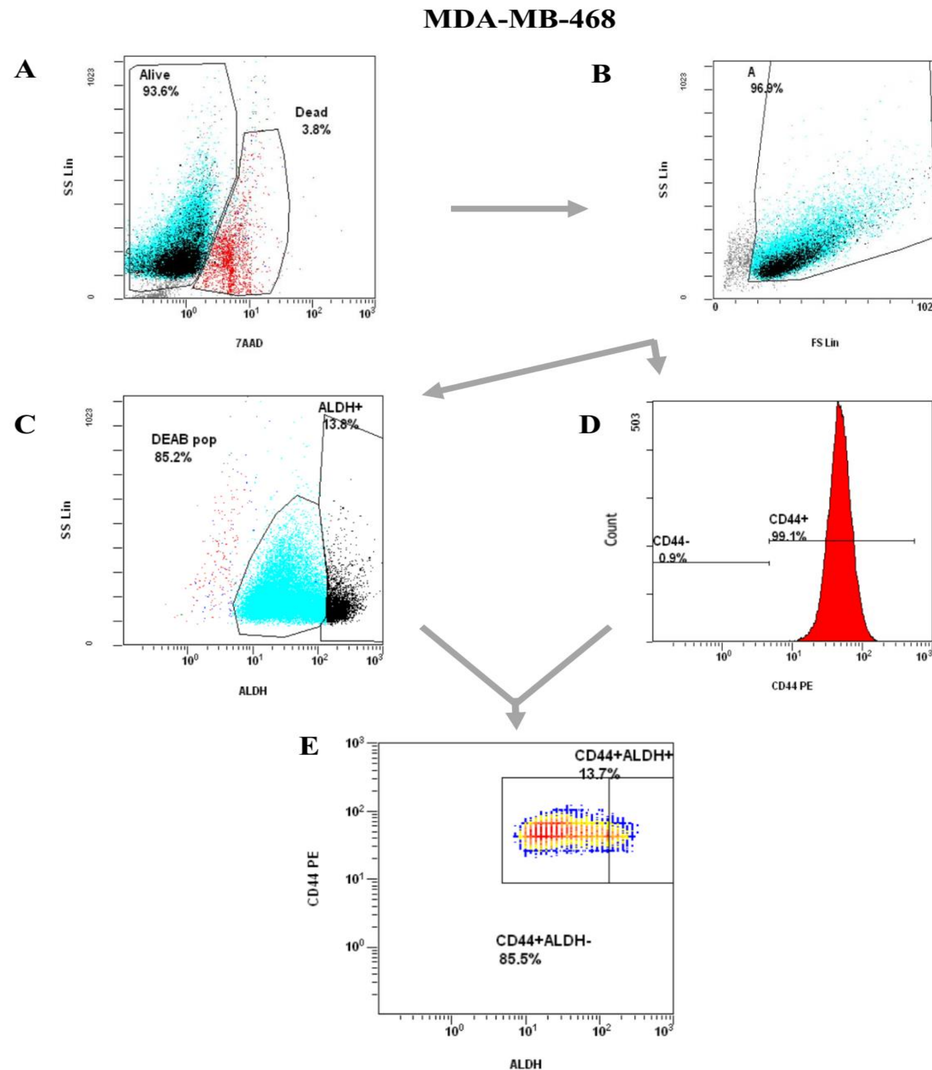


Figure 4. Representative flow cytometry gating strategy for Analysis of ALDH activity and/or CD44 cell surface expression in adherent MDA-MB-468 human breast cancer cells. Adherent whole cell populations of MDA-MB-468 breast cancer cell lines were harvested and labelled with 7-AAD, CD44-PE, and the Aldefluor™ assay kit. Analysis was performed using a three-colour multi-parameter gating strategy on a Beckman Coulter EPICS XL-MCL flow cytometer. **(A)** Viable cells were identified based on 7-AAD exclusion, and **(B)** gated based on forward scatter. Cells satisfying viability criteria were then assessed for **(C)** ALDH activity relative to a DEAB control, and **(D)** CD44 expression relative to a cells only control. **(E)** Breast cancer cells expressing both high ALDH activity and CD44 expression were used to identify the ALDH^{hi}CD44^{+} phenotype. Analysis performed using 50,000 events.

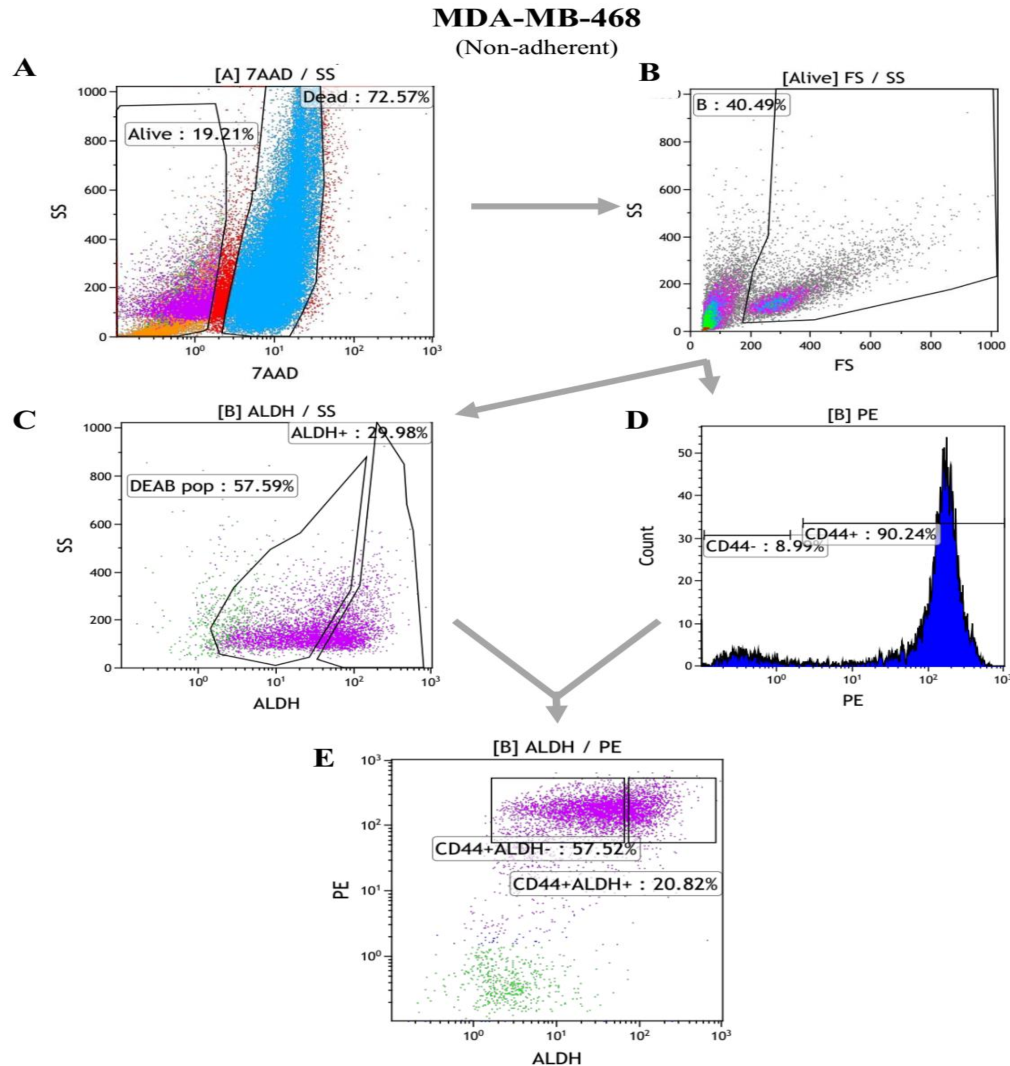


Figure 5. Representative flow cytometry gating strategy for analysis of ALDH activity and/or CD44 cell surface expression in non-adherent MDA-MB-468 human breast cancer cells. Non-Adherent cell populations of MDA-MB-468 breast cancer cell lines generated after exposure to organ-CM were harvested and labelled with 7-AAD, CD44-PE, and the Aldefluor™ assay kit. Analysis was performed using a three-colour multi-parameter gating strategy on a Beckman Coulter EPICS XL-MCL flow cytometer. **(A)** Viable cells were identified based on 7-AAD exclusion, and **(B)** gated based on forward scatter. Cells satisfying viability criteria were then assessed for **(C)** ALDH activity relative to a DEAB control, and **(D)** CD44 expression relative to cells only control. **(E)** Breast cancer cells expressing both high ALDH activity and CD44 expression were used to identify the $ALDH^{hi}CD44^{+}$ phenotype. Analysis performed using 500,000 events.

4.4 RT-qPCR Analysis

MDA-MB-468 and SUM159 cells exposed to lung-CM, BM-CM, or basal media for 24 hours were harvested (≤ 5 x 10^5 cells) and lysed using RLT lysis buffer (Qiagen, Germany). Total RNA extraction was performed using a column based RNA purification method (RNeasy® Micro kit, Qiagen, Germany). RNA quality and concentration was determined using the NanoDrop One apparatus (Thermo Fisher Scientific, USA), and RNA was stored at -80°C.

4.4.1 Analysis of ALDH1A1, ALDH1A3, and CD44 Gene Expression

Subsequent cDNA synthesis was completed (Invitrogen, USA), combined with Supergreen Mastermix (Wisent Bioproducts, CA) and custom primer sets that were designed to detect ALDH1A1, ALDH1A3, and CD44 mRNA expression (**Table 2**). Samples were loaded onto 96-well plates and loaded onto the Stratagene Mx3000p instrument. The thermal profile setup began at 95 °C for 5 min to allow cDNA to denature, followed by 40 cycles at 95 °C for 15 seconds and 60 °C for 30 seconds. Relative quantification was performed using a standard curve method with serial dilutions (1, 1:10, 1:100, 1:1000, 1:1 x 10^4). Data were analyzed using ΔCT values and transcript levels normalized to the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH). MXPro software (Agilent, CA, USA) was used for qPCR data analysis.

4.4.2 Quantitative RT-PCR Human Cancer Stem Cell® Array

RNA samples from MDA-MB-468 human breast cancer cells were harvested (~5 x 10^5 cells) after 24 hour treatment with basal media or lung-CM. Cells were then lysed using RLT lysis buffer (Qiagen, Germany), followed by total RNA extraction performed using column based RNA purification (RNeasy® Micro Kit, Qiagen, Germany). RNA quality

Table 2. Gene List and Primer Sequences

Gene	Primer Sequence	Source
ALDH1A1	Forward: 5' – CGT TGG TTA TGC TCA TTT GGA A – 3' Reverse: 5' – TGA TCA ACT TGC CAA CCT CTG T – 3'	Integrated DNA Technologies (IDT)
ALDH1A3	Forward: 5' – ATG CGG ATT GCC AAA GAG GA – 3' Reverse: 5' – AGC CAA CTT CAG GGC TTT GT – 3'	Integrated DNA Technologies (IDT)
CD44	Forward: 5' – GGG TGT ACA TCC TCA CAT CCA A – 3' Reverse: 5' – GCT CAC GTC ATC ATC AGT AGG G – 3'	Integrated DNA Technologies (IDT)
GAPDH	Forward: 5' – TTG CCC TCA ACG ACC ACT TTG T– 3' Reverse: 5' – AGG GGT CTT ACT CCT TGG AGG C– 3'	Integrated DNA Technologies (IDT)

and concentration was determined using the NanoDrop One apparatus (Thermo Fisher Scientific, USA), and stored at -80°C. Subsequent cDNA synthesis was completed using the RT^2 First Strand Kit (Qiagen, Germany), followed by RT-qPCR preparation using RT^2 SYBR Green ROX qPCR Mastermix (Qiagen, Germany). Samples were then loaded onto preset 96-well RT^2 Profiler™ PCR Array Human Cancer Stem Cells arrays (Qiagen, Germany). The thermal setup began at 95 °C for 5 min to allow cDNA to denature, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 30 seconds. RT-qPCR was performed on the Stratagene Mx3000p instrument, with analysis of ΔCT values performed using MXPro software (Agilent, CA, USA) and Qiagen's online Data Analysis Center. All transcript levels were normalized to the internal GAPDH control.

4.5 Cell Viability Assays

4.5.1 Trypan Blue Exclusion

MCF7, MDA-MB-468, MDA-MB-231, and SUM159 cells were cultured (37°C, 5% CO_2) and doubling time was analyzed to determine initial seeding densities that result in 60% tissue confluency after 48 hours incubation at 37°C (2×10^5, 1×10^5, 7.5×10^4, and 7.5×10^4 cells respectively). Cells were seeded on 6-well plates and grown for 48 hours, allowing cells to re-adhere and adjust to *in vitro* conditions. Cells were then washed with PBS x 2 and exposed to lung-CM, BM-CM, or basal media for an additional 48 hrs. Viability of resulting non-adherent breast cancer cell subpopulations was performed by collecting 10 μL of media and combining with an equal volume of Trypan Blue (1X, St. Louis, MO, USA). Total number of cells (live and dead) was enumerated using a hemocytometer under a light microscope and percentage of viable cells was determined. Representative images of floating and adherent cells were obtained using an inverted Olympus IX70 microscope.

4.5.2 LIVE/DEAD® Assay

Based on trypan blue cell viability analysis, MCF7 and MDA-MB-468 human breast cancer cell lines were chosen as candidate cell lines to confirm non-adherent cell viability using the fluorometric LIVE/DEAD® assay (Life Technologies, Burlington, ON, CA) based on staining with two dyes. Calcein-acetoxymethyl (calcein-AM) is a polyanionic dye

that is initially non-fluorescent, but once permeating the cell membrane of viable cells, becomes enzymatically converted to its fluorescent form by ubiquitous intracellular esterase activity. Conversely, ethidium homodimer-1 (EthD-1) functioned as a marker for cell death. In living cells, with a functional plasma membrane, EthD-1 is excluded from entering the cell. Once cells become damaged and the plasma membrane's integrity is lost, EthD-1 is able to penetrate and bind to nucleic acids which induce a 40-fold increase in fluorescence in dead cells.

Cells were seeded onto T-75 tissue culture flasks at 3 x 10^6 and 2 x 10^6 cells, MCF7 and MDA-MB-468 respectively. Cells were cultured for 48 hours, allowing cells to adjust to the *in vitro* conditions. Cells were then washed with PBS x 2 and exposed to lung-CM, BM-CM, or basal control media for an additional 48 hours. Media containing non-adherent cells were harvested, centrifuged (5 minutes at 1000g), and washed in PBS x 3. The collected cell pellet containing non-adherent cells became the experimental sample to be tested for viability. From the adherent subpopulation, 2 x 10^6 viable cells were collected, centrifuged (5 minutes at 1000g), washed in PBS x 3, and split into two individual tubes labelled "live" and "dead". The "live" tube provided a positive control. The "dead" tube was centrifuged (5 minutes at 1000g) and the cell pellet was treated with 100µL IntraPrep Reagent 1: Fixation (IntraPrep Permeabilization Reagent, Beckman Coulter, USA), and covered at room temperature for 15 minutes to induce cell death. These "dead" cells provided a negative control for the LIVE/DEAD® assay. A working solution of 2 µM calcein-AM and 4 µM of EthD-1 LIVE/DEAD® reagent were combined. Using a 96-well plate, 100µL of working solution was combined directly with 100µL of sample (1:1) in each required well. Samples were covered and incubated at room temperature for 30 minutes.

4.5.2.1 LIVE/DEAD® Fluorescent Imaging

After incubation, 200µL of sample was loaded onto glass microscope slides and covered with a 22mm glass coverslip. Images were acquired at 10x magnification using an upright Olympus Provis microscope (Olympus) coupled with a Retiga 2000R charge-coupled

device camera (QImaging, BC, Canada). Fluorescent images were captured using a Red-Green-Blue filter fitted to the Retiga 2000R camera.

4.5.2.2 LIVE/DEAD® Fluorescence Measurement of Viability

After incubation, the 96-well plate containing samples were inserted into a Synergy H4 Hybrid Reader (BioTek, USA). Sample excitation/emission (Ex/Em) wavelengths were adjusted to 485nm/530nm and 530nm/645nm for each of the live/dead reporters, calcein-AM and EthD-1 respectively. Sample fluorescence values were recorded and percent viability (% Live Cells) was calculated.

4.6 Mammosphere Assay

MCF7 and MDA-MB-468 cells were cultured and doubling time was calculated to determine initial seeding densities that result in 60% tissue confluency after 48 hours. Based on doubling time, 3×10^6 and 2×10^6 cells were seeded on T-75 tissue culture flasks (Corning, USA) and cultured for 48 hours respectively, allowing cells to adjust to the *in vitro* conditions. Samples were then washed with PBS and exposed to lung-CM, BM-CM, or basal media for an additional 72 hrs. Respective adherent and non-adherent cell subpopulations were isolated from each treatment condition and counted manually using a hemocytometer and trypan blue exclusion. Approximately 5×10^5 and 3×10^5 viable non-adherent cells could be expected from each T-75 tissue culture flask following 72-hour lung-CM treatment of MDA-MB-468 and MCF7 cells, respectively. Basal and BM-CM treatment rendered nearly 1.5×10^5 viable non-adherent cells in either cell line. For each subpopulation and treatment condition, 1×10^3 viable cells were resuspended in mammosphere media (500mL DMEM:F12, 2.5mL Insulin [1mg/mL], 400µL EGF [25µg/mL], 200µL bFGF [25 µg/mL], 20mL of 10% BSA, 2mL B27). Subsequent serial limiting dilutions of cells were carried out (1000 cells/well to 0.001 cells/well) and seeded onto a 96-well ultra-low attachment plates. Samples were monitored for mammosphere growth over 21 days, with media replaced periodically to account for nutrient depletion and evaporation. Upon reaching endpoint, an inverted Olympus IX70 microscope was used to image and analyze mammospheres. Criteria used to distinguish presence of

mammosphere formation was based upon identification of clusters containing ≥ 5 cells. Mammosphere formation efficiency was calculated by scoring each well for the presence or absence of mammospheres (N=3), with subsequent analyses performed using L-Calc™ software (Stem Cell Technologies, Vancouver, BC).

