THE ROLE OF ALDH^{hi}CD44^{+} CELLS IN BREAST CANCER METASTASIS AND THERAPY RESISTANCE

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Abstract

Breast cancer is a leading cause of death in women, due primarily to ineffective treatment of metastatic disease. Despite being a lethal process, metastasis is surprisingly inefficient, with less than 0.5% of cancer cells able to successfully navigate the metastatic cascade, indicating that only a small proportion of cancer cells possess the necessary characteristics to establish metastases. Cancer stem cells (CSCs) have recently been identified in leukemia and solid tumors; however, the role of CSCs in breast cancer metastasis and therapy resistance remains poorly understood. In the present study, sub-populations of cells demonstrating stem-cell like characteristics (high expression of CSC markers and/or high aldehyde dehydrogenase [ALDH] activity, an enzyme involved in stem cell self-protection) were identified in MDA-MB-435, MDA-MB-231, MDA-MB-468 breast cancer cells, but not in MCF-7 cells. When isolated and compared to $ALDH^{low}CD44^{-}$ cells, $ALDH^{hi}CD44^{+}$ cells demonstrated increased metastatic behaviours *in vitro*. Furthermore, following injection into immunocompromised mice, $ALDH^{hi}CD44^{+}$ cells showed enhanced tumorigenicity and metastasis compared to $ALDH^{low}CD44^{-}$ cells, indicating that stem-like $ALDH^{hi}CD44^{+}$ cells may be important mediators of breast cancer metastasis. $ALDH^{hi}CD44^{+}$ cells from MDA-MB-231 and MDA-MB-468 cells that were exposed to chemotherapy or radiotherapy also demonstrated significantly increased cell survival relative to $ALDH^{low}CD44^{-}$ cells, potentially through an ALDH1-dependent manner. ALDH1 expression has previously been correlated with poor patient outcome and incidence of metastatic disease. To test a potential functional role for ALDH1 expression in metastasis and therapy resistance, the ALDH1 isozymes ALDH1A1 or ALDH1A3 were knocked down using siRNA in MDA-MB-468 and SUM159 breast cancer cell lines. $ALDH1A1^{low}$ cells demonstrated decreased metastatic abilities *in vitro* and *in vivo*; whereas $ALDH1A3^{low}$ cells demonstrated minimal changes in metastatic abilities *in vitro* but showed a significant reduction in metastatic capacity *in vivo*. Additionally, $ALDH1A1^{low}$ cells but not

ALDH1A3^{low} cells demonstrated increased sensitivity to both chemotherapy and radiation. Collectively, these data suggest that ALDH1 expression demonstrates a functional role in breast cancer metastasis and therapy resistance. Thus, drug development that targets ALDH1-expressing tumor cells may represent a novel therapeutic strategy to treat metastatic breast cancer patients in the future.

Keywords

Breast Cancer, Metastasis, Cancer Stem Cells, Therapy Resistance, Aldehyde Dehydrogenase, CD44, All-*trans*-Retinoic Acid

Co-Authorship Statement

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Abbreviations

7-AAD	7-aminoactinomycin D
AATP	AML associated translocation products
ABC	ATP-binding cassette
Akt	Human homolog of viral AKR mouse thymoma protein
ALDH	Aldehyde dehydrogenase
AML	Acute myeloid leukemia
αMEM	Alpha-minimum essential medium
ANOVA	Analysis of variance
APC	Allophycocyanin
APL	Acute promyelocytic leukemia
ATCC	American Type Culture Collection
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATRA	All-*trans* retinoic acid
BAA	BODIPY™- aminoacetate
BAAA	BODIPY™- aminoacetaldehyde
BAPN	Beta-aminopropionitrile
Bcl-2	B-cell lymphoma 2
BCRP	Breast cancer resistance protein
BM	Bone marrow
Bmi-1	B lymphoma Mo-MLV insertion region 1 homolog
BSA	Bovine serum albumin
CAK	Cyclin-dependent kinase activating kinase
CAM	Chorioallantoic membrane
CARET	Beta-carotene and retinol efficacy trial
CB	Cord blood
CD	Cluster of differentiation
CDH2	N-cadherin
CDH1	E-cadherin
C/EBPα	CCAAT-enhancer binding proteins alpha
C/EBPε	CCAAT-enhancer binding proteins epsilon
Chk1,2	Checkpoint 1,2

CK8/18/19	Cytokeratin 8/18/19
CML	Chronic myeloid leukemia
CON	Control
CP	Cyclophosphamide
CRABP2	Cellular retinoic acid binding protein 2
CSC	Cancer stem cell
CXCR4	C-X-C chemokine receptor type 4
CYP26	Cytochrome P450 26
DCIS	Ductal carcinoma *in situ*
DEAB	Diethylaminobenzaldehyde
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DRP	Drug resistance protein
EMT	Epithelial-mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal regulated kinases
EtOH	Ethanol
ESA	Epithelial specific antigen
FABP5	Fatty acid binding protein 5
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FoxO	Forkhead box O
G_0	Resting phase of the cell cycle
G_1	Gap 1 phase
G_2	Gap 2 phase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBM	Glioblastoma multiform
GFP	Green fluorescence protein
GSEA	Gene set enrichment analysis
GSI	Gamma secretase inhibitor
GSTpi	Glutathione-S-transferase pi
Gy	Gray

HH	Hedgehog
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cells
HIF	Hypoxia inducible factor
HNSCC	Head and neck squamous cell carcinoma
HER2	Human epidermal growth factor receptor 2
HPF	High powered field
H&E	Hematoxylin and eosin
Klf4	Krueppel-like factor 4
LC50	Lethal concentration-50
LCIS	Lobular carcinoma *in situ*
LOX	Lysyl oxidase
LRIG1	Leucine-rich repeats and immunoglobulin-like domains
LSC	Leukemic stem cells
M	Mitotic phase of the cell cycle
MAPK	Mitogen-activated protein kinase
MAT-1	Menage-a-trois-1
MDR	Multidrug resistance
m.f.p	Mammary fat pad
MGMT	O-6-methylguanine-DNA methyltransferase
mRNA	Messenger-ribonucleic acid
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
N-CoR	Nuclear receptor co-repressor
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NICD	Notch intracellular domain
NK	Natural killer
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
NOD/SCID-IL2γ	Non-obese diabetic/severe combined immunodeficiency-IL2 gamma receptor
OCT	Optimal cutting temperature
Oct 3/4	Octamer-binding transcription factor 3/4
PBS	Phosphate buffered saline
PDK-1	Phosphoinositide-dependent kinase-1
PE	Phycoerythrin