4.7 Proteomic Analysis of Lung-CM

Proteomic analysis was carried out by Dr. Ying Xia in the Allan lab, in collaboration with Dr. Gilles Lajoie (Department of Biochemistry). Lung-CM samples (N=3) were concentrated and fractionated by 1D-SDS-PAGE followed by in-gel tryptic digestion before injection into an Ultra-High Performance Liquid Chromatography instrument interfaced with a LTQ Orbitrap Elite mass spectrometer (MS). Samples were scanned for 150 min using the data-dependent acquisition scan mode, selecting the 4 most abundant ions from each survey for fragmentation and MS/MS detection, combined with iterative exclusion (IE-MS) of previously scanned ions. Raw data was analyzed using "in-chorus" protein identification methods employing X!Tandem, SpectraST, and PEAKS search engines to allow comprehensive identification and increased statistical confidence in independently identified proteins across different platforms. Classification analyses of identified lung-CM proteins were performed using the PANTHER® Classification System (Geneontology Consortium).

4.8 Statistical Analysis

All experiments were performed with at least three biological replicates (N=3), with technical replicates (n=3) carried out internally for each biological replicate. Statistical analyses were conducted using GraphPad Prism 6.0 (San Diego, CA, USA), with the exception of the gene arrays analyzed with Qiagen's online Data Analysis Center. Data were presented as the mean ± standard error of the mean (SEM). A two-way analysis of variance (ANOVA) was used to compare multiple means across different groups. Dunnet's post-hoc test was used to confirm significance. Values <0.05 were considered statistically significant.

5 RESULTS

5.1 Exposure to lung-conditioned media decreased ALDH activity and CD44 expression in human breast cancer cells

Both the lung and bone have shown high susceptibility to the formation of secondary tumours resulting from breast cancer metastasis[52,56]. Here, we analyze phenotypic differences between two different human breast cancer cell lines, including the highly metastatic SUM159 (HER2+) and the weakly metastatic MDA-MB-468 (TN) cell lines. Assessment of phenotypic variation was assessed by flow cytometry analysis for ALDH enzymatic activity and CD44 cell surface expression, producing the $ALDH^{hi}CD44^{+}$ stem-like cell phenotype. Contrary to our original hypothesis, 72-hour exposure to lung-CM induced a significant decrease in the percentage of adherent cells with $ALDH^{hi}$ and/or $ALDH^{hi}CD44^{+}$ phenotype in both MDA-MB-468 (4.4 ± 2.4%; 4.2 ± 2.1%) and SUM159 (9.2 ± 0.4%; 9.1 ± 0.4%) cell lines, relative to basal media controls ($p<0.05$) **(Figure 6A and B)**. SUM159 cells showed a significantly decreased $ALDH^{hi}$ and/or $ALDH^{hi}CD44^{+}$ phenotype after 72 hours BM-CM treatment (11.8 ± 0.8 %; 12.0 ± 0.9%) relative to basal media controls ($p<0.05$) **(Figure 6B)**. Further, lung-CM treatment significantly decreased CD44 expression in both MDA-MB-468 and SUM159 cell lines (99.1 ± 0.2% and 71.2 ± 2.1%, respectively), relative to basal media controls (99.7 ± 0.1% and 99.9% ± 0.0%, respectively) ($p<0.05$) **(Figure 6A and B).** BM-CM had no effect on CD44 expression in either cell line.

5.2 Exposure to lung-conditioned media increased gene expression of ALDH1A3 in human breast cancer cells

Next, we examined mRNA expression for ALDH1A1, ALDH1A3, and CD44 by RT-qPCR. In contrast to the flow cytometry results for decreased CD44 expression and ALDH activity, both MDA-MB-468 **(Figure 7A)** and SUM159 **(Figure 7B)** cell lines exposed to lung-CM exhibited significantly increased CD44 (4.0 ± 1.1-fold [MDA-MB-468] and 1.8

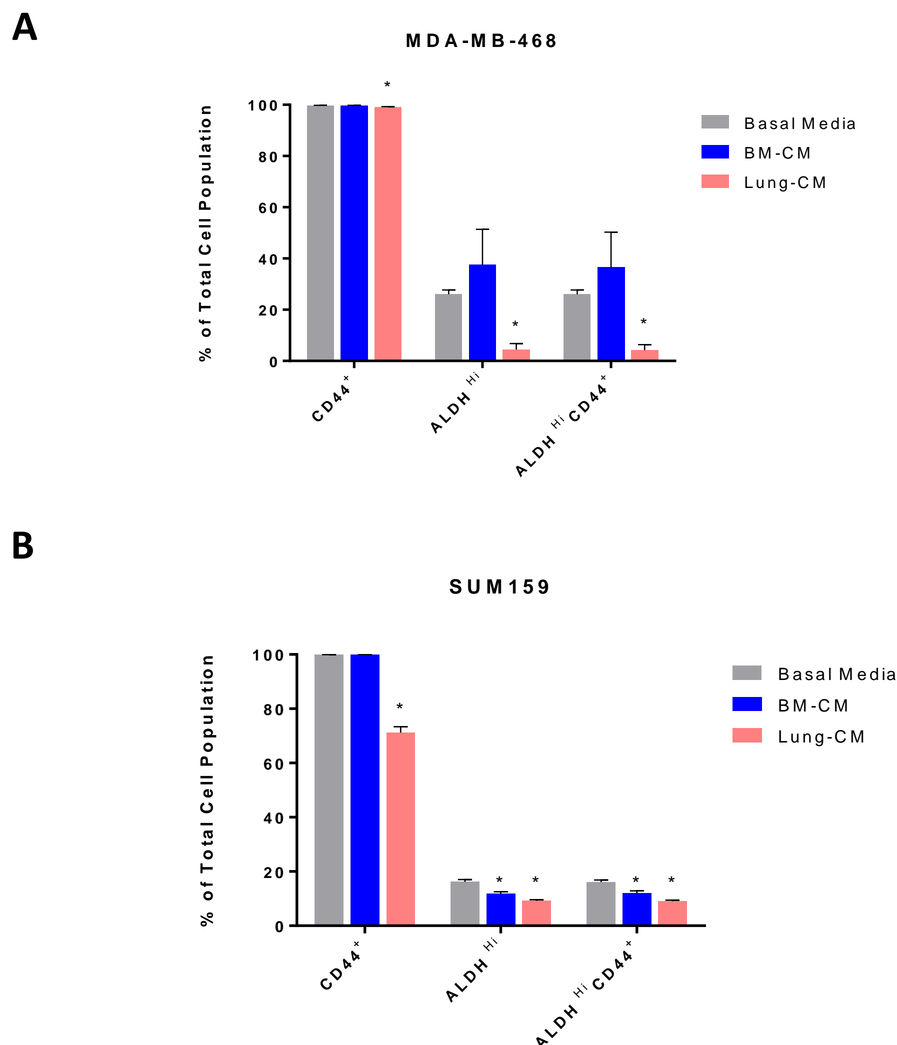


Figure 6. Exposure to lung-conditioned media decreased ALDH activity and CD44 expression in human breast cancer cells. ***(A)*** MDA-MB-468 and ***(B)*** SUM159 cells were exposed to lung-conditioned media (lung-CM), bone marrow conditioned media (BM-CM), or basal media (DMEM:F12 + Mito$^+$) over 72 hours in culture (37°C, 5% CO_2) without media replacement. Adherent cells were harvested and analyzed by flow cytometry for ALDH activity and CD44 expression, using the Aldefluor™ assay and CD44 antibody respectively. Experiments were performed a minimum of three times and statistical analyses were performed using one-way ANOVA and Dunnett's post hoc test. Data is presented as mean ± SEM. All significant values (*) are relative to the negative control basal media treatment group ($p<0.05$, N=3).

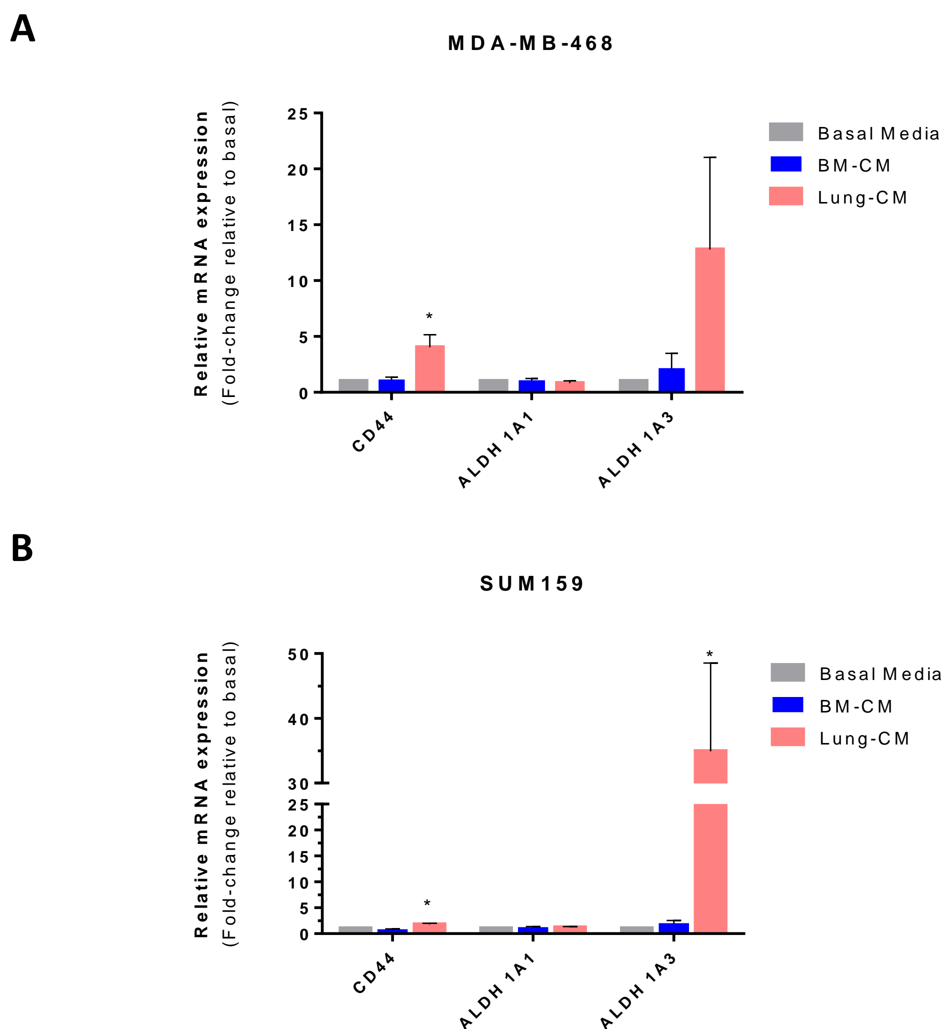


Figure 7. Exposure to lung-conditioned media increased CD44 and ALDH1A3 mRNA expression in human breast cancer cells. ***(A)*** MDA-MB-468 and ***(B)*** SUM159 cells were exposed to lung-conditioned media (lung-CM), bone marrow conditioned media (BM-CM), or basal media for 24 hours in culture (37°C, 5% CO_2) without media replacement. Cells were harvested and RNA was analyzed by RT-qPCR to assess expression of CD44, ALDH1A1 and ALDH1A3. All analyses were normalized to GAPDH expression and shown as fold-changes relative to basal media (DMEM:F12 + Mito$^+$) controls. Experiments were performed a minimum of three times and statistical analyses were performed using one-way ANOVA and Dunnett's post hoc test. Data is presented as mean ± SEM. All significant values (*) are relative to the negative basal media treatment group ($p<0.05$, N=3).

± 0.2-fold [SUM159]) mRNA expression compared to cells exposed to basal media controls ($p<0.05$). Further, ALDH1A3 mRNA expression was significantly upregulated in the SUM159 cell line following treatment with lung-CM (34.9 ± 13.6-fold), relative to basal media control ($p<0.05$) **(Figure 7B)**. Treatment with BM-CM did not lead to significant changes in gene expression. Gene expression of ALDH1A1 was not significantly affected by organ-CM treatments using either cell line. There was no significant difference in relative CD44 mRNA expression levels between either adherent or non-adherent MDA-MB-468 cell subpopulations, and the SUM159 cell line, in response to basal media **(Appendix 3)**. Moreover, the SUM159 cell line exhibited the lowest ALDH1A1 and ALDH1A3 relative mRNA expression levels compared to both adherent and non-adherent MDA-MB-468 cell subpopulations following basal media exposure ($p<0.05$) **(Appendix 3)**.

5.3 Treatment with lung-CM induced a viable, non-adherent breast cancer subpopulation

During the course of the phenotypic experiments, we observed that a subpopulation of non-adherent human breast cancer cells was produced following exposure to lung-CM, and to a lesser extent following exposure to BM-CM. Using trypan blue exclusion **(Figure 8A)**, we quantified the production of non-adherent cells using four different human breast cancer cell lines (MDA-MB-468, MDA-MB-231, MCF7, and SUM159). Surprisingly exposure to lung-CM induced a viable non-adherent subpopulation when compared to basal and BM-CM treatment. Moreover, we demonstrated that the ability of lung-CM to induce a viable non-adherent subpopulation was cell line specific, where both of the less aggressive cell lines (MCF7 and MDA-MB-468) demonstrated a viable non-adherent subpopulation in the presence of lung-CM (10.8 ± 0.9% and 7.5 ± 0.6 % of whole population, respectively) relative to basal media (0.3 ± 0.3% and 1.3 ± 0.3 % respectively) ($p<0.05$) **(Figure 8B)**. In contrast, the most aggressive SUM159 cells did not produce a non-adherent subpopulation, irrespective of media conditions. Exposure to BM-CM or basal control did not result in a prominent non-adherent subpopulation, however, viable non-adherent cells were consistently produced among MCF7, MDA-MB-468, and MDA-MB-231 cells exposed to BM-CM or basal media. Further analysis was performed using the LIVE/DEAD® assay to

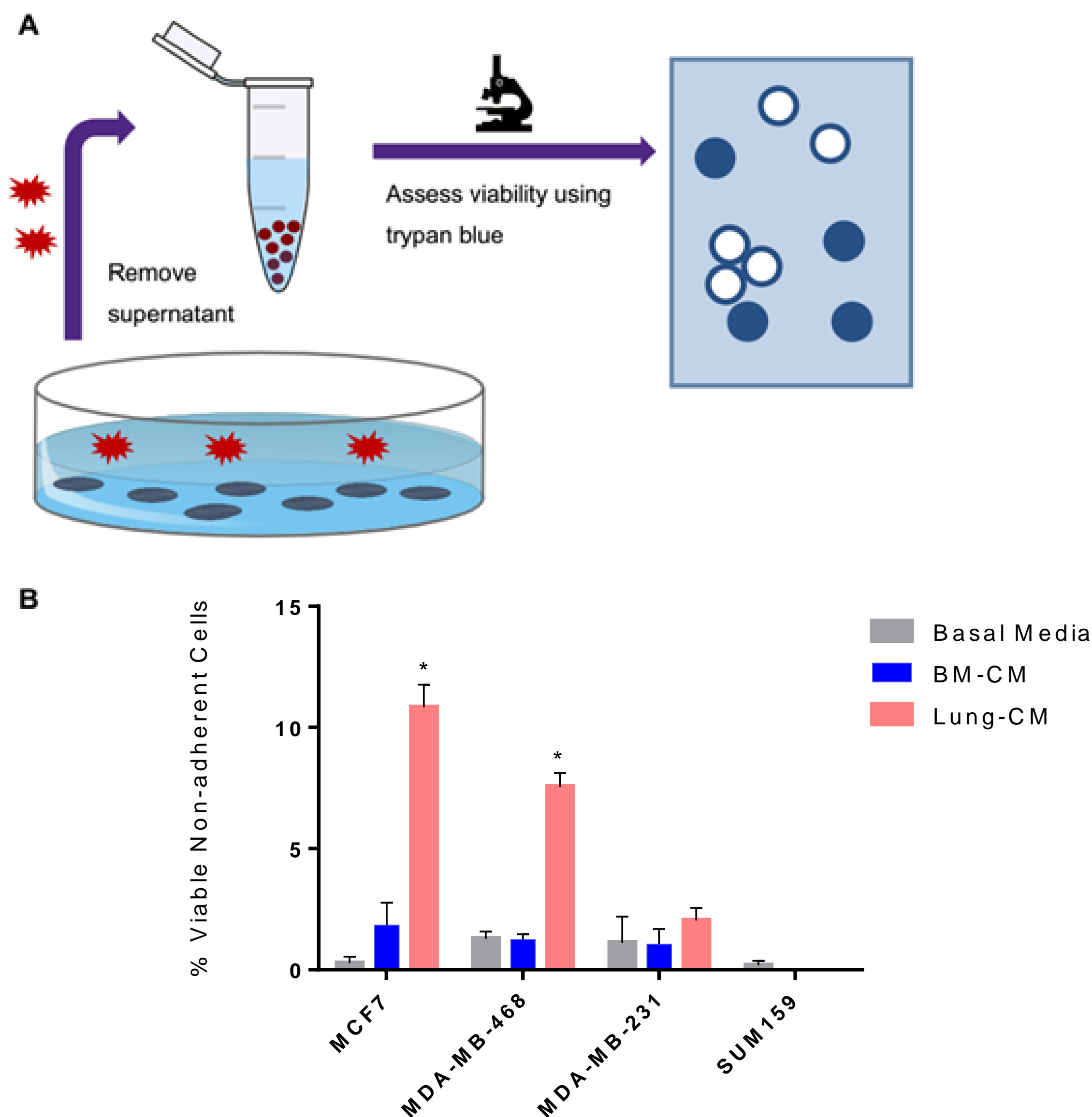


Figure 8. Exposure to lung-conditioned media supported the production of viable non-adherent breast cancer cells assessed by trypan blue exclusion. MCF7, MDA-MB-468, MDA-MB-231, and SUM159 human breast cancer cells were exposed to lung-CM, BM-CM, and basal media for 48 hours in culture (37°C, 5% CO_2) without media replacement. Media was collected, centrifuged at 1000g for 5 minutes, and non-adherent cells were manually counted using a hemocytometer to assess cell viability via trypan blue exclusion. ***(A)*** Schematic of experimental approach. ***(B)*** Analysis of viable non-adherent cells identified in MCF7, MDA-MB-468, MDA-MB-231, and SUM159 human breast cancer cells following 48-hour exposure to lung-CM, BM-CM, or basal media. Experiments were performed a minimum of 3 times and statistical analyses were performed using one-way ANOVA and Dunnett's post hoc test. Data is presented as mean ± SEM. All significant values (*) are relative to the negative control basal media treatment group ($p<0.05$, N=3).

quantify viability based on a more sensitive fluorometric approach. Based on trypan blue exclusion findings, MCF7 and MDA-MB-468 cell lines were chosen as candidates for further analysis. Exposure to lung-CM significantly increased the percentage of viable, non-adherent cells produced by both MCF7 and MDA-MB-468 cell lines (11.5 ± 1.2 % and 34.9 ± 1.9 % respectively), relative to basal media control ($p<0.05$) **(Figure 9A)**. Further, treatment with BM-CM significantly decreased the viable non-adherent subpopulation produced by the MCF7 cell line, but not the MDA-MB-468 cell line. (5.3 ± 0.1% and 8.8 ± 0.2 % respectively) ($p<0.05$) **(Figure 9A)**. Both viable and non-viable cells were evident through fluorometric analyses **(Figure 9B)**.

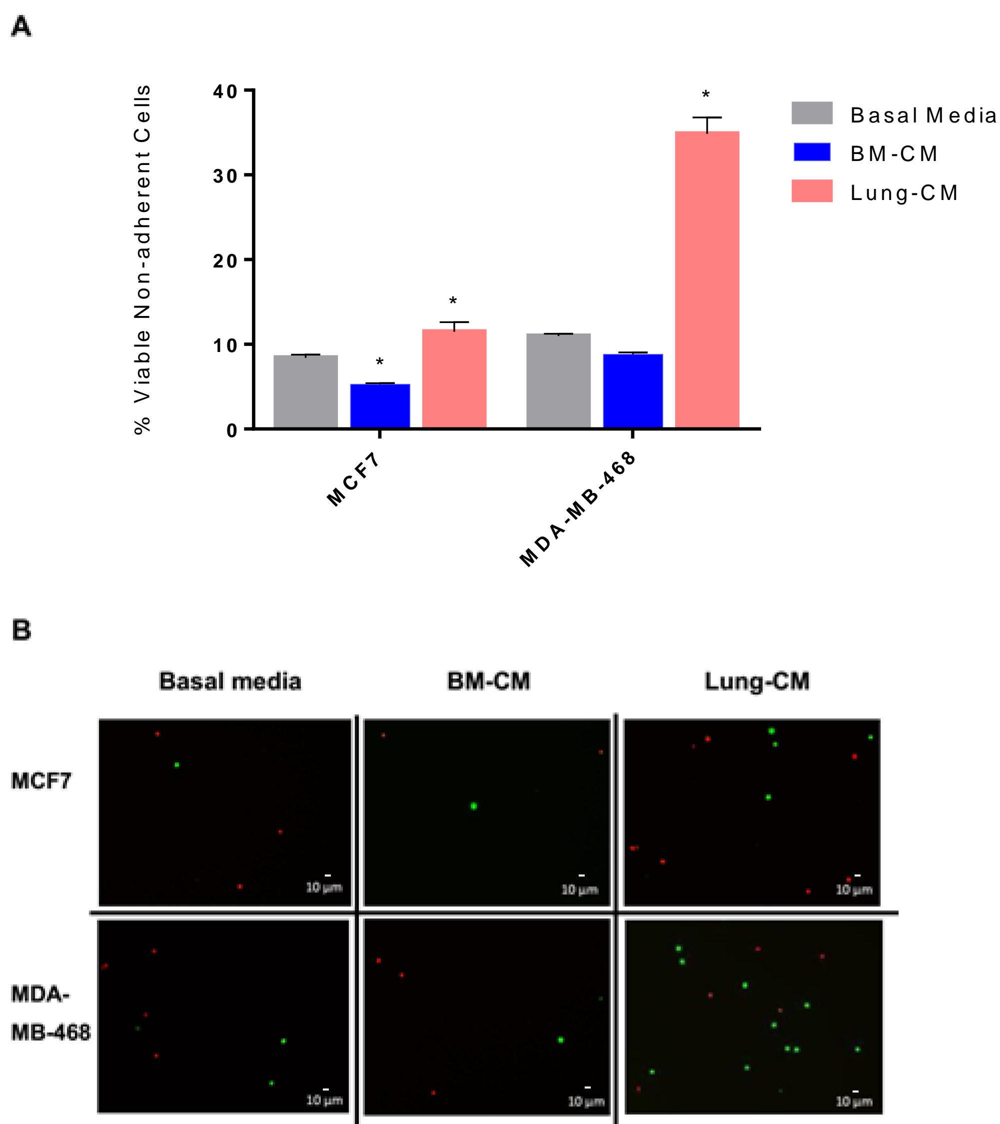


Figure 9. Exposure to lung-conditioned media supported the production of viable non-adherent breast cancer cells assessed by Live/Dead® assays. MCF7 and MDA-MB-468 human breast cancer cells were exposed to lung-CM, BM-CM, or basal media for 48 hours without media replacement. Media was collected, centrifuged at 1000g for 5 minutes, and non-adherent cells were manually counted using a hemocytometer to assess cell viability via the Live/Dead® cell viability assay as per the manufacturer's instructions. ***(A)*** Analysis of cell viability in non-adherent cells in MCF7 and MDA-MB-468 human breast cancer cells following 48-hour exposure to lung-CM, BM-CM, and basal media. ***(B)*** Representative images (10x magnification) used to carry out fluorometric analysis. Experiments were performed a minimum of three times and statistical analyses were performed using one-way ANOVA and Dunnett's post hoc test. Data is presented as mean ± SEM. All significant values (*) are relative to the negative control basal media treatment group ($p<0.05$, N=3).

5.4 Lung-CM and BM-CM reduced CD44 expression, ALDH activity, and ALDH1A1 gene expression in non-adherent human breast cancer cells.

Recent evidence suggests the emergence of cancer cell subpopulations with reduced adhesive characteristics originating from traditionally adherent breast, ovarian, and colon cancer cell lines express heightened tumourigenic capacity, both *in vitro* and *in vivo*[140,141]. To assess whether there were differences in ALDH and/or CD44 phenotypes between adherent and non-adherent breast cancer cell subpopulations, MDA-MB-468 cells were cultured in lung-CM, BM-CM, or basal media for 72 hours, and the non-adherent and adherent subpopulations were isolated and analyzed by flow cytometry and RT-qPCR. Contrary to our expectations, flow cytometry **(Figure 10A)** revealed that exposure to lung-CM significantly decreased ALDH activity and CD44 expression, alone or in combination, within the non-adherent subset (57.7 ± 2.5 % [CD44], 6.0 ± 1.1 % [ALDH], and 5.1 ± 0.9 % [$ALDH^{hi}CD44^{+}$]) compared to the non-adherent cells exposed to basal media (86.2 ± 2.9 % [CD44], 28.2 ± 0.9 % [ALDH], 23.3 ± 1.5 % [$ALDH^{hi}CD44^{+}$]) (β; $p<0.05$) Furthermore, this decrease in ALDH activity and CD44 expression, alone or in combination, was also significantly reduced relative to the adherent population exposed to basal control media (99.7 ± 0.1 % [CD44], 26.1 ± 1.6 % [ALDH], 27.6 ± 0.2 % [$ALDH^{hi}CD44^{+}$]) (*; $p<0.05$). Moreover, CD44 expression was significantly decreased in non-adherent cells (57.7 ± 2.5 %), compared to their adherent counterpart after exposure to the same lung-CM (99.1 ± 0.2 %) (α; $p<0.05$). Treatment with BM-CM significantly decreased CD44 expression in the non-adherent subpopulation (78.3 ± 2.5 %), relative to the adherent counterpart receiving the same BM-CM treatment (99.7 ± 0.1 %) ($p<0.05$). There was no effect on ALDH activity, or the $ALDH^{hi}CD44^{+}$population, after treatment with BM-CM **(Figure 10A)**.

To further investigate phenotypic differences between adherent and non-adherent subpopulations after exposure to lung-CM or BM-CM, RT-qPCR analysis was performed to assess ALDH1A1, ALDH1A3, and CD44 mRNA expression (**Figure 10B**). We

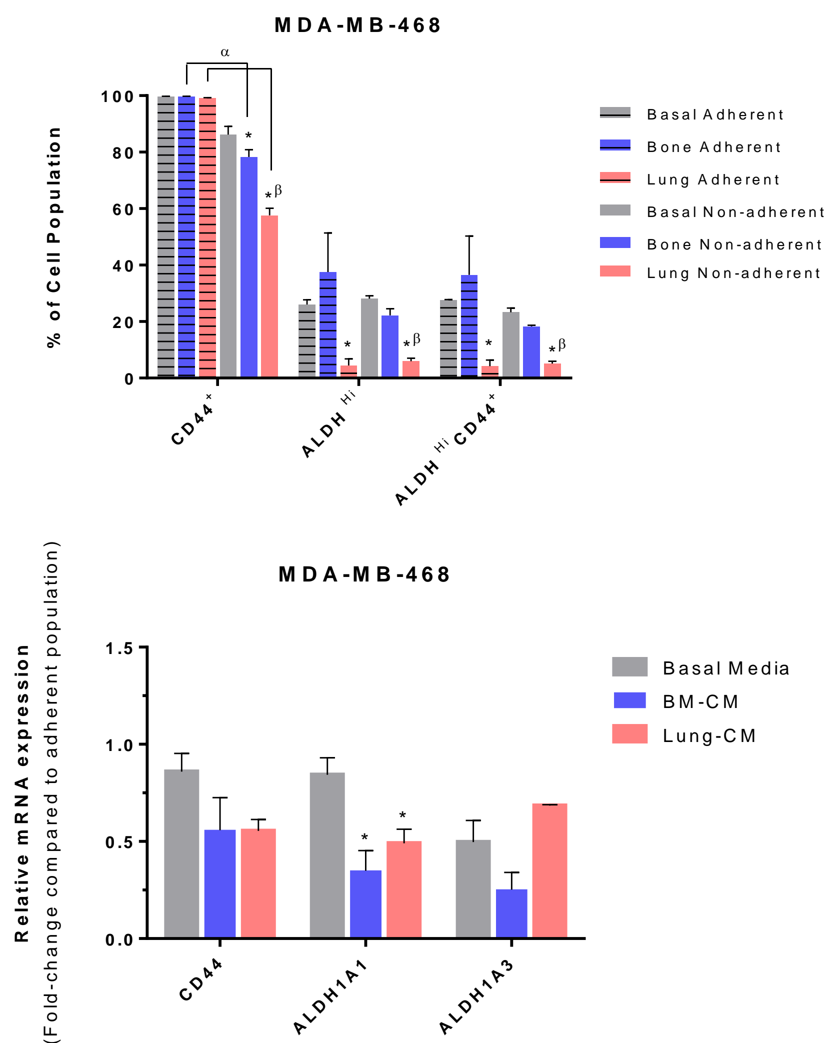


Figure 10. Exposure to lung-conditioned media reduced CD44 expression, ALDH activity, and ALDH1A1 gene expression in MDA-MB-468 human breast cancer cells. The MDA-MB-468 human breast cancer cells were exposed to lung-CM, BM-CM, or basal media (DMEM:F12) for **(A)** 72 hours or **(B)** 24 hours in culture (37°C, 5% CO_2) without media replacement. Non-adherent and adherent subpopulations were harvested and analyzed by **(A)** flow cytometry or **(B)** RT-qPCR. Data are presented as mean ± SEM. Experiments were performed a minimum of 3 times and statistical analyses were performed using a 2-way ANOVA with Dunnett's post hoc test. Significant values are relative to the adherent (*), non-adherent (β) basal media treatment group, or the adherent subpopulation of the respective treatment (α) ($p<0.05$, N=3).

observed that ALDH1A1 mRNA expression was significantly decreased after 24-hour exposure to both BM-CM or lung-CM (0.4 ± 0.1-fold and 0.5 ± 0.1-fold, respectively), relative to basal media (0.8 ± 0.1-fold) ($p<0.05$). There was no significant difference in ALDH1A3 and CD44 mRNA expression between the adherent and non-adherent subpopulations exposed to the same organ-CM (**Figure 10B**).

5.5 Lung-conditioned media impaired mammosphere formation by non-adherent human breast cancer cells.

To assess whether stem-like function was affected by lung-CM or BM-CM, both adherent and non-adherent cell subpopulations were subjected to a limiting-dilution mammosphere formation assay. This assay enables the cells either poised for mitotic division or already dividing to form non-adherent clusters, using a variety of activated stem cell-associated signaling pathways to do so. MDA-MB-468 and MCF7 cell lines were exposed to lung-CM, BM-CM, or basal media for 72 hours, and the non-adherent and adherent subpopulations were plated in limiting dilutions using the mammosphere assay and cultured for 21 days. Interestingly, non-adherent MCF-7 and MDA-MB-468 cells were incapable of forming mammospheres following exposure to lung-CM (0.0 ± 0.0 freq. and 0.0 ± 0.0 freq., respectively), relative to the non-adherent subpopulation receiving basal treatment (0.008 ± 0.002 freq. and 0.001 ± 0.000 freq., respectively) ($p<0.05$) **(Figure 11A and B)**. Conversely, exposure to basal media or BM-CM supported mammosphere formation by both cell lines, irrespective of cell subpopulation. Regarding the adherent subpopulations, treatment with organ-CM did not significantly affect mammosphere frequency in either MCF7 or MDA-MB-468 cell lines. Further, treatment with BM-CM significantly decreased mammosphere frequency among only the non-adherent MCF7 cell subpopulation (0.001 ± 0.000 freq.), relative to the non-adherent subpopulation receiving basal treatment (0.008 ± 0.002 freq.) ($p<0.05$) **(Figure 11A)**.