PI3K	Phosphoinositide 3-kinase
PLZF	Promyelocytic leukemia zinc finger protein
PM	Phosphoramide mustard
PML	Acute progranulocytic leukemia: retinoic acid receptor alpha
PMSF	Phenylmethylsulfonyl fluoride
PPARδ/γ	Peroxisome proliferator-activated receptor-delta/gamma
PTCH1	Patched-1
PVDF	Polyvinylidene difluoride
qRT-PCR	quantitative reverse transcription-polymerase chain reaction
RA	Retinoic acid
RARα	Retinoic acid receptor alpha
RARβ	Retinoic acid receptor beta
RARγ	Retinoic acid receptor gamma
Rb	Retinoblastoma
ROS	Reactive oxygen species
RT	Radiation therapy
RXR	Retinoic X receptor
S	DNA synthesis phase of the cell cycle
SC	Stem cell
SCF	Stem cell factor
SDF-1	Stromal-derived factor 1
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SHH	Sonic hedgehog
siRNA	Small interfering ribonucleic acid
SMO	Smoothened
SMRT	Silencing mediator of retinoic acid and thyroid hormone receptor
SOX2	SRY (sex determining region Y)-box 2
SP	Side population
STAT3	Signal transducer and activator of transcription-3
STAT5	Signal transducer and activator of transcription-5
TBST	Tris buffered saline + Tween-20
TGF-β	Transforming growth factor beta
TNFα	Tumor necrosis factor-alpha

tRNA	Transfer-ribonucleic acid
uPAR	Urokinase-type plasminogen activator receptor
VCAM-1	Vascular cell adhesion protein-1
VEGFR1	Vascular endothelial growth factor receptor-1
YB1	Y-Box protein-1

Chapter 1

Introduction

A version of this chapter has been published as three separate review papers/book chapters:

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Croker AK and Allan AL. Chapter 23: Future Directions: Cancer Stem Cells as Therapeutic Targets. In: Allan AL, ed., *Cancer Stem Cells in Solid Tumors*. Springer Science, New York, NY, 2011:403-429.

1.1 Breast cancer

Breast cancer represents the most commonly diagnosed and the second highest cause of cancer-related deaths in North American women[1-3]. Cancer is a complex disease that arises as a result of multiple genomic mutations causing a disruption of normal cellular homeostasis. These mutations allow malignant cells to gain unlimited proliferative capacity and the ability to invade surrounding tissues and vasculature through the acquisition of key characteristics, including self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evasion of apoptosis[4,5]. Underlying factors allowing for this malignant transformation include inherent genomic instability due to loss of DNA repair mechanisms; tumor-promoting inflammation; tumor cell evasion of immune response; plasticity of cellular metabolism; and the ability of tumor cells to recruit beneficial stromal cells to both the primary and the secondary tumor sites[4,5].

Breast cancer originates from the transformation of breast epithelial cells found either lining the milk ducts or in the milk-producing lobules of the breast. Lobules and ducts are formed

from three lineages of cells in two germ layers: the myoepithelial layer is common to both structures and forms the basal layer, while ductal epithelial cells line the ducts, and alveolar epithelial cells synthesize the milk within the lobules[6,7]. While still confined within the duct or lobule of origin, breast tumors are classified as ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS), respectively. When breast cancers are diagnosed at the *in situ* stage, treatments are highly effective[8-10]. Unlike DCIS (the precursor for invasive ductal carcinoma), LCIS has not been shown to be a direct precursor for invasive disease, but rather is an overall risk factor for developing breast cancer. Therefore, most patients diagnosed with LCIS do not undergo treatment, but instead are subjected to increased surveillance for signs of new malignant lesions[8,10]. A patient's prognosis worsens when the tumor invades adjacent tissues and gains the potential to metastasize. Clinically, it is metastatic disease that is associated with the majority of breast cancer-related deaths[11-13].

1.2 Metastasis

Metastasis is a sequential, multi-step process that results in cancer cells escaping from the primary tumor and causing malignant disease at distant sites in the body (*Figure 1.1*). During the initial stages, cancer cells intravasate from the primary tumor into the circulation or lymphatic system. Subsequently, the cells must survive and migrate through the body, adhere at a secondary site, and extravasate from the circulation and into the secondary tissue. Tumor cells must then initiate and maintain growth in a secondary site to form micrometastases, which, following angiogenesis at the secondary site, can form macroscopic, clinically relevant macrometastases[11,13].

1.2.1 Metastatic inefficiency

Based on the complexity of the metastatic process, it seems unlikely that all cancer cells successfully complete all the steps necessary to form clinically relevant metastases. Indeed, metastasis is characterized as a highly inefficient process, and interestingly, not all the steps