5.6 Exposure to lung-CM increased mRNA expression related to migration and decreased mRNA expression of CSC markers.

Next, a discovery-based approach was employed to uncover genes affected by lung-CM treatment relevant to human CSC function. Both non-adherent and adherent MDA-MB-468 cell subpopulations were exposed to basal or lung-CM for 24 hours, followed by RT-

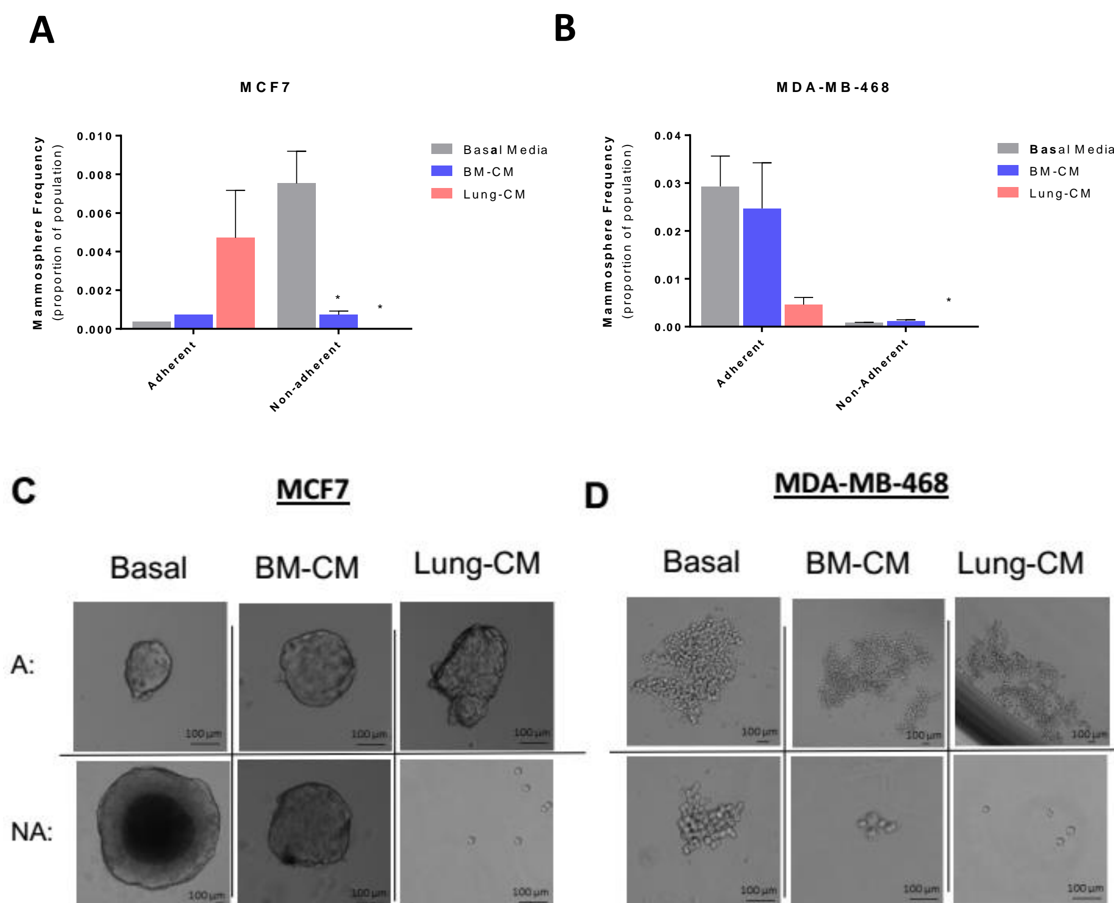


Figure 11. Exposure to lung-conditioned media impaired mammosphere formation by non-adherent human breast cancer cells. ***(A)*** MCF7 and ***(B)*** MDA-MB-468 human breast cancer cells were exposed to lung-CM, BM-CM, and basal media over 72 hours in culture (37°C, 5% CO_2) without media replacement. Adherent and non-adherent cell subpopulations were harvested and viable cells were subjected to a mammosphere formation assay over 21 days in culture. Cells were seeded in serial limiting dilution fashion onto 96-well ultra-low attachment plates. Mammosphere formation was assessed based on the presence or absence of clusters containing ≥ 5 cells per well, followed by mammosphere frequency calculated using L-Calc software. Representative images of mammospheres formed, or, absence of mammosphere formation by adherent and non-adherent ***(C)*** MCF7 and ***(D)*** MDA-MB-468 cell subpopulations. All images were taken at 10X magnification using an inverted Olympus IX70 microscope. Experiments were performed a minimum of 3 times and statistical analyses were performed using one-way ANOVA and Dunnett's post hoc test. Data is presented as mean ± SEM. All significant values (*) are relative to the respective basal media treatment in the adherent or nonadherent subpopulation ($p<0.05$, N=3).

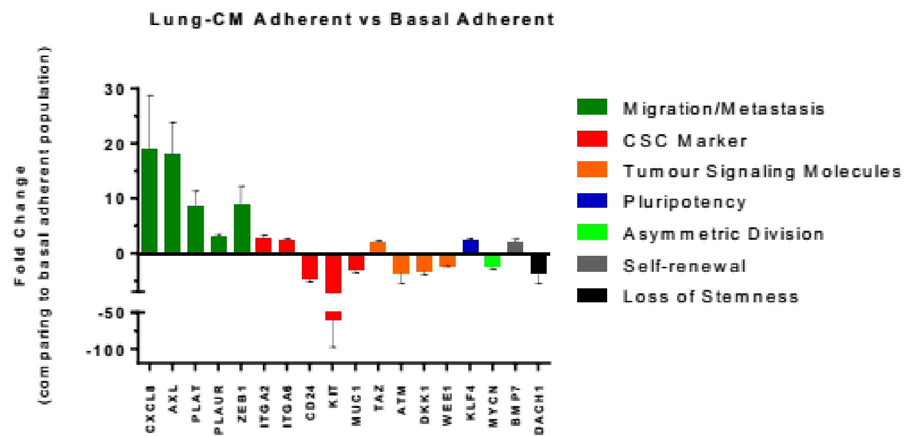
qPCR analyses. We observed the expression of five genes of interest were affected by treatment with lung-CM, relative to basal treatment, including C-X-C Motif Chemokine Ligand 8 (CXCL8), Cluster of Differentiation 24 (CD24), Mucin 1 (MUC1), Ataxia Telangiectasia Mutated (ATM), and WEE1 G_2 Checkpoint Kinase (WEE1) **(Figure 12, Tables 3,4)**. Exposure to lung-CM in both adherent and non-adherent subpopulations significantly increased gene expression of the metastasis/migration associated gene, CXCL8 (19.0 ± 8.8-fold [adherent] and 3.6 ± 1.0-fold [non-adherent]), relative to the same subpopulations exposed to basal media ($p<0.05$) **(Figure 12)**[142]. Average Ct values between adherent and non-adherent MDA-MB-468 cell subpopulations following 24-hour exposure to lung-CM or basal media are provided **(Appendix 4)**.

Conversely, exposure to lung-CM consistently decreased gene expression of two CSC markers, CD24 and MUC1, in both adherent and non-adherent subpopulations (-4.6 ± 0.7-fold [adherent CD24] and -3.2 ± 0.3-fold [non-adherent CD24]; -2.9 ± 0.6-fold [adherent MUC1] and -6.1 ± 0.9-fold [non-adherent MUC1]), as well as two genes related to tumour signaling molecules, ATM and WEE1 (-3.5 ± 1.9 fold [adherent ATM] and -4.9 ± 0.3-fold [non- adherent ATM]; -2.2 ± 0.2-fold [adherent WEE1] and -4.8 ± 0.7-fold [non-adherent WEE1]), relative to the same subpopulations exposed to basal media ($p<0.05$) **(Figure 12)**[143-146].

5.7 Lung-CM contained proteins related to migration, adhesion, and stemness

Finally, mass spectrometry analysis was performed to assess the protein content within lung-CM (relative to basal media) in order to gain insight into which effectors may be contributing to the observed phenotype and behavior of breast cancer cells. Overall, 1,721 unique proteins were found in lung-CM. Using the PANTHER® classification system, lung-CM proteins were organized based on extracellular (13.4%) or intracellular (86.6%) localization **(Figure 13A)**. Among proteins related to the extracellular space, further classification was performed to divide proteins based on association with the extracellular region (7.4%), membrane-bound proteins (3.5%), cellular junctions (1.3%), and

A



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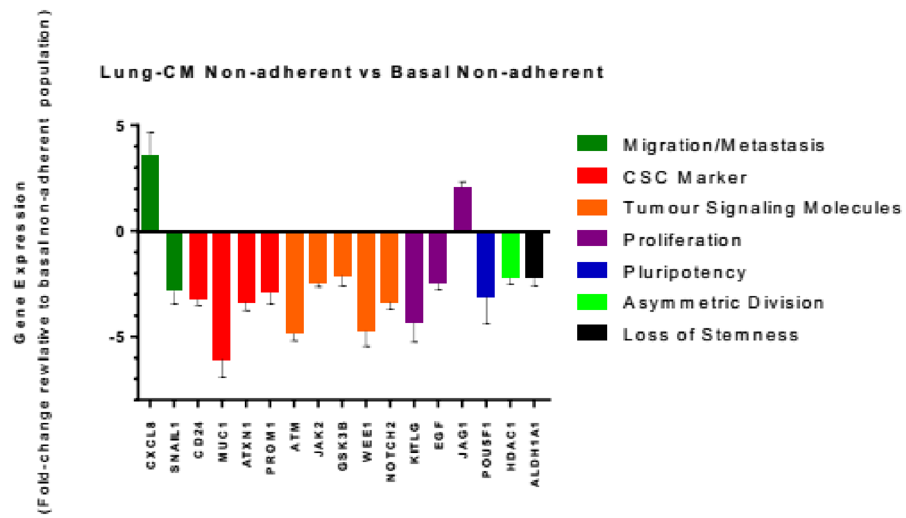

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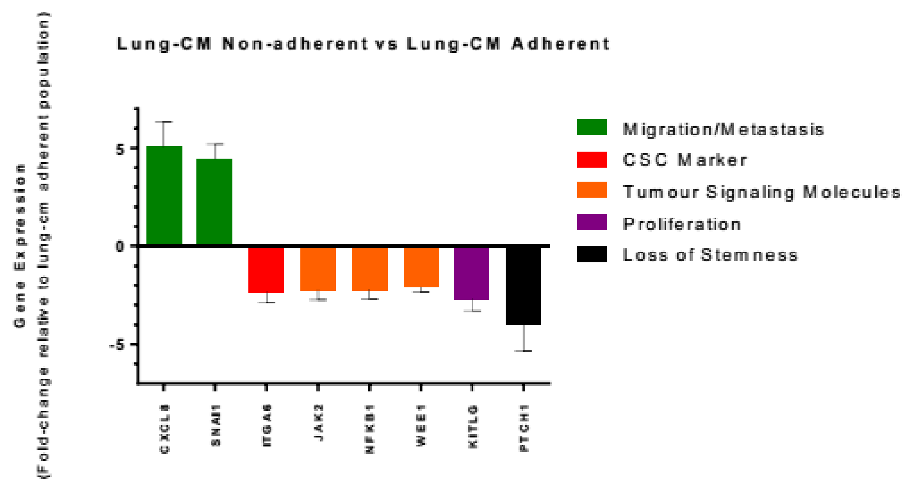

Figure 12. Exposure to lung-conditioned increased mRNA expression related to migration and decreased mRNA expression of CSC markers. MDA-MB-468 human breast cancer cells were exposed to basal media or lung-CM for 24 hours. Adherent and non-adherent subpopulations were harvested and RNA was extracted for use with Human Cancer Stem Cell RT_2 Profiler PCR® arrays. ***(A)*** Adherent cells exposed to lung-CM versus adherent cells exposed to basal media. ***(B)*** Non-adherent cells exposed to lung-CM versus non-adherent cells exposed to basal media. ***(C)*** Non-adherent versus adherent cells exposed to lung-CM. The listed genes exhibited a statistically significant and at least 2-fold change following normalization to GAPDH ($p<0.05$, N=3). Analyses were performed using Qiagen's Data Analysis Center® software.

Table 3. CXCL8, CD24, MUC1, ATM and WEE1 emerge as key genes affected by lung-CM treatment. Gene expression analysis was acquired from RT-qPCR of Human Cancer Stem Cell® gene arrays. Findings represent differences in gene expression (> 2-fold) induced by treatment with lung-CM in either adherent or non-adherent subpopulations, relative to basal treatment. Arrows reflect direction of gene expression fold-change significance.

	Migration/Metastasis						CSC Markers							Tumour Signaling Molecules							Proliferation			Pluripotency		Asymmetric Division		Self-renewal	Loss of Stemness		
	CXCL8	AXL	PLAT	PLAUR	ZEB1	SNAIL1	ITGA2	ITGA6	CD24	KIT	MUC1	ATXN1	PROM1	TAZ	ATM	JAK2	DKK1	WEE1	GSK3B	NOTCH2	KITLG	EGF	JAG1	KLF4	POU5F1	MYCN	HDAC1	BMP7	ALDH1A1	DACH1	PTCH1
Lung A vs Basal A	↑	↑	↑	↑	↑		↑	↑	↓	↓	↓			↑	↓		↓	↓						↑		↓		↑		↓	
Lung N vs Basal N	↑					↓			↓		↓	↓	↓		↓	↓		↓	↓	↓	↓	↓	↑		↓		↓		↓		

A = Adherent *NA = Non-adherent*

Table 4. Function of CXCL8, CD24, MUC1, ATM, and WEE1. Genes of interest were chosen for further characterization based on response to lung-CM treatment, irrespective of adherent or non-adherent cell subpopulation.

Classification	Gene of Interest	Function
Migration/Metastasis	**C-X-C Motif Chemokine Ligand 8 (CXCL8)**	Encodes interleukin 8 (IL8) protein expression. As a proinflammatory chemokine, IL8 can induce chemotaxis of immune-related cells to a target site[147,148].
CSC Markers	**Cluster of Differentiation 24 (CD24)**	CD24 encodes a glycoprotein that is anchored via a glycosyl phosphatidylinositol (GPI) link to the cell surface. CD24 protein functions as a cell adhesion molecule, and its loss is associated with a stem-like cancer cell phenotype[62].
	Mucin 1 (MUC1)	MUC1 encodes for cell surface glycoprotein with an active extracellular domain due to O-linked glycosylation. The cytoplasmic tail of MUC1 functions as an oncoprotein through interactions with tumour promoting pathway and is often overexpressed in certain cancers. The protein can also localize to the nucleus for interaction with WNT signaling[149].
Tumour Signaling Molecules: Cell Cycle Control	**Ataxia Telangiectasia Mutated (ATM)**	ATM encodes for serine/threonine kinase, which belongs to the PI3/PI4-kinase family. Together with ATR, ATM is considered a master regulator of cell cycle checkpoints. ATM has a central role in repair of double-stranded DNA breaks[150]. Further, elevated ATM expression has been associated with favorable patient prognosis[151].
	WEE1	WEE1 encodes for a nuclear kinase that is part of the Ser/Thr protein kinase family. Through the inhibition of CDK1, WEE1 is a negative regulator of entry into mitosis (G2 – M)[146]. In cancer, impaired WEE1 has led to the loss of DNA-damage induced apoptosis and aberrant mitosis[146].

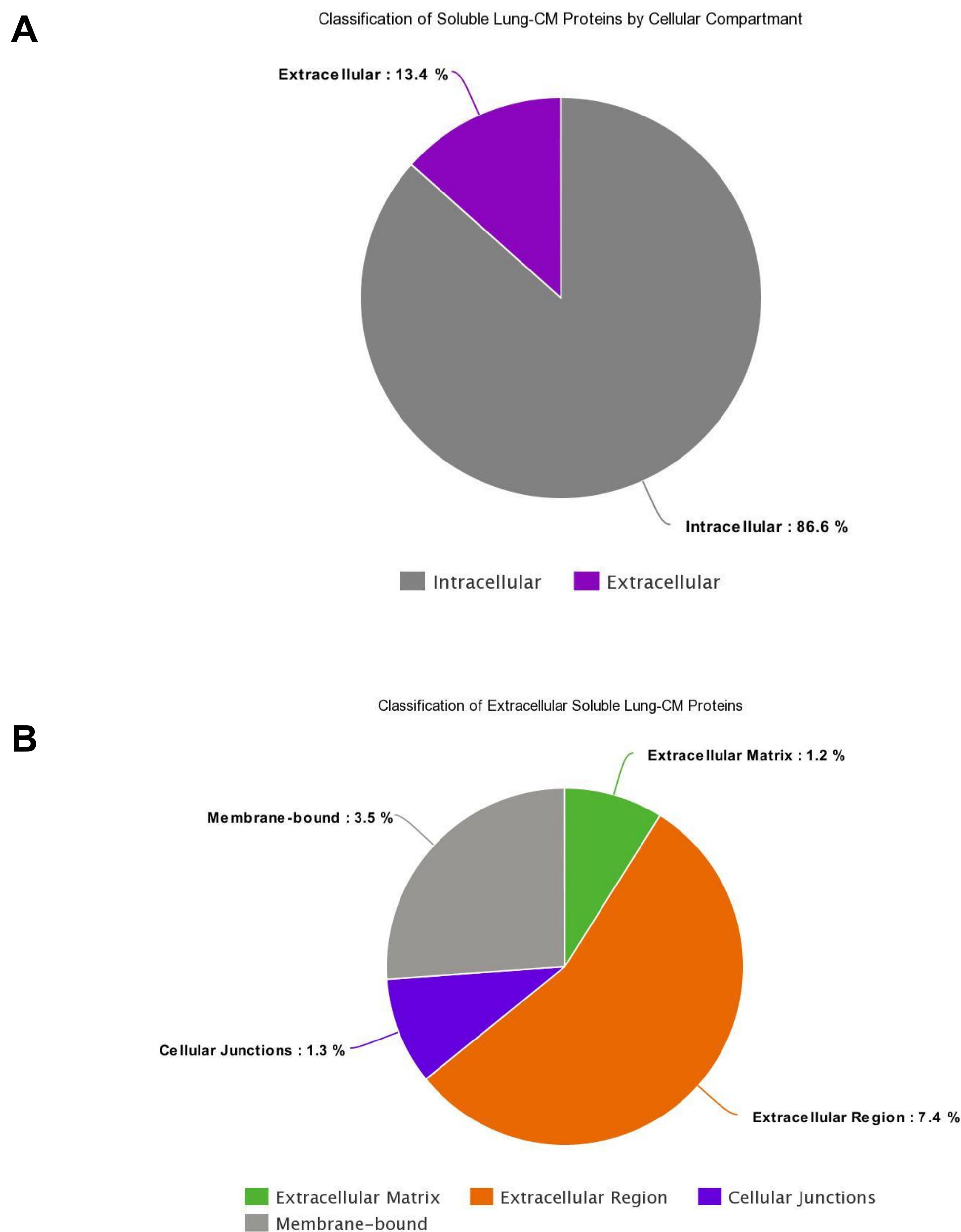

Figure 13. Classification of intra- and extracellular proteins found in lung-CM. Mass spectrometry analysis (N=3) revealed 1,721 proteins that are unique to the lung-CM relative to basal media. ***(A)*** Among these proteins, 13.4% are classified as extracellular and 86.6% are classified as intracellular compartmentalized proteins. ***(B)*** Proteins belonging to the extracellular compartment are of particular interest as they may be secreted from the lung as soluble proteins and impose an effect on human breast cancer cells. Among the proteins belonging to the extracellular compartment, further classification associated these proteins with the extracellular region (7.4%), membrane-bound (3.5%), cellular junctions (1.3%), and extracellular matrix (1.2%). Analyses were performed using PANTHER® Classification System software.

extracellular matrix (1.2%) **(Figure 13B)**. From the soluble extracellular compartment, osteopontin (OPN), periostin (POSTN), a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10), and β-catenin were of particular interest as they have strong associations with cell migration and adhesion pathways[136,152-154]. Upon analysis of the intracellular compartment, several proteins associated with angiogenic VEGF, stemness (WNT/NOTCH), and migratory (Ras/Rho) pathways were observed to be present[155-157]. When assessing proteins related to CD44, ADAM10 was present in the lung-CM, and absent in the basal media **(Table 5, Figure 14A)**[152]. Among intracellular proteins, 6 proteins were found to be related ALDH/RA signaling pathway (**Table 5**) including retinol binding protein 1 (RBP1), alcohol dehydrogenase 1 (ADH1), aldehyde dehydrogenase 1A1 and 1A7 (ALDH1A1 and ALDH1A7), cytosolic retinoic acid binding protein 2 (CRABP2) and fatty acid binding protein 5 (FABP5) **(Figure 14B)**[158,159].

Table 5. Function of proteins within lung-CM related to ALDH/RA signaling pathway and CD44 cleavage.

Potential Interaction with:	Proteins of Interest	Function
ALDH/RA Pathway	**Retinol Binding Protein 1 (RBP1, CRBP1)**	Involved in retinol transport once the vitamin A alcohol has entered the cell (functions intracellularly)[160].
	Retinol Binding Protein 4 (RBP4)	Major role in retrieving retinol from liver storage, and transporting to peripheral tissue through systemic circulation (functions within blood plasma)[161].
	Alcohol Dehydrogenase 1 (ADH1)	Enzyme involved in oxidation of retinol to retinal, an aldehyde. Required for clearance of excess retinol, which could result in retinol toxicity[162].
	Aldehyde Dehydrogenase 1A1 (ALDH1A1)	Enzyme responsible for the further oxidation of retinal to retinoic acid (RA). Enzyme activity has significant implications regarding tumour development and stem cell maintenance[92].
	Aldehyde Dehydrogenase 1A7 (ALDH1A7)	ALDH1 family isoenzyme involved in RA synthesis. Has been associated with olfactory and respiratory tissues[163].
	Cytosolic Retinoic Acid Binding Protein 2 (CRABP2)	Binds to, and translocates RA into the nucleus for activation of RAR/RXR transcription machinery[159]. Has anticarcinogenic effects associated with cell apoptosis, differentiation, and growth arrest[159].
	Fatty Acid Binding Protein 5 (FABP5)	Can also bind to RA and translocate into the nucleus for activation of PPAR pathway[159]. Has procarcinogenic effects associated with cell survival and proliferation[159].
CD44 Cell Surface Expression	**Disintegrin and Metalloproteinase Domain-containing Protein 10 (ADAM10)**	ADAM10 is a proteolytic enzyme that is capable of preferentially cleaving CD44[152].

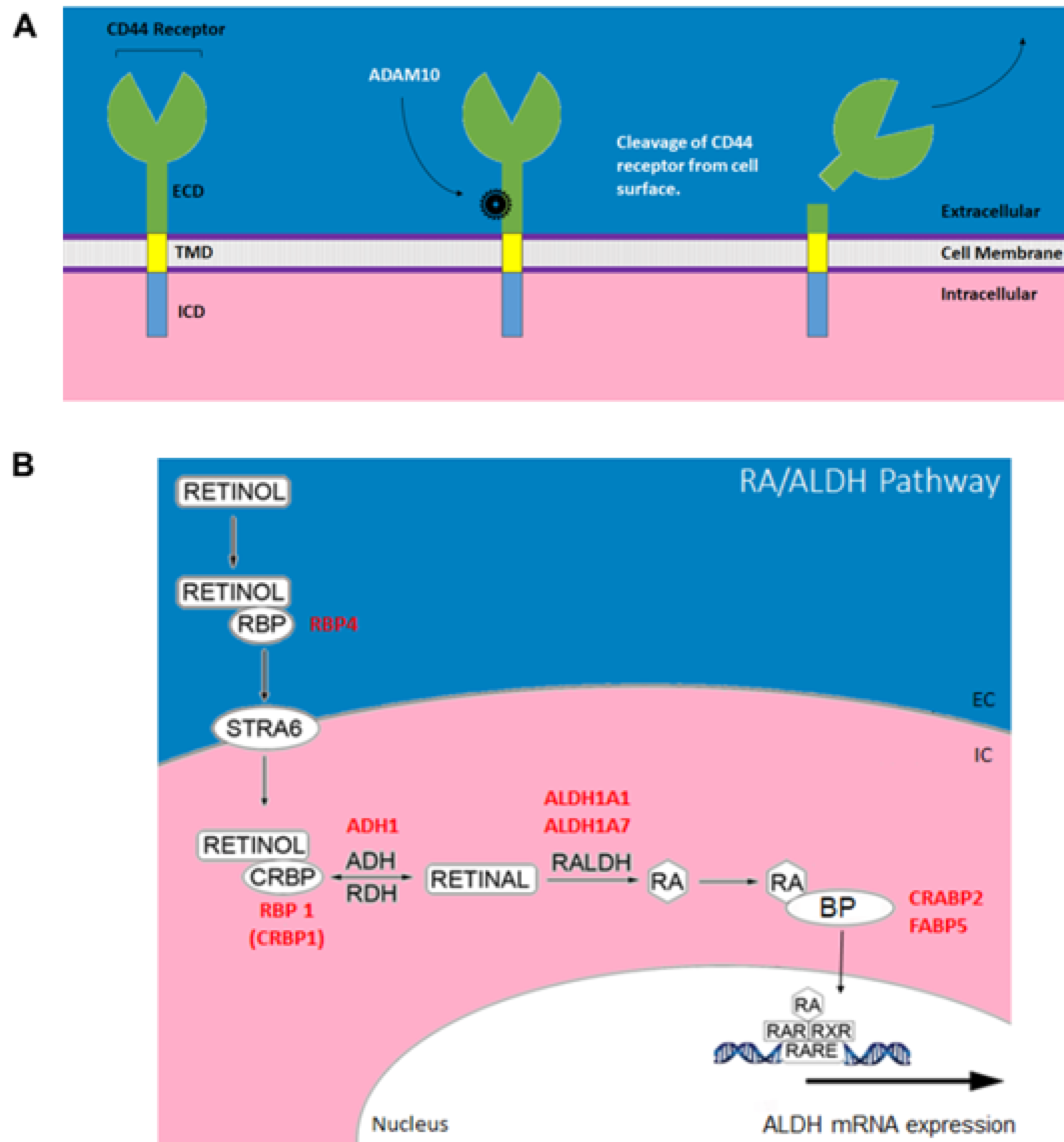

Figure 14. Mass spectrometry analysis of lung-CM identifies key proteins related to the ALDH/RA and CD44 pathways. Mass spectrometry analysis was carried out to investigate proteins contained with the lung-conditioned media and their potential relationship with the phenotypic and functional behavior of breast cancer cells. ***(A)*** The CD44-related disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) cleaves CD44 at the extracellular ectodomain. ***(B)*** Several proteins relevant to the ALDH/RA pathway (*labelled in red*) were found to be present within the lung-CM. Those proteins included: retinol binding protein 1 and 4 (RBP 1 + 4), alcohol dehydrogenase 1 (ADH 1), aldehyde dehydrogenase 1A1 and 1A7 (ALDH1A1 and ALDH1A7), cytosolic retinoic acid binding protein 2 (CRABP2), and fatty acid binding protein 5 (FABP5). There may be a potential mechanism for these soluble proteins to be internalized by cancer cells and utilized as exogenous machinery.

6 DISCUSSION

Among Canadians, the mortality rate associated with breast cancer has declined by 44% since its peak in 1986[1]. Such a drastic decrease can be attributed to improved early-stage tumour detection, by government-funded mammography screenings, made readily available to Canadians in 1992[164]. Although mortality rates have decreased, the incidence rates have not decreased. Since 1988, the age-standardized incidence rates have remained high among women and seen little change in this trend as of late[1]. This stagnant incidence rate has maintained breast cancer's position as the third most common cancer among Canadians, making up 13% of all cancers and 25% of cancers in women[1]. What is more dismal, after receiving adjuvant chemotherapy for the primary tumour, patients who developed metastatic disease within 10 years of treatment were met with a near unanimously fatal outcome, a statistic that has not changed in the last 30 years[165].

Breast cancer is currently a treatable disease, but a lack of effective therapies in the metastatic setting render breast cancer largely incurable once the cancer has disseminated beyond the breast. This is due in part to tumour heterogeneity which has presented a major obstacle for the research and clinical communities, making cancer biology complex and a generic treatment regimen difficult to achieve[166]. For patients with endocrine-responsive tumours, hormonal therapy has demonstrated modest improvements in overall patient survival[167]. When endocrine-receptors are absent, hormonal therapy becomes ineffective, and systemic chemotherapy is necessary to target a wide range of rapidly proliferating cell types[167]. Often, tumourigenic cells acquire resistance to primary treatment strategies and become resistant to therapy, allowing the metastatic processes to persist[166,167]. The issue concerning current therapies is that they do not eradicate all cancer cells within a patient, neglecting cells that may exhibit a decreased rate of proliferation. The emerging CSC model suggests that a rare population of slow-proliferating, tumourigenic cells are capable of repopulating a heterogeneous tumour and contribute to disease recurrence and evasion of conventional therapies[168]. Several studies have identified subpopulations of potential stem-like cancer cells using traditional stem cell markers, such as ALDH, alone or in combination with different intrinsic and extrinsic factors. In breast cancer, stem-like cells have been phenotypically identified as having high enzymatic ALDH activity and CD44

cell surface expression, however targeted treatments have been unsuccessful due the transient and dynamic phenotype of stem-like cancer cells[61,62,169]. Understanding the role of stem-like and/or metastasis-initiating cancer cells during tumour progression and/or metastatic development is critical for establishing effective treatment strategies to target these rare and aggressive populations.

The significance of CSCs in breast cancer metastasis and secondary tumour formation is important to consider in the context of preferential patterns of organ tropism. Massagué and colleagues contributed significantly to knowledge about organ-specific breast cancer metastasis when they observed that intrinsic genes within breast cancer cells can mediate metastasis to the lung, bone marrow, and brain[47-49]. Although profound, these findings did not address the role of the organ microenvironment, nor the role of CSCs in organ tropism of breast cancer metastasis. Efforts in our laboratory aimed to address these voids by demonstrating the potential for soluble proteins generated by the lung and BM microenvironments to promote migratory and proliferative behaviour in stem-like $ALDH^{hi}CD44^{+}$ breast cancer cells *in vitro,* and increased incidence of spontaneous metastasis of these stem-like cells to lung *in vivo* [52,70,136]. These initial investigations suggested a role for the lung and bone marrow microenvironments in supporting growth, migration and metastasis of $ALDH^{hi}CD44^{+}$ breast cancer cells. However, it was unclear whether the lung and/or bone microenvironments could additionally promote the acquisition of a stem-like phenotype and function within breast cancer cell populations, and this thesis focused on this question. We utilized an *ex vivo* model of lung- and bone-conditioned media and hypothesized that exposure to these lung or bone "microenvironments" would increase the proportion of breast cancer cells expressing the stem-like $ALDH^{hi}CD44^{+}$ phenotype and enhance stem-like cell behaviour.

6.1 Summary of Key Experimental Findings

The key experimental findings of this thesis are summarized in **Table 6** and listed below.

1. Exposure to lung-CM decreased the frequency of MDA-MB-468 and SUM159 breast cancer cells expressing the stem-like $ALDH^{hi}CD44^{+}$ phenotype.

2. Exposure to lung-CM increased the frequency of a viable, non-adherent subpopulation in MDA-MB-468 and MCF7 human breast cancer cells.
3. Exposure to lung-CM decreased CD44 expression in non-adherent MDA-MB-468 human breast cancer cells.
4. Exposure to lung-CM impaired mammosphere formation by non-adherent MDA-MB-468 and MCF7 human breast cancer cells.
5. Lung-CM contained proteins related to migration, adhesion, and stemness.
6. Exposure to lung-CM resulted in increased mRNA expression related to migration and decreased mRNA expression of "cancer stem cell (CSC)" markers.

Table 6. Summary of results assessing adherent and non-adherent cell subpopulations treated with BM-CM or lung-CM.

Type of Analysis	Experiments	Lung-CM		BM-CM	
		Adherent	Non-Adherent	Adherent	Non-Adherent
Phenotypic	Flow Cytometry: **ALDH activity and/or CD44 expression**	↓ ALDH activity ↓ CD44 expression ↓ ALDH^{Hi}CD44^{+}	↓ ALDH activity ↓ CD44 expression ↓ ALDH^{Hi}CD44^{+}	↓ ALDH activity (SUM159 Only) ↓ ALDH^{Hi}CD44^{+} (SUM159 Only)	↓ CD44 expression
	Gene Expression: **ALDH1A1 ALDH1A3 CD44**	↑ CD44 ↑ ALDH1A3	↓ ALDH1A1	No Effect	↓ ALDH1A1
	Human Cancer Stem Cell Gene Array	↑ CXCL8 ↓ CD24, MUC1, ATM, Wee1		Unknown	
	Mass Spectrometry	↑ ALDH/RA and CD44 related proteins			
Functional	**Trypan Exclusion**	Unknown	↑ Viability in non-adherent subpopulation	Unknown	No Effect
	LIVE/DEAD® Assay				↓ Viability in non-adherent subpopulation (MCF7 Only)
	Mammosphere Formation	No Effect	↓ Mammosphere Formation Efficiency	No Effect	↓ Mammosphere frequency in non-adherent subpopulation (MCF7 Only)

6.2 Lung-CM inhibits stem-like phenotype and behaviour in adherent human breast cancer cells

Contrary to expectations, we observed that exposure of MDA-MB-468 and SUM159 breast cancer cells to lung-CM decreased the proportion of cells expressing an $ALDH^{Hi}CD44^{+}$ phenotype. Of the parameters that comprise our chosen stem-like phenotype, ALDH activity was significantly decreased in response to lung-CM treatment in both cell lines, while CD44 expression was only decreased in the SUM159 cell line. Treatment with BM-CM also decreased the $ALDH^{Hi}CD44^{+}$ phenotype, however, this decrease was modest in comparison to lung-CM. This discovery was unanticipated given that lung and BM tissue are highly targeted sites of metastasis, together with accumulating evidence suggesting that $ALDH^{hi}CD44^{+}$ cells play a key role in driving breast cancer progression[52,72,170,171].

To further investigate the influence of lung-CM, we performed mRNA analysis of CD44, and two major ALDH isoenzymes, ALDH1A1 and ALDH1A3[72]. Due to the inherent delay associated with gene transcription prior to achieving functional protein, RT-qPCR analysis was performed after 24-hour exposure to organ-CM. Interestingly, mRNA transcription analyses demonstrated a significant upregulation of CD44 mRNA expression after 24-hour treatment with lung-CM, in both MDA-MB-468 and SUM159 cell lines. This increase in CD44 mRNA expression was not consistent with our flow cytometry results measuring decreased CD44 cell surface expression. This is not surprising as CD44 cleavage often occurs at the cell surface during locomotion and migration, suggesting a potential positive feedback system driving CD44 mRNA expression following treatment with lung-CM.[172] Further, ALDH1A3 mRNA expression was also increased following treatment with lung-CM in the SUM159 cell line only. As ALDH activity was largely decreased by lung-CM treatment following flow cytometry analysis, this warranted further investigation to measure ALDH protein level. However, western blot analysis of ALDH1A3 protein did not indicate a significant difference in protein levels between basal media and lung-CM treatments, suggesting potential post-transcriptional or post-translational modifications that may interrupt the production of functional ALDH1A3 enzyme (**Appendix 2**). In the future, further analysis of terminal protein localization and the extent of ubiquitination on the ALDH1A3 protein may help better understand the opposing data (e.g. whether proteins

are destined for lysosomal degradation)[173]. Another factor that may contribute to a loss of ALDH activity is stem cell differentiation or maturation. Although high ALDH activity is associated with the detection of a stem-like phenotype, ALDH can induce differentiation through the production of RA and subsequent downstream signaling pathways. Therefore, treatment with lung-CM could induce stem-like cells to differentiate, resulting in decreased ALDH activity. In a recent study, Muramoto *et al* found that inhibition of the ALDH1 enzyme impeded the differentiation of murine hematopoietic stem cells (HSCs), resulting in a 9-fold expansion of radioprotective cells[174]. These HSCs both maintained their stem-like state and maintained radioprotection through inhibition of ALDH activity. Another study performed by Hessman *et al* demonstrated that decreased ALDH1 protein expression in primary and metastatic colorectal cancer samples correlated with advanced and metastatic cancers, while high ALDH1 expression was associated with non-metastatic tumours[175]. Together with findings in the literature, our recent data suggests a loss of stem-like phenotype could result in increased metastatic capacity.