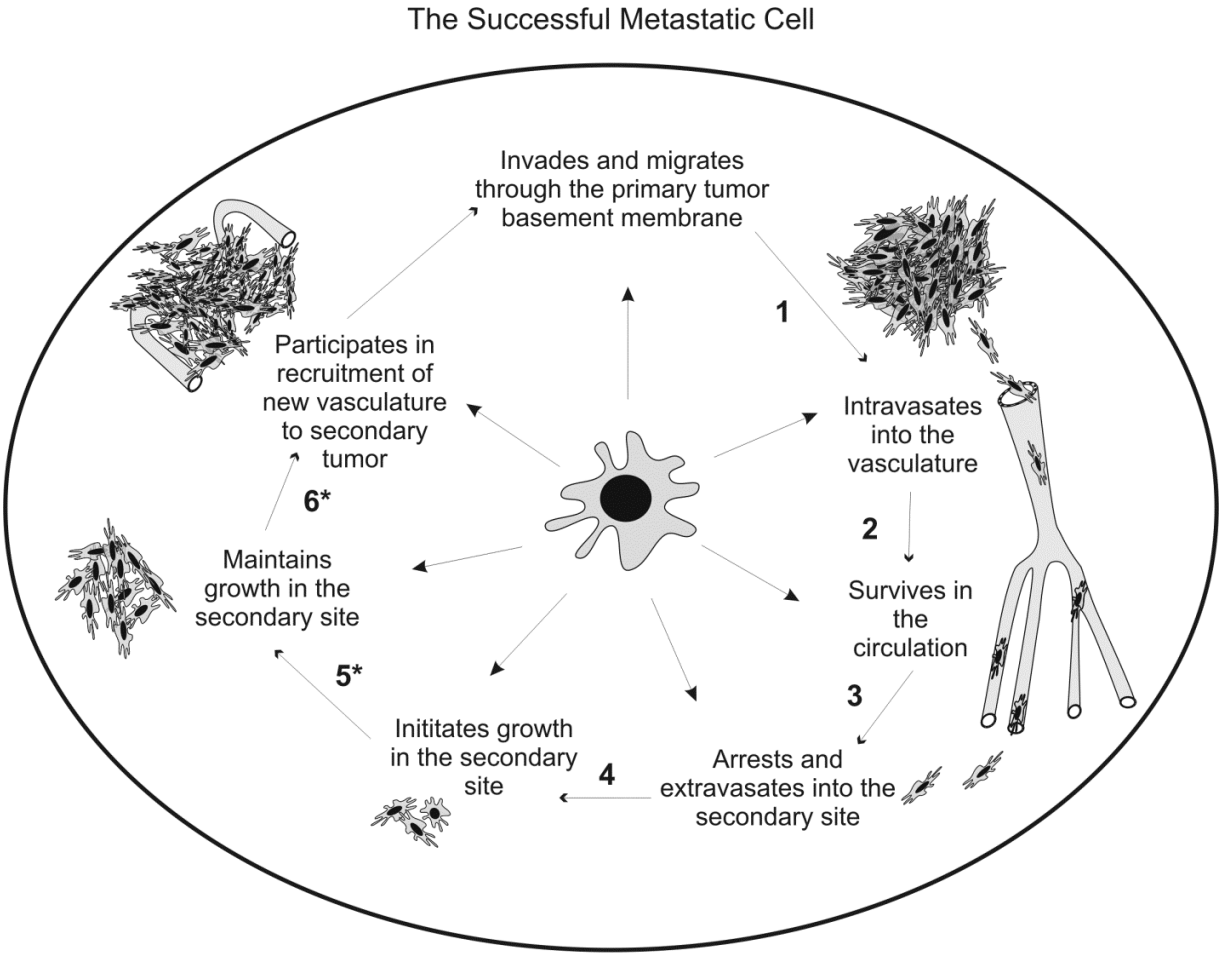


Figure 1.1. Breast cancer metastasis. The successful metastatic cell must carry out a number of sequential steps in order to form clinically relevant metastases. Based on the complexity of the metastatic process, it seems unlikely that all cancer cells would be able to successfully complete all the steps necessary to form macrometastases. Indeed, it is known that metastasis is a highly inefficient process, and that not all the steps of the metastatic process are equally inefficient. The principal rate limiting steps are *__5__: initiation of growth into micrometastases; and *__6__: maintenance of growth into macrometastases; whereby less than ~2% of solitary cells are able to initiate growth and less than ~0.02% of cells are able to maintain growth into clinically relevant metastases. From Croker and Allan, *JCMM*, 2008[14].

of the metastatic process are equally inefficient[11,15,16]. Previous studies by Luzzi et al. (1998) used *in vivo* video microscopy to follow the fate of metastatic melanoma cells in an experimental model of metastasis. They observed that cancer cells were very proficient at intravasating, migrating through the circulation, adhering at a secondary site and extravasating back out of the vasculature. In fact, almost 90% of metastatic cells could successfully complete these early steps in the metastatic process; however, only ~2% of disseminated cells were able to form micrometastases, and only ~0.02% of cells were able to develop into vascularized macrometastases[15]. These results suggest that only a subset of tumor cells is capable of successfully traversing the entire metastatic cascade, and that the initiation and maintenance of cancer cell growth at a secondary site represents the rate limiting step in metastasis (*Figure 1.1*).

1.2.2 Organ-specific metastasis

Regulation of growth at the secondary site can differ depending on to which organ the tumor cells metastasize, and many cancers show an organ-specific pattern of metastasis. For example, colorectal cancers preferentially metastasize to the liver, prostate cancer often metastasizes to bone, and breast cancer favours metastasis to regional lymph nodes, bone, liver, brain and lungs[11,13]. It was originally believed that many metastases could be explained purely by circulatory patterns[17]. According to this mechanical hypothesis, since most cancer cells are much larger than blood cells, cancer cells are forced to arrest in the capillary bed of the first organ they encounter in the circulation, and thus form metastases wherever they are stopped[11,17].

Several theories, however, have challenged this idea by proposing that there are additional, molecular level mechanisms which explain why and how cancer cells can arrest and grow in ‘favourite’ metastatic sites. The most central of these theories is the ‘seed and soil’ theory of metastasis, first proposed in 1889 by Stephen Paget[18]. Paget predicted that cancer cells (the ‘seed’) can survive and proliferate only in secondary sites (the ‘soil’) that produce

growth factors appropriate for that type of cell, and this theory has largely withstood the test of time[19]. In a meta-analysis of published autopsy study data, Weiss showed that, in many cases, metastases detected at autopsy were in proportion to the blood flow from the primary tumor site to the secondary organ[20]. However, in some cases, more metastases (notably breast cancer metastasis to bone) or fewer metastases were detected than would be expected by blood flow alone, indicating that the 'soil' or microenvironment in the secondary organ is likely very important[18-20]. Another concept, often called the homing theory, proposes that different organs produce chemotactic factors (i.e. chemokines, such as stromal cell-derived factor 1 [SDF-1]) which can attract specific types of tumor cells to 'home' to and arrest in a particular organ[21-23]. It is likely that all these theories could be correct simultaneously. Since metastasis is such an inefficient process, and since the inefficiency lies in the growth of cancer cells in the secondary tissue, it is possible that the primary method of dissemination is mechanical (i.e. blood flow patterns) and/or dependent on chemotactic factors, and whether or not a tumor will form depends on if the metastatic microenvironment is suitable to sustain tumor growth.

1.2.3 Epithelial-mesenchymal transition (EMT)

A theory that has gained favour in recent years is the idea that epithelial-mesenchymal transition (EMT) can contribute to the metastatic process. First identified in embryonic development, EMT involves conversion of epithelial cells to a mesenchymal phenotype via loss of polarity and cell–cell contacts and dramatic cytoskeletal remodelling[24]. Cells undergoing EMT also acquire expression of mesenchymal proteins and develop an enhanced ability to migrate, thus assisting in cell distribution throughout the embryo and organ development.

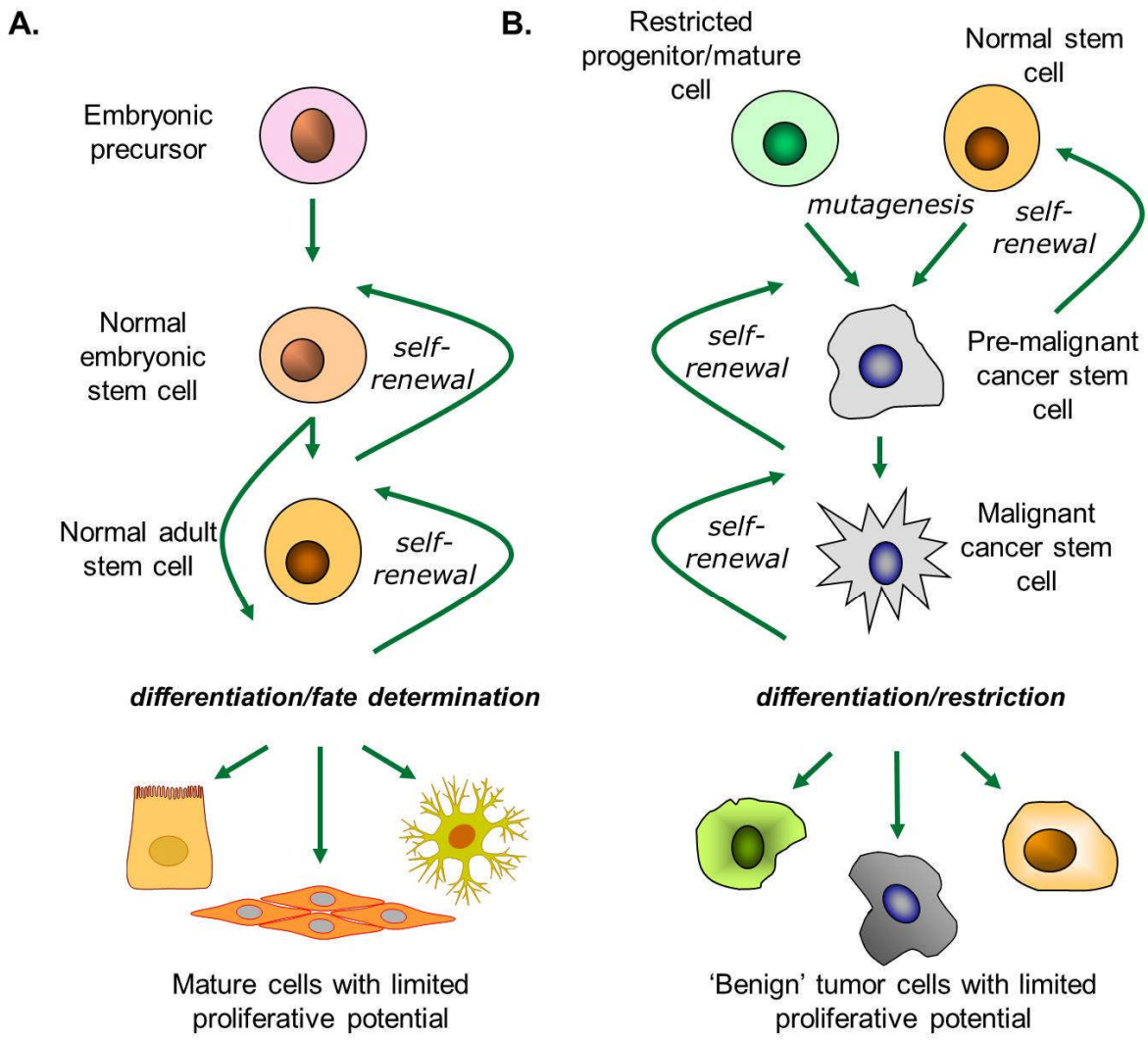
In cancer, it is believed that epithelial tumor cells may be able to activate this primitive developmental program, thus converting differentiated epithelial cancer cells into de-differentiated cells that possess more mesenchymal characteristics[25]. The EMT phenotype in cancer has been associated with a decrease in tumor growth, increased resistance to apoptosis, increased motility and invasiveness, and enhanced metastatic ability[26]. These

phenotypic transitions are reversible, and it is hypothesized that once tumor cells have reached their destination, they may transform back into an epithelial phenotype in order to facilitate tumor growth in the secondary site[27]. EMT is characterized by the expression of various factors responsible for mediating this process at the molecular level. The growth factor transforming growth factor-β (TGF-β) has been shown to induce reversible EMT, along with Wnt pathway proteins (in particular β-catenin), Notch, and Hedgehog signalling pathways, which often act in a sequential manner to induce EMT[27-31]. Additionally, transcription factors such as Snail and NF-κB have been shown to confer apoptotic resistance to tumor cells undergoing EMT[32,33]. Given the developmental origin of EMT, it is interesting but not surprising that many of these molecules have also been associated with stem cell maintenance[25]. In fact, metastatic cells share many cellular and molecular characteristics of cells important to early development, and growing evidence suggests that many cancers may contain small subsets of 'stem-like' cells or 'cancer stem cells' that are responsible for tumor initiation and progression.

1.3 The cancer stem cell hypothesis

Two theories have been proposed that attempt to explain how tumors develop[34,35]. The stochastic model suggests that every cell within a tumor is a potential tumor-initiator, but that entry into the cell cycle is governed by a low probability of stochastic events. According to this model, all cells have the same ability to initiate tumorigenic growth since each cell has an equal ability to be tumorigenic. This is in contrast to the hierarchy theory, which proposes that only a small subset of tumor cells are capable of initiating tumorigenic growth, but that these cells do so at a high frequency. According to this theory, it should be possible to identify the cells responsible for tumor formation because not all the cells have the same phenotypic characteristics. This heterogeneous hierarchy within a tumor represents the basis for the cancer stem cell hypothesis (*Figure 1.2*).

Figure 1.2. The cancer stem cell hypothesis. A. In a normal system, stem cells can self-renew to maintain the stem cell population. They can also differentiate to give rise to various types of mature cells with limited proliferative potential. **B.** The cancer stem cell hypothesis posits that a cancer stem cell (the origin of which is still highly debated: possibly a stem or progenitor cell, or a mature cell that dedifferentiates to become more stem cell-like) can also self-renew to maintain the cancer stem cell population, but can also differentiate to give rise to 'benign' tumor cells with limited proliferative potential that make up the bulk of the tumor. Adapted from Pardal et al., 2003[36].