In addition to phenotypic analysis by flow cytometry, the enrichment of stem-like cells through spheroid formation has become a useful technique to measure stem-like behavior of cancer cell populations. Introduced in 1992 by Reynolds and Weiss to assess proliferation, self-renewal, and multipotency of neural precursor cells, the neurosphere assay has since been repurposed for use in a variety of models, particularly CSC biology[176]. Adapted by Dontu and colleagues, the mammosphere assay utilizes non-adherent and non-differentiating culture conditions to evaluate individual breast cancer cells on their ability to self-renew/proliferate in suspension, forming multicellular mammospheres *in vitro*[177]. In the current study, we set out to investigate the mammosphere-forming capacity of adherent MCF7 and MDA-MB-468 human breast cancer cells after exposure to lung- or BM-CM. We did not observe a significant effect on mammosphere formation frequency in either adherent MCF7 or MDA-MB-468 cells following exposure to lung-CM or BM-CM. Both cell lines were capable of forming mammospheres irrespective or organ-CM received, suggesting that factors within the lung-CM or BM-CM did not interfere with stem-like functions such as mammosphere formation among the adherent cell subpopulation.

Taken together, exposure to lung-CM decreased the proportion of cells expressing the stem-like $ALDH^{hi}CD44^{+}$ phenotype, however did not affect functional stem-like behaviour of human breast cancer cells. This suggests that the lung microenvironment does not promote stem-like phenotype in adherent breast cancer cells, and instead, may inhibit it.

6.3 Lung-CM induced a viable, non-adherent breast cancer cell subpopulation with decreased stem-like phenotype and function

During the course of our studies, we qualitatively observed that exposure to lung-CM induced a non-adherent breast cancer cell subpopulation. Both the MDA-MB-468 and SUM159 cell lines have been characterized as adherent in culture, leaving the possibility of a thriving non-adherent subpopulation among these cells unlikely[178,179]. Interestingly, upon trypan exclusion analysis, a viable, non-adherent MDA-MB-468 subpopulation was confirmed after exposure to lung-CM. This finding was not nearly as pronounced after either BM-CM or basal control treatments, and completely absent in the more metastatic SUM159 cell line, irrespective of organ-CM. Since only the MDA-MB-468 cell line produced a non-adherent subpopulation, we performed a screening of two additional human breast cancer cell lines, MDA-MB-231 and MCF7, to assess if other traditionally adherent cell lines would generate a similar non-adherent subpopulation following exposure to lung-CM. Both additional cell lines produced a non-adherent subpopulation, however of the four cell lines tested, the least aggressive MCF7 and MDA-MB-468 cell lines most efficiently produced viable, non-adherent cells. We next performed a LIVE/DEAD® viability assay on the two candidate cell lines, MDA-MB-468 and MCF7 to confirm our trypan exclusion results. Upon fluorometric analysis, both MDA-MB-468 and MCF7 cells demonstrated a significant increase in viable, non-adherent cells after lung-CM treatment. The observation that the induction of viable non-adherent cells was most pronounced after treatment with lung-CM suggests that components of the lung microenvironment may interact with adherent breast cancer cells in a manner that induces cell detachment.

To accurately compare differences between the stem-like phenotype of adherent and non-adherent breast cancer cell subpopulations, we performed additional flow cytometry analyses to assess ALDH activity and CD44 cell surface expression after treatment with

organ-CM. Again unexpectedly, we observed that exposure of the non-adherent MDA-MB-468 cell subpopulation to lung-CM further decreased CD44 expression relative to the adherent subpopulation, while ALDH activity, and the proportion of cells expressing the stem-like $ALDH^{hi}CD44^{+}$ phenotype, remained relatively low and unchanged between both adherent and non-adherent subpopulations. When comparing BM-CM treatment between adherent and non-adherent MDA-MB-468 cells, we also observed a decrease in CD44 expression within the non-adherent subpopulation, yet this decrease was limited in comparison to the lung-CM response. Consistent with our analyses of protein expression, gene expression analysis revealed that ALDH1A1 gene expression in the non-adherent MDA-MB-468 subpopulation was significantly downregulated after exposure to both lung- and BM-CM. Thus, while the proportion of non-adherent and adherent cells expressing the stem-like $ALDH^{Hi}CD44^{+}$ phenotype was largely unaffected, CD44 expression was consistently decreased in the non-adherent cell subpopulation relative to the adherent counterpart, especially after treatment with lung-CM. In a recent study by Ngan *et al*, loss of E-cadherin and CD44 expression were significantly correlated with poor survival in colorectal cancer patients[180]. In addition, a study conducted by Sugino *et al* demonstrates that a loss in CD44 expression resulted in tumour cell detachment from the basal membrane, and subsequent invasion by cancer cells[181].

Next, we wanted to address potential differences in stem-like function between non-adherent and adherent breast cancer cell subpopulations after exposure to organ-CM. Research published by House *et al* demonstrated a non-adherent subpopulation in both ACI-23 and OVCAR-5 human ovarian cancer cell lines that more readily produce spheroids *in vitro*, and larger tumours *in vivo*[140]. Furthermore, a Morata-Tarifa *et al* found trypsin sensitive (non-adherent) human breast and colon cancer cells demonstrated increased sphere-forming capacity *in vitro*, when compared to their trypsin-resistant (highly adherent) counterparts[141]. We performed a similar experiment whereby we exposed adherent and non-adherent subpopulations of both MDA-MB-468 and MCF7 breast cancer cells to organ-CM, and subjected them to the mammosphere formation assay in limiting dilutions. Contrary to the findings of House *et al* Morata-Tarifa *et al*, our data does not demonstrate increased mammosphere formation by non-adherent cells. Instead, we observed that treatment with lung-CM significantly impaired mammosphere formation in

both cell lines, while treatment with BM-CM decreased mammosphere formation in non-adherent MCF7 cells. Interestingly, both MCF7 and MDA-MB-468 non-adherent cell subpopulations exposed to lung-CM were incapable of forming mammospheres after 21 days in culture. As a cell adhesion molecule, CD44 expression is significant in making cell-cell and cell-ECM interactions. Ponti et al demonstrated the necessity for CD44 expression in successful mammosphere formation when they documented 95-96% of cells within mammospheres derived from MCF7 and three primary cells lines were $CD44^{+}/CD24^{-}$[182]. Their findings suggest that the inability of our non-adherent breast cancer cells to form mammospheres could be attributed to the observed loss of cell surface CD44 expression. Taken together with our previous studies and our observations here that the non-adherent breast cancer subpopulation was less stem-like than the adherent subpopulation in the presence of lung-CM, our findings suggest that while CD44 and stemness are likely important for the earlier steps of metastasis, they may not be necessary once breast cancer cells become established in the lung microenvironment[52,70].

6.4 Lung-CM contained proteins related to migration, adhesion, and stemness

Distant metastases account for nearly 90% of cancer-related deaths, yet the processes leading to the development of distant tumours is the most poorly understood aspect of cancer pathogenesis[183,184]. Accumulating data now suggests that breast cancer, leukemia, sarcoma, and kidney cancer have a preferential pattern of metastasis towards the lung tissue, while other cancers affecting the colon, head-and-neck, and pancreas also reach the lungs, but in a non-specific manner[118,185]. The lung microenvironment is composed of insoluble and soluble components, both of which have unique roles in tumourigenesis. The insoluble lung microenvironment is composed of several structural ECM proteins such as collagens, elastin, fibronectin, laminin, and proteoglycans that together, represent nearly 65% of the lung tissue[186-188]. The majority of tissue infrastructure in the lung is provided by these ECM components, and often commandeered by tumour cells as the porous and elastic environment is well suited for metastatic colonization[189-191]. Similar to the insoluble lung microenvironment, ECM components such as collagens, fibronectins, and laminins are present in varying levels within the insoluble structures of cancellous bone

matrices[192,193]. Considering these similarities, investigation of the soluble lung microenvironment could provide insight relative to the insoluble component. Composed of more than 60 cell types, a variety of unique secretions are produced by cells of the lung which may have a pivotal role in mediating preferential metastases[194]. Findings by Chu *et al* and Pio *et al* demonstrated that soluble proteins within the lung and BM microenvironments induced preferential migration in a chemotactic manner. In particular, both studies noted an increased migratory capacity among the stem-like ALDH^{hi}CD44^{+} breast cancer cell population towards both lung and BM microenvironments, supporting Croker *et al* finding of increased spontaneous metastases by ALDH^{hi}CD44^{+} breast cancer cells to the lung *in vivo*[52,70,136]. To begin to uncover potential mechanisms that may be involved in preferential metastases to the lungs, our goal was to start to investigate the composition of lung-CM and assess how this may be influencing stem-like phenotype and characteristics of human breast cancer cells.

Investigation of our lung-CM treatment was initiated by Chu *et al.* through protein array analysis. Over 70 unique proteins were observed in the lung-CM that have an association with migration, proliferation, adhesion, and metastasis[52]. Much of this work highlighted the role of the lung microenvironment as a chemoattractant, where OPN was suggested to have significant roles in breast cancer migration towards the lungs[52]. To provide a more unbiased analysis of the composition of lung-CM, we utilized mass spectrometry and found a total of 1,721 soluble proteins unique to the lung-CM, that were absent in the basal treatment. The clear majority, 86.6%, were intracellular-derived proteins, likely originated from the lung tissue as an artifact of the dissociation process required to cultivate lung-CM. The remaining 13.4% of proteins belong to the extracellular compartment, making this proportion of proteins a key area for investigation. As our treatment conditions required human breast cancer cells to be cultured with organ-CM, proteins that would normally be associated with the extracellular space *in vivo* are prone to interact with seeded breast cancer cells *in vitro*. Among the proteins identified, key mediators of migration and adhesion including OPN, POSTN, ADAM10, and β-catenin were present.

6.4.1 Osteopontin

Confirming findings by Chu *et al*, lung-derived OPN was present in the lung-CM and has been previously been implicated in cell-matrix interactions that promote cell motility, invasion, and angiogenesis[195-197]. Using a melanoma model, Kumar *et al* found the knockout of OPN in mice decreased tumour growth, impaired angiogenic processes, and stunted metastatic potential[195]. Findings by Pio *et al* also support the importance of BM-derived OPN in promoting breast cancer cell migration and mammosphere formation *in vitro* among whole cell and stem-like $ALDH^{hi}CD44^{+}CD24^{-}$ breast cancer cell populations[136]. Moreover, experiments involving exogenous overexpression of OPN have demonstrated its role as a negative regulator of HSC self-renewal and localization within BM, while OPN-null mice displayed a clear expansion of the HSC population in murine BM[130,198]. With lung-derived OPN present within lung-CM, the negative regulation of HSC maintenance by OPN suggests this function could potentially extend to CSC regulation, inhibiting expansion of stem-like cells when OPN is present within the microenvironment. Thus, OPN's association with metastatic processes and regulation of stem cell fate may contribute to the reduction in $ALDH^{Hi}CD44^{+}$ phenotype and stem-like function demonstrated in our study.

6.4.2 Periostin

With implications in tumourigenesis, the identification of POSTN within lung-CM is also an interesting finding as its secretion has been found to originate from both tissue stromal cells and infiltrating cancer cells[120]. Abnormally high levels of POSTN have been reported both experimentally and clinically in various cancers of the breast, ovary, and liver[199,200]. In the pulmonary tissue, lung fibroblasts secrete POSTN into the extracellular space to transmit signals from the ECM to cells via interactions with surface receptors such as integrins, mediating cell motility, adhesion, and proliferation[201]. It is well documented that POSTN promotes tumour cell invasion and metastasis through the integrin/PI3/AKT pathway, promoting the development of various cancers[153,202]. In particular, POSTN interaction with integrin $\alpha v\beta 3$ expressed on the endothelium of alveolar micro-vessels cells has been shown to mediate fibroblast or malignant cell migration[203,204]. Sasaki *et al* utilized in situ RNA hybridization to identify high POSTN gene expression was not occurring from

within the breast cancer cells, but instead originating from stromal cells directly adjacent to the bulk tumour[205]. Although many studies postulate the source of POSTN being the cancer cell, very few cancer cell lines have demonstrated significant POSTN mRNA levels *in vitro*, suggesting that stroma-derived POSTN may be of interest during tumourigenesis[206]. The finding that lung-CM contains POSTN derived from the pulmonary stroma suggests that its presence could be a factor that promotes metastasis and suppresses stem-like characteristics.

6.4.3 β-Catenin

The presence of β-catenin within the lung-CM is worthy of further investigation as it has been implicated as a factor involved in determining stem cell fate[207,208]. Regulated by extracellular Wnt ligand interactions with the Frizzled receptor family, the canonical Wnt/β-catenin signaling pathway allows for the intracellular accumulation of β-catenin resulting in induction of stem cell differentiation[209]. Aberrant fluctuation of cytosolic β-catenin has been associated with malignancy in various organs, and several studies report that basal-type TN breast cancers expressing unusually high levels of β-catenin have worse overall survival[154,210,211]. Although its production occurs intracellularly, recent findings have demonstrated that β-catenin can be packaged in exosome-like vesicles and transported into the extracellular space or circulation[212,213]. Further, Dovrat *et al* demonstrated that human embryonic kidney cells (HEK293) not only produced extracellular vesicles containing β-catenin, but that these vesicles were prooncogenic as the adjacent target cells receiving the exosome translocate β-catenin to the host nucleus and activates Wnt-stimulated transcription[212]. Since our model of the lung microenvironment involves mechanical dissociation of murine lung tissue, if exosomal structures containing β-catenin had formed, such a vesicle could be lysed and its contents released into the lung-CM for interaction with human breast cancer cells *in vitro*. Thus, potential uptake of β-catenin by human breast cancer cells could lead to accumulation within the cell, activating canonical Wnt signaling to induce differentiation among CSCs, resulting in a decreased stem-like $ALDH^{hi}CD44^{+}$ phenotype.

6.4.4 Proteins that Influence Cell Detachment and Extracellular Transport

To address the potential role of the lung microenvironment in mediating the transition of adherent human breast cancer cells into a non-adherent subpopulation, both β-catenin and ADAM10 have demonstrated mechanisms involved in cellular detachment. With the possibility that β-catenin internalization by breast cancer cells could have potentially occurred in our *ex vivo* model, previous studies have shown that β-catenin overexpression in Madin-Darby Canine Kidney (MDCK) cells induced a transformation of these traditionally highly adherent epithelial cells towards a mesenchymal phenotype with distinct cellular extensions[214]. Furthermore, several studies have confirmed that MDCK cell survival is anchorage-dependent, however Orford *et al* demonstrate that overexpression of β-catenin resulted in 75% of transfected MDCK cells to maintain viability in suspension for at least 16 hours, avoiding cell death by anoikis[214,215]. These findings support a potential mechanism whereby exogenous β-catenin uptake could induce an epithelial-to-mesenchymal transition that promotes anchorage-independent survival of single cells.

Likewise, when considering the role of ADAM10 in promoting anchorage-independence, several studies have reported the ADAM family of proteases to cleave the extracellular domains of transmembrane proteins. In particular, ADAM10 specifically cleaves CD44 at the ectodomain in order to direct cell migration[152]. As a cell adhesion molecule, CD44 expression plays an important role in cell-cell and cell-ECM interactions[216]. Perhaps the initial induction of a non-adherent breast cancer cell subpopulation and the inability of these cells to form mammospheres after exposure to lung-CM could be attributed, at least in part, to the loss of cell surface CD44 expression via ADAM10 cleavage. In a recent study performed by Mullooly *et al*, inhibition of ADAM10 with small interfering RNA (siRNA) resulted in significantly decreased invasion and migration of MDA-MB-231 human breast cancer cells *in vitro*[217]. Moreover, using 117 primary tumour extracts they demonstrated that elevated ADAM10 protein expression correlates with high-grade, aggressive breast tumours[217]. Their results suggest that in the absence of ADAM10, CD44 mediated cell-cell and cell-ECM interactions remain intact, resulting in a decrease in

migratory and invasive capacity of human breast cancer cells. In addition, a mechanistic model of cell motility proposed by Nagano *et al* highlights the importance of ADAM10 after its activation by stretch-activated calcium ion (Ca^{2+}) channels that promptly result in the cleavage of CD44 at the trailing end of the cell[172]. Taken together, both β-catenin and ADAM10 have previously been shown to promote cellular detachment and anchorage-independent growth. As both proteins are soluble, can exist in the extracellular space, and are present within our lung-CM model, potentially one or both proteins may be involved in the induction of the observed non-adherent breast cancer subpopulation.

6.4.5 Potential Influence of Intracellular Proteins

Lastly, considering that potential mechanisms exist allowing intracellular proteins to be packaged and exported from the cell in the form of exosomal vesicles, it is important to consider what implications this may pose for proteins that are exclusive to the intracellular space. Among the intracellular proteins that made up 86.6% of the proteins detected in the lung-CM, six proteins overlap with key regulators of the ALDH/RA signaling pathway. These proteins include: RBP1, ADH1, ALDH1A1, ALDH1A7, CRABP2, and FABP5. As previously discussed, it is not uncommon for cells to internalize and utilize proteins from the extracellular microenvironment. By expressing an endogenous ligand that mimics endocytic criteria for the receptor of interest, small molecules and proteins can be internalized into the cell[218]. In a groundbreaking discovery led by Sansone and colleagues, the horizontal transfer of the entire mitochondrial genome was packaged and transported to neighboring cells via extracellular vesicles[219]. Further, they determined that primary breast cancer samples from patients receiving hormonal therapy were deficient in oxidative phosphorylation, and that murine-derived mitochondrial DNA (mtDNA) packaged into extracellular vesicles successfully restored metabolic function in these cancer cells, inducing their exit from a dormant state[219]. Moreover, they demonstrated that this phenomenon also exists in stem-like cancer cells, as mutated mtDNA transfer from hormone therapy resistant cells into hormone therapy sensitive cells resulted in elevated self-renewal capacity[219]. Whether this mechanism of internalization is feasible with respect to ALDH/RA components in our model system, and if so, would render functional proteins has yet to be investigated.