(*adapted from Pardal et al.; 2003*)

In 1855, Rudolph Virchow suggested that cancer cells behaved in a similar manner to embryonic-like cells[37]. However, it was not until 1994 that the first solid evidence supporting the CSC hypothesis emerged[38]. Bonnet and Dick discovered that acute myeloid leukemia (AML) originated from a hierarchy of tumor-initiating stem-like cells, where only the most primitive cells ($CD34^+CD38^-$ cells) were able to initiate and sustain the leukemia[39]. After isolating $CD34^+CD38^-$ cells and "non-CSCs" ($CD34^-CD38^-$ cells) and injecting them into NOD/SCID mice, it was observed that that only the $CD34^+CD38^-$ cells were able to initiate and sustain the leukemia[39,40]. These findings sparked tremendous interest in discovering stem-like cells in solid tumors, and there is now evidence supporting the existence of CSC populations in brain, breast, prostate, colon, and pancreatic tumors, as well as in melanoma[41-48].

1.3.1 Origin of the CSC: normal stem cell gone bad?

The CSC has been clearly defined by Clarke et al. as "a cell within a tumor that possesses the capacity to self-renew and to produce the heterogeneous lineages of cancer cells that comprise the tumor"[49]. It is important to note that this definition does not include the cellular origin of the CSC, and this remains one of the most elusive and highly debated questions surrounding the CSC hypothesis[49,50]. If these cells arise from normal stem cells, then cancer cells could requisition the existing stem cell regulatory pathways for self-renewal. On the other hand, if these cells arise from mature, differentiated cells, oncogenic mutations would be required to drive de-differentiation and self-renewal. The fact that multiple mutations are necessary for a cell to become tumorigenic and/or metastatic has implications for the cellular origin of cancer cells[4,5]. It can be argued that mature cells have a very limited lifespan, and thus it is unlikely that all the necessary mutations could be acquired during the relatively short life of these cells; however, it has also been demonstrated that the actual number of mutations required to form a pluripotent stem cell from an adult fibroblast is surprisingly small. Forced expression of Oct-4, c-Myc, Sox2 and klf4 in adult fibroblasts results in cells that are both morphologically similar to embryonic stem cells, and have stem cell-like replicative potential[51].

In contrast, the infinite self-renewal capacity of normal stem cells means these cells may be the only cells that survive long enough to accumulate the necessary mutations[52]. It has been suggested that stem or progenitor cells must be the initial targets for malignant transformation since the CSCs need to be able to self-renew, and it would be more difficult for a differentiated cell to regain self-renewal properties through mutations. However, since the presence of stem cells in adult tissue is extremely rare, progenitor cells (which retain partial self-renewal capacity but are more abundant and more proliferative than stem cells in adult tissue) may be the cells from which CSCs are derived[50,53,54]. In further support of this idea, microarray analyses have demonstrated that the gene signatures of CSCs are more similar to the stem cells of the tissue in which the primary tumor originates than to the non-CSCs of the tumor[55-57].

Therefore, although it is presently unclear whether tumors initiate from a stem or progenitor cell that has accumulated genetic mutations towards a more cancerous phenotype, or whether a cancerous cell somehow de-differentiates to become more stem cell-like, what is clear is that this stem-like population in tumors is important for the initiation and maintenance of tumor growth. Focusing research attention on these populations would thus be very beneficial for understanding cancer biology and potentially discovering new therapeutic targets to combat cancer.

1.3.2 Breast cancer stem cells

In breast cancer, stem-like cells were first identified based on $CD44^{+}CD24^{-/low}lin^{-}$ cell surface marker phenotype. When these cells were isolated from primary tumors or pleural effusions from breast cancer patients and injected into the mammary fat pad of immunocompromised mice, as few as 100 $CD44^{+}CD24^{-/low}lin^{-}$ cells were sufficient to cause a tumor in these mice, whereas injections of tens of thousands of cells that did not express that phenotype failed to cause tumor formation[41]. Subsequent studies by Ginestier et al. (2007) demonstrated that ALDH1 is a marker of stem/progenitor cells of both the normal human breast and also breast carcinomas[58]. They demonstrated that $ALDH^{+}$ cells represent a subpopulation

of normal breast epithelium with the broadest lineage differentiation potential capable of self-renewal, and demonstrated the highest ability to grow *in vivo* in a xenotransplantation animal model[58]. In breast tumors, cells with high ALDH activity contained the tumorigenic cell fraction able to self-renew and to recapitulate the heterogeneity of the parental tumor[58]. Furthermore, this group compared the tumorigenic potential of the original $CD44^+CD24^{-/low}lin^-$ CSC phenotype, the $ALDH1^+$ phenotype, and also the combination of the two ($ALDH^+CD44^+CD24^{-/low}lin^-$ cells). Interestingly, the results indicated that $ALDH^-CD44^+CD24^{-/low}lin^-$ cells were not tumorigenic, even upon injection of up to 50,000 cells. $ALDH^+$ cells, on the other hand, were able to form tumors with injections of 1,500 cells. However, with $ALDH^+CD44^+CD24^{-/low}lin^-$ cells, as few as 20 cells injected into the mammary fat pad were sufficient to cause a tumor, indicating that an ALDH-enriched population of $CD44^+CD24^{-/low}lin^-$ cells may be the most tumorigenic cells within a breast tumor[58].

Although there is solid evidence that a subpopulation of stem-like cells can contribute to breast tumor initiation, the functional role of such cells in breast cancer metastasis and treatment remains poorly understood, and this represents the central topic of this thesis.

1.4 Parallels between stem cell behaviour and metastatic behaviour

As discussed earlier, it is well known that metastasis in an inefficient process. At present, this inefficiency is explained in terms of the need for cancer cells to find the proper microenvironment, since initiation and maintenance of tumor growth in secondary organs are the most inefficient steps of the metastatic process. We and others believe that there may be an additional or alternative explanation for this inefficiency, one related to the CSC hypothesis[50,52,53,59,60].

Rare cells drive metastatic progression, a fact illustrated by the observation that less than 1% of disseminated cancer cells are able to form clinically relevant macrometastases[15].

Application of the CSC hypothesis to metastasis therefore suggests that this rare subset of cells within a primary tumor that is capable of re-initiating growth to form metastases in distant sites may in fact be CSCs. It also suggests that the inefficiency of metastasis may be due both to the rarity of CSCs and the unsuitable habitats presented by different organ microenvironments. This is supported by the observation that metastatic cells and stem cells share a number of key properties, including an unlimited capacity for self-renewal; the requirement for a specific 'niche' or microenvironment to grow (and, juxtapositional with that, a self-protective ability to survive and grow in harsh environments); use of the SDF-1/CXCR4 axis for migration; the ability to undergo long periods of cell dormancy/quiescence; enhanced resistance to apoptosis; and an increased capacity for drug resistance (*Table 1.1*).

1.4.1 The niche/microenvironment

Normal stem cells require a specific niche or microenvironment in order to grow and survive[61-63]. The stem cell niche is an anatomically defined space that has been identified in many different tissue types, and it serves to regulate stem cell number and function as well as to modulate stem cells under conditions of physiologic change. The niche cells and the microenvironment they create allow the niche to maintain the stem cell pool and prevent its differentiation, while at the same time also directing tissue growth and renewal through more differentiated daughter progenitor cells[63]. Furthermore, the niche provides protection to stem cells. In those tissues where the niche is well defined, the stem cell may be practically enveloped by differentiated cells which work to house and interact with the stem cells. For example, in the mammary gland, putative stem and progenitor cells are sequestered from both the basement membrane and the lumen in structurally specialized spaces. The presence of a niche within the epithelium can provide nourishment, yet exclusion from molecules that may cause differentiation or mutation[61]. Besides protective effects, the stem cell niche can also play a role in determining cell fate. The physical orientation of a stem cell in its niche can affect the symmetry of cell division, and niche-forming cells can be stimulated by growth factors to

Table 1.1 **Parallels between stem cell behaviour and metastatic behaviour**[21,22,64-80]. From Croker and Allan, *JCMM*, 2008[14].

Stem Cell Property	Potential Molecular Factors Involved	Proposed Therapeutic Strategy
Requirement for specific "niche" or microenvironment: • Growth versus differentiation • Maintenance of the CSC pool	• TGF-β • Other factors?	• Differentiation therapy
Cellular quiescence/dormancy • Regulating periods of quiescence/proliferation in order to maintain the SC/CSC pool • May play a role in controlling solitary metastatic cells	• Kruppel • FoxO • Rb • Notch-1 • Myc • Lrig1 • Cell Cycle Regulators (p21, p27, p38, ERK, uPAR)	Treatment with agents that block quiescence or promote proliferation: • Lrig1 • Myc • Cell cycle regulators
Use of the SDF-1/CXCR-4 axis: • Homing to secondary sites • Adhesion, migration, invasion	• SDF-1 • CXCR4 • Other factors?	• Treatment with agents that target CXCR4 (i.e. TN14003, AMD3100)
Resistance to apoptosis and protection from cellular insult: • Maintenance of the CSC pool • Drug resistance • Resistance to DNA damage	• TGF-β • Hedgehog (HH) • Bmi-1 • Bcl-2 • Notch-1 • ABC transporters (i.e. ABCC1, ABCB1, ABCG2) • DNA checkpoint proteins (i.e. Rad17, Chk1, Chk2) • Other factors?	Treatment with agents that target: • HH signaling (i.e. cyclopamine) • Bmi-1 • Bcl-2 • Notch-1 (i.e. γ secretase inhibitors) • DNA checkpoint proteins • ABC transporters (i.e. verapamil)

produce ligands that act on stem cell receptors such as Notch to initiate stem cell mitosis or specify differentiation[61,62].

Metastatic cells, like normal stem cells, require a particular niche to grow. This was elegantly demonstrated by Kaplan et al. (2005), who showed that bone marrow-derived VEGFR1$^+$ hematopoietic cells (HPC) can home to tumor-specific pre-metastatic sites and form cellular clusters before the arrival of metastatic tumor cells in mice[81]. At these sites, the bone marrow-derived cells express several primitive haematopoietic markers, such as CD34, CD116, c-Kit and Sca-1, which help in maintaining their progenitor cell status within the tissue parenchyma in the pre-metastatic niche. These VEGFR1$^+$ HPCs alter the local micro-environment, which leads to activation of integrins and chemokines (such as SDF-1) that promote attachment, survival, and growth of tumor cells. When treated with an anti-VEGFR1 antibody, the supportive pre-metastatic cell clusters were abolished and metastasis was prevented, indicating that these clusters play an important role in the metastatic process[81-84]. Additional studies have shown that other niche cells such as bone marrow-derived mesenchymal stem cells have been shown to localize to breast carcinomas, and seem to be involved in cancer metastasis. When mesenchymal stem cells were mixed with non-metastatic MCF-7/Ras human breast cancer cells, their metastatic potency increased in immune-compromised mice[85]. Furthermore, breast cancer cells stimulated chemokine secretion from the mesenchymal cells, which acted in a paracrine fashion on cancer cells to enhance their motility, invasion and metastasis.

Lysyl oxidase (LOX) and its regulator, hypoxia inducible factor (HIF), may also be factors that influence the tumor microenvironment or niche to favour metastasis. It is known that patients with high LOX-expressing tumors (i.e. increased hypoxia in tumors) have decreased survival due to more aggressive metastasis. Secreted LOX is thought to be responsible for the invasive properties of hypoxic human cancer cells through FAK activity and cell matrix adhesion[86]. Interestingly, when LOX was inhibited (using β-aminoproprionitrile [BAPN] or an

antibody targeting LOX), metastasis was eliminated in mice, but there was no effect on the primary tumor[86]. This suggests that hypoxic environments and the induced expression of LOX and HIF are important in the metastatic setting. Normal stem cells also survive and proliferate in hypoxic environments, typically maintaining an undifferentiated state that is important to preservation of the stem cell pool[51].