Overall, the lung microenvironment is an abundant source of exogenous proteins that influence human breast cancer cell function. In the current study, we have identified several intra- and extracellular soluble proteins within lung-CM such as OPN, POSTN, β-catenin, and ADAM10 that may have specific roles in propagating cell migration, detachment from a monolayer, anchorage-independent growth, and differentiation. Interestingly, some of these exogenous proteins have been implicated in reducing stem-like phenotype and behaviour in experimental models, findings that are in keeping with our results. In addition, we have highlighted potential internalization mechanisms that could potentiate the packaging of intracellular machinery into extracellular vesicles for successful delivery into a recipient cell.

6.5 Lung-CM increases gene expression related to migration and decreases expression of CSC markers

The stochastic model of cancer development speculates that the accumulation of random pro-oncogenic mutations within a cell is the source for aberrant growth patterns and subsequent formation of homogenous tumours[220]. Consequently, the approach to cancer therapy has remained relatively archaic, utilizing cytotoxic agents as a means for targeting a group of homogenous, highly proliferative cells, without discriminating between healthy and malignant cells[221]. Systemic chemotherapy is highly efficient at inducing apoptosis in cells that are actively undergoing mitosis, but ineffective at targeting quiescent cells that are arrested in G_0-G_1 phase of the cell cycle[221]. Despite the phenotypic heterogeneity of tumour cells documented since the earliest days of cancer cell biology, the concept of intra-tumour heterogeneity has gained very little traction until recently[222,223]. Mounting evidence suggests that a bulk tumour is far from homogenous, and instead, is composed of a myriad of distinct cell types that coordinate with each other to maintain tumour homeostasis and drive tumour development[224]. The emerging hierarchical CSC hypothesis has received a lot of attention as it challenges the stochastic model and provides a viable explanation for intra-tumour heterogeneity. The first studies to support the CSC model of cancer development in solid tumours was performed by Al-Hajj and colleagues when they successfully isolated a population of stem-like $CD44^+CD24^-$ breast cancer cells that could recapitulate a breast tumour in 8 of 9 mice tested[62]. As few as 100 stem-like $CD44^+CD24^-$

breast cancer cells could reconstitute a tumour, while significantly higher numbers of non-stem-like breast cancer cells were incapable of tumour formation[62]. Since its introduction, several studies have reported an association with the hematopoietic stem cell marker, ALDH, and its relation to the CSC model. In particular, ALDH activity in tumour cells has been demonstrated experimentally both *in vitro* and *in vivo* to increase invasive potential, migratory capacity, chemoprotection, and self-renewal in various types of solid tumours[61,70,76,171,225,226].

Clinically, breast cancer dissemination has been well documented to metastasize in an organ-specific pattern, often targeting the lung, BM, liver, brain and lymph nodes. Chu *et al* brought to light the importance of the soluble organ microenvironment, as they demonstrated that lung and BM microenvironments were especially capable of promoting migration of stem-like $ALDH^{Hi}CD44^{+}$ breast cancer cell phenotype *in vitro*[52]. Research by Croker *et al* lent support to these findings as they reported increased incidence of spontaneous lung metastases by stem-like $ALDH^{Hi}CD44^{+}$ breast cancer cells *in vivo*[70]. Together, work conducted by Chu *et al* and Croker *et al* suggest that the lung microenvironment *supports* the metastasis of stem-like $ALDH^{hi}CD44^{+}$ human breast cancer cells. The work presented in this thesis aimed to further build on these findings by investigating the potential role of the lung microenvironment in *promoting* a stem-like phenotype once the breast cancer cells reach the secondary site of metastasis. In doing so, we discovered that lung-CM not only decreased the proportion of cells expressing the stem-like $ALDH^{hi}CD44^{+}$ phenotype, but also reduced the expression of several other genes related to stemness. Utilizing a discovery based approach, arrays composed of human cancer stem cells genes highlighted the effect lung-CM treatment has on human breast cancer cells. Collectively, we identified five genes that were affected by lung-CM treatment in both adherent and non-adherent cell subpopulation. Notably, genes related to a stem-like phenotype in breast cancer cells, CD24 and MUC1, were significantly downregulated in response to lung-CM. As a heavily glycosylated adhesion molecule, CD24 has been implicated in progression and metastatic spread of several cancers. In a meta-analysis conducted by Lee *et al*, CD24 expression was more frequently and highly expressed in malignant tumours of the breast and ovaries, relative to their benign counterparts[227]. Moreover, elevated levels of CD24 expression have been associated with

tumour progression and metastasis when investigating its role as a molecular marker of CSCs[143]. Although it remains a controversial subject, some studies have reported a decrease in CD24 protein expression in stem-like progenitor cells relative to differentiated cells[143]. We also observed a decrease in MUC1 gene expression following exposure to lung-CM. With its role in the expansion of pluripotent human embryonic stem cells, MUC1 has been shown to be overexpressed in both ER+ and ER- breast cancers, in addition to its association with breast cancer cell side populations identified via exclusion of Hoechst 33342 stain[144]. Together with a decrease in stem-like $ALDH^{hi}CD44^{+}$ breast cancer cell phenotype and impaired mammosphere formation, the decrease in CD24 and MUC1 gene expression in response to the lung microenvironment supports the idea that stemness is being lost in these human breast cancer cells.

Despite observations that support diminished stem-like phenotype and behaviour, treatment with lung-CM affected the expression of several other genes related to increased aggressiveness. For example, both ATM and WEE1 were significantly downregulated in response to lung-CM. These genes represent a class of tumour signaling molecules involved in cell cycle control and have been implicated numerous times to have a role in cancer development. ATM is a serine/threonine kinase that has a central role in recognition of DNA damage, and responds accordingly by repairing double-stranded DNA breaks. Interestingly, in a study investigating 385 patients with gastric cancer, Han *et al* report a downregulation of ATM mRNA expression within tumour samples, relative to adjacent healthy tissue[150]. In addition, Kaplan-Meier analysis demonstrated patients with ATM-negative tumours had a drastically lower survival rate compared to ATM-positive tumours[150]. Similarly, increased ATM gene expression in breast carcinomas has been associated with a favorable patient outcome and prognosis[151]. WEE1 is also a serine/threonine kinase that is involved in regulating G_2-M cell cycle checkpoint. The main role of WEE1 is to arrest mitotic entry in response to DNA damage, and its impairment has led to the loss of DNA-damage induced apoptosis and aberrant mitosis, however its role in carcinogenesis remains controversial[146]. Studies that have successfully inhibited WEE1 activity report an anticarcinogenic role in basal and TN breast cancer cells, while WEE1 overexpression in both melanoma and vulvar squamous cell carcinoma have been associated with poor disease-free survival and malignancy[228-231]. Although there is not a

clear consensus on the role of WEE1 in tumour development, it is likely that a decrease in gene expression of ATM and/or WEE1 may contribute to heightened malignancy due to loss of cell cycle control, despite the decrease in stemness.

Of the five genes of interest that were consistently affected by lung-CM treatment in both adherent and non-adherent breast cancer subpopulations, the only gene that exhibited an increase in expression was metastasis/migration related CXCL8, a precursor to interleukin 8 (IL8). Originally identified as a monocyte-derived factor, IL8 has been reported to recruit and activate neutrophils to site of inflammation, as well as the propagation of epithelial-to-mesenchymal transition in human cancer cells[147,148]. In breast cancer, IL8 expression is significantly increased in more aggressive ER- subtypes and has been reported to promote metastasis through increased cell invasion and angiogenesis[148,232]. Again, this data suggests that although lung-CM diminishes stem-like phenotype and behavior, it may still support metastatic capacity through mechanisms such as increased motility and cell recruitment.

Many studies have documented the importance of ALDH activity in combination with cell surface markers, such as CD44, to isolate populations of cancer cells that behave in an aggressive manner both *in vitro* and *in vivo*. The unexpected findings of this thesis do not discredit the tumourigenic potential of stem-like cell populations, but instead suggest that stem-like characteristics are not compulsory for tumour development once in the secondary lung microenvironment. Our gene array analysis demonstrates that CSC-related genes were downregulated, along with the downregulation of tumour signaling molecules involved in cell cycle regulation. Moreover, the upregulation of CXCL8 suggests the soluble lung microenvironment may promote cell migration. Taken together, although stemness of breast cancer cells is decreased after exposure to lung-CM, these cells may still retain metastatic capacity in order to drive disease progression in a CSC-independent manner.

6.6 Possible Limitations of the Study

The model system used in this thesis is based on an *ex vivo* representation of the soluble organ microenvironment, and with this, is an imperfect model that relies on several assumptions.

The first assumption is that breast cancer metastasis is primarily influenced by the soluble organ microenvironment. Several studies have recently addressed the importance of the insoluble organ microenvironment in providing the necessary infrastructure for tumour development, with parameters such as scaffold composition, density, pore size, and elastic modulus affecting *in vitro* tumour development[233,234]. Thus, to more accurately depict the role of the entire organ microenvironment regarding preferential metastasis and stemness *in vitro*, it would be necessary to incorporate both soluble and insoluble components.

The second assumption is that the composition of the lung-CM media will truly reflect the soluble lung microenvironment. During the process of generating lung-CM, murine lungs are mechanically dissociated to allow soluble proteins to be secreted into the culture medium. Although the proteins that make their way into the conditioned media are specific to the lung tissue, they are not necessarily all natively secreted proteins and many would generally remain confined within the cell of origin *in vivo*. During the dissociation process, cells of the lung are forcibly lysed and their contents released into the culture medium, resulting in a lung-CM that is enriched in both intracellular and extracellular related proteins. We observed that 86.6% of proteins within the lung-CM belong to the intracellular compartment. This is not an issue with BM-CM as its generation requires several passages that likely discard any intracellular contents that may be present due to cell lysis. An improved method for generating lung-CM would be one that is less prone to cell lysis, such as using bead mill homogenizers[235].

The third assumption is that breast cancer cells and their associated stem-like characteristics will behave in a similar manner when exposed to lung-CM derived from either healthy or diseased lung tissue. As our study utilizes healthy murine tissue to generate lung and BM-CM, there is the potential that we are not presenting a fully accurate model for metastasis to secondary organs in a host that first developed a primary tumor.

The concept of metastatic priming has been recently introduced and supported by several studies; whereby the presence of a primary tumour may "prime" the microenvironment of a distant secondary organ prior to the arrival of metastatic cancer cells[236-238]. Permitting tumour development following orthotopic injection of human breast cancer cells into the mammary fat pad of mice could allow potential "priming" mechanism to influence the microenvironments of secondary sites of metastasis.

The fourth assumption is with regards to our animal model and generation of organ-CM. Primarily, we utilized an immunocompromised murine model to generate our organ-CM treatments as future studies would utilize the inherent NOD/SCID mutation to improve rates of human tissue engraftment *in vivo*. Due to their reduced innate immunity (NOD mutation) and complete T- and B-cell deficiency (SCID mutation), these mutations may have noticeable effects on the lung tissue[239]. Within the lower respiratory tract, alveolar macrophages represent the largest population of leukocytes in healthy lung tissue capable of ingesting microbes, and, initiating an immune response by presenting cell surface antigens[240]. Thus, it is likely these alveolar macrophages (among other immune cells) are significantly reduced in our model of the healthy lung and may be beneficial to utilize an immunocompetent model instead. Further, CO_2 asphyxiation was carried out during euthanasia. This method helps to maintain our lung samples intact post-euthanasia, however may damage the microarchitecture within the lung tissue. It has been reported that rapid asphyxia with CO_2 results in alveolar atrophy and hemorrhaging within murine lungs, thus compromising the native state of healthy lungs within our model[241]. An alternative approach could be euthanization by retroorbital ketamine-xylazine injections[242]. Although this method may appear distasteful, it is more humane than other IV injections and better conserves the integrity of lung tissue by avoiding asphyxiation[242].

The final assumption concerns the use of immortalized cell lines. Although cell lines are routinely used in the cancer research community, their behaviour and intrinsic mechanisms can become altered over successive passages and the foreign *in vitro* culture conditions. The breast cancer cell lines used in this study were once derived from individual primary patient samples, however their immortalization is linked to inherent mutations that allow for continuous growth outside of their native microenvironment. Preferably, a model

utilizing primary breast cancer cells would more accurately depict the underlying biological processes of cancer pertaining to phenotype and behaviour[243].

6.7 Future Directions

While this thesis investigated several important questions pertaining to stem-like breast cancer phenotype and function in response to the lung and bone microenvironments, there are several avenues of investigation that need to be addressed in future studies to better understand the role of these organ microenvironments in metastasis.

First, as lung-CM treatment decreased cell surface CD44 expression and induced a non-adherent breast cancer subpopulation incapable of mammosphere formation, it is important to investigate whether these cells have lost adhesive properties through CD44-mediated interactions. A potential resolution could be to coat tissue culture plastic with a known CD44 receptor ligand, such as OPN, and monitor whether non-adherent breast cancer cells retain their capacity to re-adhere when provided suitable conditions. If the breast cancer cells remain non-adherent after exposure to a known ligand, one can infer that loss of cell surface CD44 may not be responsible for the non-adherent phenotype.

Second, as we only investigated the composition of the lung-CM, it is necessary to perform similar analysis on the BM-CM. Understanding which factors are present within the BM-CM would provide insight into the effects that BM-CM imposed on our adherent and non-adherent breast cancer cell subpopulations. Furthermore, by assessing the composition of BM-CM, we would be able to effectively compare which proteins are similar or different between the lung-CM and BM-CM models.

Third, considering the BM is a rich stem cell niche for hematopoietic stem cells, it would be interesting to investigate whether CSC-related genes are affected in breast cancer cells after treatment with BM-CM. Using a similar human cancer stem cell gene array used for lung-CM analysis, some insight would be provided with regards to changes in CSC gene expression and whether similar genes were affected by both lung-CM and BM-CM.

Finally, future studies must move these findings into an *in vivo* model. Recapitulating the native organ microenvironment *in vitro* is a significant feat, and often, those who attempt its representation experience shortcomings. Although many studies highlight the tumourigenic and metastatic potential of stem-like cancer cells, our study indicates that the

stem-like phenotype may be diminished once breast cancer cells are exposed to the lung microenvironment. However, the induction of a viable, non-adherent population of breast cancer cells with gene expression patterns suggestive of increased migratory and/or metastatic capacity warrants further investigation of the true functional effect of these cells *in vivo*, independent of the CSC context.

6.8 Final Conclusions

The purpose of this thesis was to test the hypothesis that the lung and/or bone microenvironments could promote stem-like phenotype and function in human breast cancer cells. As metastasis is the leading cause of cancer-related deaths, it is of utmost importance to strengthen our understanding of tumour dissemination and development at distant secondary sites. Prior to this study, our lab has focused on the "getting there" perspective of metastasis and have demonstrated that the lung microenvironment can promote chemotactic migration in both whole cell populations and sorted $ALDH^{hi}CD44^{+}$ stem-like human breast cancer cell populations. Here, we have begun investigation into the "establishment" stage of tumour metastasis. In particular, this thesis has directed attention to breast cancer cell stemness and plasticity, investigating whether the secondary microenvironment can mediate stem-like attributes associated with the CSC theory of cancer development.

Taken together, our findings (summarized in **Table 6**) did not support the hypothesis of this thesis. Treatment with lung-CM decreased stem-like characteristics, namely ALDH and CD44 phenotype. Further, these cells were not capable of producing mammospheres, which is also a common *in vitro* measure of stemness. Despite these findings, evidence in the literature also supports the notion that cancer cells with decreased ALDH activity and CD44 expression can be more tumourigenic and correlate with advanced stage cancers. Further, our data suggests that the migration related gene, CXCL8, is upregulated after lung-CM treatment, while CSC markers (CD24 and MUC1) and tumour signaling molecules (ATM and WEE1) are downregulated. Previous studies in our lab provide evidence that lung-CM *supports* existing $ALDH^{hi}CD44^{+}$ cells, with respect to migration and growth, however the findings of this thesis indicates that neither lung-CM or bone-CM actually *promote* stemness of breast cancer cells. Nonetheless, we did uncover an intriguing non-adherent, viable subpopulation of breast cancer cells that are induced by lung-CM, and this population will be important to investigate further in the future to determine if and how the lung microenvironment may promote survival and migration of breast cancer cells using mechanisms distinct from CSC pathways.

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8 APPENDICES

Appendix 1. Approved animal use protocol

AUP Number: 2009-064
PI Name: Allan, Alison
AUP Title: Role of ALDH+/CD44+ stem-like cells in breast cancer progression and treatment
Approval Date: 10/27/2017

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2009-064:9: entitled " Role of ALDH+/CD44+ stem-like cells in breast cancer progression and treatment" 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.