There has been some controversy over whether or not CSCs require specific niches to grow. However, in leukemia, studies have shown that leukemia stem cells actually occupy a similar region as normal hematopoietic stem cells (the perioendosteal region), suggesting that CSCs may in fact require a niche to protect and maintain their tumor initiating/tumor sustaining capacity[63]. In the situation of metastasis, we might consider that the tumor microenvironment in a secondary organ is in fact a 'metastatic niche'. Initially, it was believed that the microenvironment where a metastatic cell ended up either passively supported tumor development and facilitated tumor formation, or it did not support growth, and no tumor was able to form. There is now evidence to suggest that the tumor and secondary organ microenvironments actively contribute to the growth and invasion of metastatic cells, and that non-CSCs may actually contribute to the creation of the CSC niche[35,62,63,76,81,87]. In metastasis, the niche could be supporting the establishment and expansion of CSCs either through normal or dysregulated signalling. For example, TGF-β has been shown to inhibit the proliferation of quiescent hematopoietic stem cells[68], so perhaps non-CSCs in leukemia control TGF-β secretion to dysregulate CSC division, thus influencing the balance between CSC proliferation, self-renewal, differentiation, and senescence. In solid cancers, it could be hypothesized that CSCs in metastatic sites are similarly regulated by inappropriate signalling from the metastatic niche[88,89].

Currently, the similarities between stem cell niches in different tissues remains poorly understood, in particular with regards to whether tissue-specific stem cells can be regulated by stem cell niches in other organs. This knowledge will have important implications for

understanding metastatic growth in secondary sites, including the possibility that some CSCs (i.e. breast, prostate) may favour growth in the bone marrow because it provides a particularly rich stem cell niche[52].

1.4.2 Cellular quiescence/dormancy

An interesting characteristic of metastatic cells, especially in certain cancers such as breast cancer, is their ability to remain dormant for months or even years after arriving in a secondary site. In fact, it is not uncommon for a breast cancer patient to seemingly be in complete remission from their primary tumor, only to present years later with metastatic disease[11]. Interestingly, the behavior of these dormant metastatic cells is in many ways similar to stem cell quiescence. If it is indeed true that successful metastatic cells are CSCs, then perhaps metastatic cell dormancy can be better understood by taking into consideration some of the mechanisms that regulate the balance between quiescence and activation/growth in stem cells.

Many adult tissues maintain a stem cell pool which is needed to sustain tissue homeostasis and ensure rapid response to injury. One way in which this pool is maintained is through tight control of the number of cell divisions undertaken by stem cells within the pool. Thus, stem cells go through long periods of quiescence, and only proliferate when needed for production of differentiated progeny and/or replenishment of the stem cell pool[90]. Several molecular mechanisms have been identified that contributed to this process. For example, stem cell quiescence has been associated with the Kruppel, FoxO, and Rb families of transcriptional regulators that induce reversible arrest in various lineages[91]. Microarray data has also shown that during stem cell quiescence, there is active suppression of differentiation-related housekeeping genes, and a higher expression of Notch and its downstream molecules, which are important in maintaining the stem cell pool in some tissues[91,92]. During quiescence, expression of Lrig1 ensures that stem cells are less responsive to growth factor stimulation than their differentiated progeny. It has been proposed that Lrig1 expression maintains skin stem

cells in a quiescent, non-proliferative state in part by negatively regulating Myc transcription. It has also been suggested that Myc-induced differentiation acts as a fail-safe device to prevent uncontrolled proliferation of stem cells when Lrig1 is down-regulated[93]. Interestingly, a soluble form of LRIG1 has been shown to inhibit proliferation of some cancers[94]. A similar situation has been observed in an inducible Myc transgenic mouse model of cancer, where Myc inactivation resulted in tumor dormancy, cell differentiation, and expression of cytokeratin 19 in a subset of cells. This effect however was reversible, and upon Myc reactivation tumors continued growing[95]. Although relatively little is known about what controls solitary metastatic cell dormancy, several key molecules and pathways, including p21, p27, p38, uPAR, ERK, and Myc have been implicated in maintaining this dormancy[96,97]. As these molecules are known to be cell cycle regulators or part of related signaling pathways, it is logical that the niche or microenvironment, much in the same way it regulates stem cells, is regulating these dormant metastatic cells.

Interestingly, recent evidence has indicated that the stem cell pool itself can be comprised of a heterogeneous cell population. For example, in the murine hematopoietic system, two subtypes of HSCs have been identified, a large proportion that are capable of activation for homeostasis purposes, and a smaller proportion of protected, dormant HSCs which are needed for critical response to injury and absolute maintenance of the stem cell pool. These “dormant” HSCs have the highest repopulating and self-renewing capacity of all HSCs, and are only activated in response to injury. Once repair is complete, 100% of the previously dormant HSCs return to their niche microenvironment and their dormant state[98]. HSCs have also been shown to be heterogeneous in terms of their differentiation capacity and migratory ability[99-101]. This observed stem cell heterogeneity implies that this is perhaps also the case in the CSC hierarchy. It is possible that there are primitive CSCs that remain relatively dormant, and that these cells, once activated, will have the highest self-renewing and tumor repopulating activity of all the CSCs. Furthermore, the capacity to stay dormant would allow these primitive

CSCs to stay protected from therapies aimed at eliminating replicating cells (discussed further below). If this is the case, then targeting the dormant metastatic cells would be very important in a clinical setting. This observation of stem cell heterogeneity could explain why, in the majority of metastasis models, cells are simultaneously observed at the single cell, micrometastasis and macrometastasis stages[15,102].

Chronic wound healing has been suggested as a mechanism of cancer initiation, where the dormant stem cells repeatedly become activated for tissue repair, but eventually lose the ability to differentiate and become CSCs [reviewed in [103]]. In support of this, small populations of "primed" cells within tumors have an increased ability to grow due to epigenetic silencing of the p16 promoter. This silencing leads to an increase in proliferation, invasion, and angiogenesis and a decrease in apoptosis and immune surveillance. Furthermore, loss of p16 holds cells in a primitive state, increasing stem cell self-renewal and preventing differentiation[104]. It is possible that activation of dormant metastatic cells occurs in much the same way. When the most primitive of CSCs disseminate from the primary tumor, they may stay in their proper niche (i.e., breast CSCs would stay in the mammary stem cell niche), or alternatively, migrate to a niche that meets the cells' requirements (i.e. the bone marrow). The CSCs then remain "quiescent" or "dormant" for long periods of time, perhaps enabling them to escape from prolonged cancer therapy. It may be that they stay dormant, in fact, until such a time as the normal stem cells in that niche are stimulated due to changes in the microenvironment and/or injury related to aging, toxins, or mechanical damage. The stem cells (along with the CSCs) are then activated and migrate toward the site of the injury, and the CSC, due to its previous mutations, begins dividing in a dysregulated manner, ultimately forming a metastatic tumor. Alternatively, CSCs may escape from a primary tumor and form their own niche via differentiated progeny in the secondary tissue of their choosing. In this way, the non-CSC niche (much like a normal stem cell niche) would protect the CSCs from damaging and differentiating molecules, and relay

signals to keep the CSCs in a dormant state until such a time that some stimuli awaken the CSCs and a metastatic tumor is initiated.