As per your declaration within this approved AUP, you are obligated to ensure that:
1) Animals used in this research project will be cared for in alignment with:
a) Western's Senate MAPPs 7.12, 7.10, and 7.15
http://www.uwo.ca/univsec/policies_procedures/research.html
b) University Council on Animal Care Policies and related Animal Care Committee procedures
http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm

2) As per UCAC's Animal Use Protocols Policy,
a) this AUP accurately represents intended animal use;
b) external approvals associated with this AUP, including permits and scientific/ departmental peer approvals, are complete and accurate;
c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and
d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.
e) http://uwo.ca/research/services/animalethics/animal_use_protocols.html

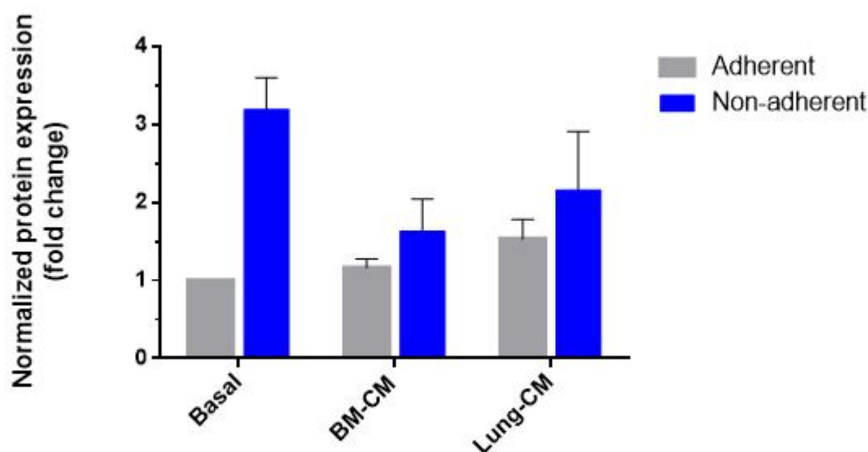
3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
a) be made familiar with and have direct access to this AUP;
b) complete all required CCAC mandatory training (training@uwo.ca); and
c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAPP 7.15,
a) Practice will align with approved AUP elements;
b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
c) UCAC policies and related ACC procedures will be followed, including but not limited to:
i) Research Animal Procurement
ii) Animal Care and Use Records
iii) Sick Animal Response
iv) Continuing Care Visits
5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,
http://www.uwo.ca/hr/learning/required/index.html

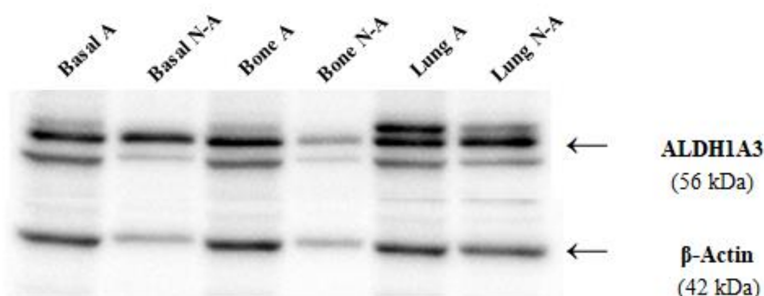
Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

Dr.Timothy Regnault,
Animal Care Committee Chair

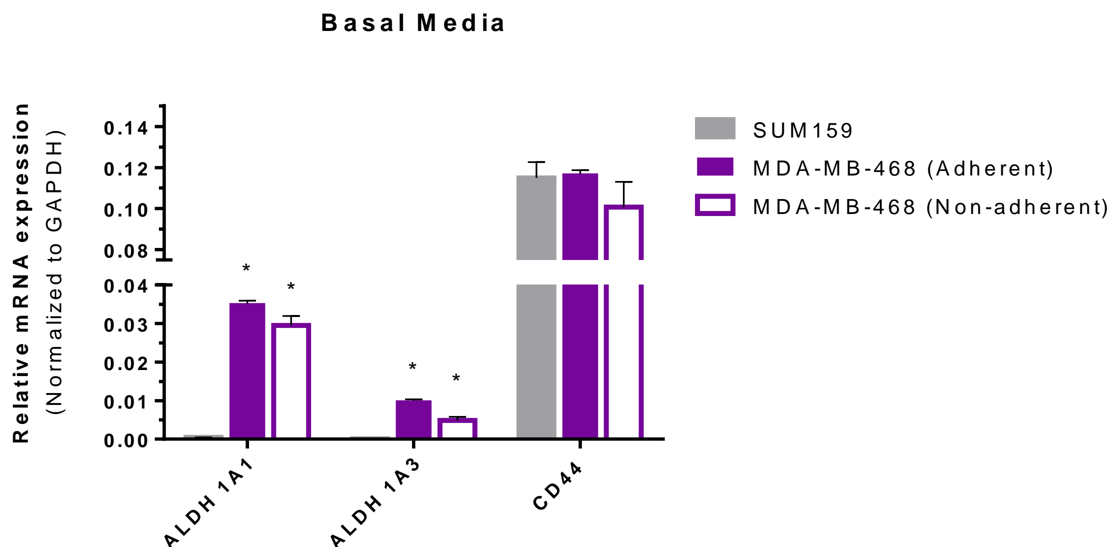
A



B



Appendix 2. Exposure to organ-CM did not affect protein expression of ALDH1A3 in human breast cancer cells. MDA-MB-468 human breast cancer cells were exposed to lung-CM, BM-CM, and basal control for 72 hours. Both adherent and non-adherent subpopulations were harvested and cell lysates utilized for analysis of ALDH1A3 protein expression by western blot. ***(A)*** Treatment with either lung-CM or BM-CM did not affect ALDH1A3 protein expression relative to basal media. All data are normalized to the basal control group and β-Actin. ***(B)*** Representative cropped image of western blot probing for ALDH1A3 (56 KDa) and β-Actin (42 KDa). Lanes are labelled based on treatment condition received (lung, bone, or basal) and associated with either adherent (A) or non-adherent (N-A) subpopulations. Experiments were performed a minimum of 3 times and analyses were performed using 2-way ANOVA and Dunnett's post hoc test. Data is presented as mean ± SEM. All significant values (*) are relative to the respective treatments adherent population ($p<0.05$).



Appendix 3. Relative mRNA expression levels of cell lines and cell subpopulations in response to basal media. MDA-MB-468 (Adherent and Non-adherent subpopulations) and SUM159 cells were exposed to basal media for 24 hours in culture (37°C, 5% CO_2) without media replacement. Cells were harvested and RNA was analyzed by RT-qPCR to assess expression of CD44, ALDH1A1 and ALDH1A3. All analyses were normalized to GAPDH expression and shown as relative expression levels. Experiments were performed a minimum of three times and statistical analyses were performed using one-way ANOVA and Dunnett's post hoc test. Data is presented as mean ± SEM. All significant values (*) are relative to the SUM159 cell line with each gene respectively ($p<0.05$, N=3).

Appendix 4. Average Ct values of MDA-MB-468 cells following qRT-PCR Human Cancer Stem Cell gene array analysis. Adherent (A) and Non-adherent (NA) cell subpopulations were analyzed and data sets are colour-coded based on subpopulations being compared: Basal-A vs Basal-NA (Black), Lung-A vs Lung-NA (Blue), Basal-NA vs Lung-NA (Orange), and Basal-A vs Lung-A (Green). Raw data acquired using Qiagen's Data Analysis Center® software.

Gene Name	Average Ct Values							
	Basal – A (Control)	Basal – NA (Sample)	Lung – A (Control)	Lung – NA (Sample)	Basal – NA (Control)	Lung – NA (Sample)	Basal – A (Control)	Lung – A (Sample)
ABCB5	40	40	40	40	40	40	40	40
ABCG2	40	40	39.53	39.68	40	39.68	40	39.53
ALCAM	25.56	26.39	26.29	28.04	26.39	28.04	25.56	26.29
ALDH1A1	25.12	25.75	25.74	27.5	25.75	27.5	25.12	25.74
ATM	30.93	30.9	32.76	33.78	30.9	33.78	30.93	32.76
ATXN1	27.01	27.78	28.4	30.12	27.78	30.12	27.01	28.4
AXL	35.02	32.52	31.51	32.87	32.52	32.87	35.02	31.51
BMI1	27.22	27.58	27.41	28.92	27.58	28.92	27.22	27.41
BMP7	30.92	31.59	30.29	31.85	31.59	31.85	30.92	30.29
CD24	25.96	27.03	28.53	29.32	27.03	29.32	25.96	28.53
CD34	40	38.81	40	39.84	38.81	39.84	40	40
CD38	35.99	34.74	35.95	37.12	34.74	37.12	35.99	35.95
CD44	22.59	23.05	22.22	23.5	23.05	23.5	22.59	22.22
CHEK1	26.6	28.08	27.15	29.19	28.08	29.19	26.6	27.15
DACH1	34.13	35.52	36.2	38.86	35.52	38.86	34.13	36.2
DDR1	26.66	27.16	26.46	28.2	27.16	28.2	26.66	26.46

DKK1	28.29	29.47	30.32	30.83	29.47	30.83	28.29	30.32
DLL1	40	37.79	38.62	39.15	37.79	39.15	40	38.62
DLL4	40	38.61	40	38.33	38.61	38.33	40	40
DNMT1	25.46	26.11	25.5	27.31	26.11	27.31	25.46	25.5
EGF	31.25	30.82	30.68	32.72	30.82	32.72	31.25	30.68
ENG	30.4	30.53	29.88	31.29	30.53	31.29	30.4	29.88
EPCAM	23.48	24.17	23.64	25.16	24.17	25.16	23.48	23.64
ERBB2	29.76	30.28	29.76	31.76	30.28	31.76	29.76	29.76
ETFA	23.78	24.83	24.75	26.29	24.83	26.29	23.78	24.75
FGFR2	29.32	29.98	29.35	31.33	29.98	31.33	29.32	29.35
FLOT2	26.37	26.75	26.28	27.95	26.75	27.95	26.37	26.28
FOXA2	40	40	39.43	39.67	40	39.67	40	39.43
FOXP1	28.96	29.32	29.41	30.82	29.32	30.82	28.96	29.41
FZD7	30.01	30	30.17	31.3	30	31.3	30.01	30.17
GATA3	29.5	30.17	29.76	31.51	30.17	31.51	29.5	29.76
GSK3B	26.29	26.65	26.62	28.26	26.65	28.26	26.29	26.62
HDAC1	25.15	25.53	25.57	27.25	25.53	27.25	25.15	25.57
ID1	27.27	28.25	25.79	27.88	28.25	27.88	27.27	25.79
IKBKB	29.59	29.54	29.29	30.59	29.54	30.59	29.59	29.29
CXCL8	32.66	29.2	29.17	28.09	29.2	28.09	32.66	29.17
ITGA2	29.89	30.04	28.81	30.13	30.04	30.13	29.89	28.81
ITGA4	38.52	40	40	40	40	40	38.52	40

ITGA6	27.36	27.75	26.44	28.72	27.75	28.72	27.36	26.44
ITGB1	26.49	27.04	26.3	28.37	27.04	28.37	26.49	26.3
JAG1	27.88	28.1	26.14	27.7	28.1	27.7	27.88	26.14
JAK2	31.17	31.56	31.22	33.46	31.56	33.46	31.17	31.22
KIT	33.24	35.12	38.82	38.85	35.12	38.85	33.24	38.82
KITLG	27.15	28.01	28.18	30.64	28.01	30.64	27.15	28.18
KLF17	37.08	34.83	35.36	36.83	34.83	36.83	37.08	35.36
KLF4	29.35	29.58	28.56	30.38	29.58	30.38	29.35	28.56
LATS1	28.38	28.78	28.65	30.5	28.78	30.5	28.38	28.65
LIN28A	34.14	33.21	35.45	36.18	33.21	36.18	34.14	35.45
LIN28B	38.76	40	40	39.16	40	39.16	38.76	40
MAML1	30.47	30.73	30.6	32.32	30.73	32.32	30.47	30.6
MERTK	34.74	33.79	33.44	35.48	33.79	35.48	34.74	33.44
MS4A1	40	40	40	40	40	40	40	40
MUC1	26.52	26.97	28.37	30.15	26.97	30.15	26.52	28.37
MYC	24.12	25.47	24.21	26.25	25.47	26.25	24.12	24.21
MYCN	31.68	33.42	33.29	36.35	33.42	36.35	31.68	33.29
NANOG	38.63	35.79	40	38.63	35.79	38.63	38.63	40
NFKB1	29.32	29.66	29.01	31.23	29.66	31.23	29.32	29.01
NOS2	38.34	35.4	36.73	39.26	35.4	39.26	38.34	36.73
NOTCH1	31.55	32.05	31.28	32.86	32.05	32.86	31.55	31.28
NOTCH2	27.18	27.81	28.14	30.15	27.81	30.15	27.18	28.14

PECAM1	36.08	35.63	36.83	38.08	35.63	38.08	36.08	36.83
PLAT	39.97	38.59	37.39	38.77	38.59	38.77	39.97	37.39
PLAUR	27.63	27.68	26.39	27.59	27.68	27.59	27.63	26.39
POU5F1	33.03	31.71	32.77	33.68	31.71	33.68	33.03	32.77
PROM1	25.86	26.44	27.03	28.54	26.44	28.54	25.86	27.03
PTCH1	33.8	35.36	34.17	37.1	35.36	37.1	33.8	34.17
PTPRC	38.74	40	40	39.32	40	39.32	38.74	40
SAV1	25.76	26.89	25.94	28.07	26.89	28.07	25.76	25.94
SIRT1	27.41	27.89	27.73	29.51	27.89	29.51	27.41	27.73
SMO	32.91	33.59	34.17	35.33	33.59	35.33	32.91	34.17
SNAI1	37.26	35.13	38.13	37.16	35.13	37.16	37.26	38.13
SOX2	40	40	40	40	40	40	40	40
STAT3	24.81	25.44	25.1	26.86	25.44	26.86	24.81	25.1
TAZ	27.96	27.92	27.29	29.02	27.92	29.02	27.96	27.29
TGFBR1	27.09	27.34	26.84	28.71	27.34	28.71	27.09	26.84
THY1	35.94	37.51	37.71	38.32	37.51	38.32	35.94	37.71
TWIST1	38.82	40	39.02	40	40	40	38.82	39.02
TWIST2	30.16	30.83	29.63	31.47	30.83	31.47	30.16	29.63
WEE1	26.15	27.06	27.7	29.88	27.06	29.88	26.15	27.7
WNT1	40	39.42	40	40	39.42	40	40	40
WWC1	26.62	27.05	26.55	28.57	27.05	28.57	26.62	26.55
YAP1	25.26	25.75	25.29	27.24	25.75	27.24	25.26	25.29

ZEB1	39.04	37.05	36.51	38.43	37.05	38.43	39.04	36.51
ZEB2	40	40	40	40	40	40	40	40
ACTB	19.57	20.46	19.95	21.43	20.46	21.43	19.57	19.95
B2M	23.37	24.49	24.66	26.14	24.49	26.14	23.37	24.66
GAPDH	21.11	22.05	21.52	22.65	22.05	22.65	21.11	21.52
HPRT1	26.48	27.41	27.43	29.04	27.41	29.04	26.48	27.43
RPLP0	19.55	20.21	20.19	21.47	20.21	21.47	19.55	20.19
HGDC	38.61	40	40	40	40	40	38.61	40
RTC	21.91	22.15	22.42	22.23	22.15	22.23	21.91	22.42
RTC	22.02	22.19	22.52	22.27	22.19	22.27	22.02	22.52
RTC	21.97	22.17	22.42	22.15	22.17	22.15	21.97	22.42
PPC	19.87	19.92	20.05	20.01	19.92	20.01	19.87	20.05
PPC	19.57	19.78	20	19.86	19.78	19.86	19.57	20
PPC	19.88	20.08	20.01	19.99	20.08	19.99	19.88	20.01

Curriculum Vitae

Ashkan Sadri

EDUCATION

Master of Science (MSc) **Sept. 2015 – Jan. 2018**
Anatomy and Cell Biology (Intended)
University of Western Ontario - London, ON, Canada

Bachelor of Medical Sciences (BMSc) **Sept. 2011 – April 2015**
Honours Specialization Medical Science
University of Western Ontario - London, ON, Canada

RESEARCH AND RELATED EXPERIENCE

Graduate Research Assistant (MSc Thesis) **Sept. 2015 – Jan. 2018**
University of Western Ontario - London, ON, Canada (Intended)
Supervisor: Dr. Alison Allan

Graduate Teaching Assistant **Sept. 2017 – April 2017**
Physiology 3130z
University of Western Ontario - London, ON, Canada

AWARDS AND ACCOMPLISHMENTS

Translational Breast Cancer Research Unit Studentship **Sept. 2015 – Dec. 2017**
Amount: $39,666

Western Graduate Student Research Scholarship **Sept. 2015 – Aug. 2017**
Amount: $9000

Dean's Honor List (2 years) **Sept. 2013 – April 2015**

The University of Western Ontario Bursary **November 2014**
Amount: $1,800

The University of Western Ontario Bursary **November 2013**
Amount: $3,200

Marion and Arthur Knight Bursary **December 2012**
Amount: $1,500

The Queen Elizabeth II Aiming for the Top Scholarship **Sept. 2011-April 2012**
Amount: $3,500

POSTER/ABSTRACT PRESENTATIONS

Role of the metastatic microenvironment in supporting and/or promoting stem-like human breast cancer cells. **June 2017**

Poster Presentation
Oncology R&E Day
London, ON, Canada

Role of the metastatic microenvironment in supporting and/or promoting stem-like human breast cancer cells. **April 2017**

Poster Presentation
American Association of Cancer Research
Washington D.C., USA

Role of the metastatic microenvironment in supporting and/or promoting stem-like human breast cancer cells. **March 2017**

Poster Presentation
London Health Research Day
London, ON, Canada

Role of the metastatic microenvironment in supporting and/or promoting stem-like human breast cancer cells. **October 2016**

Poster Presentation
Anatomy and Cell Biology Research Day
London, ON, Canada

Role of the metastatic microenvironment in supporting and/or promoting stem-like human breast cancer cells. **June 2016**

Poster Presentation
Oncology R&E Day
London, ON, Canada

ORAL PRESENTATIONS

Cancer's Journey: From Breast to Lung. **October 2015**

Retiring with Strong Minds Research Presentation
Guest Speaker
The University of Western Ontario
London, ON, Canada