1.4.3 The SDF-1/CXCR4 axis

Another similarity between metastatic cells and stem cells is their use of chemokine pathways for migration. The chemokine SDF-1 is believed to play a critical role in stem cell migration in cooperation with its receptor CXCR4[21]. SDF-1 is an ideal candidate for aiding in metastasis because its major biological effects are related to the ability of this chemokine to induce motility, chemotactic responses, adhesion, secretion of MMPs and secretion of angiopoietic factors such as VEGF in cells that express CXCR4. SDF-1 also increases adhesion of cells to VCAM-1, fibronectin, and fibrinogen by activating/modulating the function of several cell surface integrins[22]. In cancer development, fibroblast expression of SDF-1 and tumor cell expression of CXCR4 is often increased within hypoxic areas of the tumor, subsequently triggering tumor cell growth, motility, and invasiveness. Furthermore, many $CXCR4^+$ metastatic cells use the SDF-1/CXCR4 axis to migrate through the body according to an SDF-1 gradient[22,67]. In support of this, studies have demonstrated that breast cancer cells treated with a CXCR4 inhibitor showed a significantly inhibited metastatic ability[71], and intracranial glioblastoma and medulloblastoma xenografts treated with a CXCR4 antagonist (AMD3100) show reduced cell growth and increased tumor cell apoptosis[77]. This axis may also help to explain the organ-specific nature of metastatic growth, since CXCR4-expressing cancer cells may home to organs that express high levels of SDF-1. For example, breast cancer has been shown to metastasize experimentally using the SDF-1/CXCR4 axis, with CXCR4-expressing breast cancer cells preferentially metastasizing to SDF-1-expressing organs such as lymph nodes, liver, and bones[11,13,105]. Many cancers are positive for CXCR4 expression[22,67,105], and it seems, at least in breast cancer, that CXCR4 expression correlates with the CSC content, and thus the aggressiveness, of cancer cell lines. For example, relative to non-metastatic MCF-7 breast cancer cells, highly metastatic MDA-MB-231 cells have a larger proportion of CSCs and

express higher levels of CXCR4[67]. Furthermore, in pancreatic cancer, it has been shown that a $CD133^+$ CSC population was heterogeneous in nature with regards to CXCR4 expression, and that only the $CD133^+CXCR4^+$ CSCs were able to metastasize[43].

1.4.4 Resistance to apoptosis and protection from cellular insult

Resistance to apoptosis is another key property shared by both normal stem cells and highly malignant tumor cells. Stem cells can resist apoptosis by a number of mechanisms, including via TGF-β signalling or activation of the Hedgehog (HH) pathway[79]. Additionally, stem cells also express higher levels of anti-apoptotic proteins than differentiated cells, including members of the Bcl-2 family[80]. Stem cells must also resist early senescence in order to maintain the stem cell pool, a process facilitated by Bmi-1 expression. Interestingly, Bmi-1, Bcl-2, TGF-β and HH pathway components have all been shown to be up-regulated in cancer cells[65,72,74,79,80]. Furthermore, despite their limitless self-renewal capacity, normal stem cells are relatively quiescent and divide infrequently unless activated[36,66,106]. Similarly, CSCs may cycle through long periods of quiescence and short bursts of proliferation, and since most chemotherapeutic anti-cancer agents are designed to target rapidly dividing cells, this may be one mechanism by which CSCs escape cytoxicity from these drugs.

From an evolutionary point of view, normal stem cells have a number of unique properties that help protect them from cellular insult and ensure their long lifespan. For instance, normal stem cells express high levels of ATP-binding cassette (ABC) transporters that facilitate rapid efflux of toxins and drugs, but these genes get turned off in committed progenitors and mature cells[66]. These transporters include ABCB1, which encodes P-glycoprotein, and ABCG2, which encodes a protein called breast cancer resistance protein (BCRP). These two transporter proteins can help a cell efflux a large number of different chemotherapeutic agents, including doxorubicin and paclitaxel. ABCC1, ABCB1 and ABCG2 represent the three principal multi-drug resistance (MDR) genes overexpressed in tumor cells[36,107-109]. This drug resistance could be an

inherent feature of CSCs, and could help explain the high level of drug resistance in metastatic disease[52,66].

Finally, stem cells are also thought to be more resistant to DNA damaging agents than differentiated cells because of their ability to undergo asynchronous DNA synthesis and their enhanced capacity for DNA repair. During asynchronous DNA synthesis, the parental 'immortal' DNA strand always segregates with the new stem cell rather than with the differentiating progeny, thus helping to protect the stem cell population from DNA damage[110-113]. Similarly, CSCs are believed to be resistant to radiation therapy by preferentially up-regulating their DNA proofreading mechanisms in order to avoid cellular death due to DNA damage[64,75]. Studies have shown that treating a tumor with radiation can deplete the non-CSC population and increase the CSC population by 3–5 fold, thus rendering the tumor even more aggressive and resistant to treatment[64]. It is also possible that CSCs may tend to be located in the hypoxic regions of tumors, which would affect their sensitivity to radiation via the oxygen enhancement ratio. It is more likely that radioresistance is a general property of CSCs, due to their ability to more efficiently repair their DNA than non-CSCs[114].

1.5 Therapeutic implications of the cancer stem cell hypothesis

Consideration of the CSC hypothesis in the context of metastasis has far-reaching implications to the way that we not only study cancer, but also how we treat it. Although some early stage cancers can be successfully treated by surgery, radiation and/or systemic cytotoxic therapy, the majority of current therapies fail in the metastatic setting[115-118]. Metastatic cells are often highly resistant to therapy, and this is reflected by the high mortality rate observed once a primary tumor has metastasized[118]. One of the major questions raised by CSC hypothesis is whether or not current therapies are, in fact, targeting the right cells. As discussed above, it has been speculated that CSCs have the ability to avoid or resist current cancer therapies. It has

also been hypothesized that the proportion of CSCs within a tumor may correlate with the severity of the cancer[64,114,119]. Tanei et al (2009) conducted a study looking at 108 breast cancer patients who received neoadjuvant paclitaxel and epirubicin-based chemotherapy[120]. When $ALDH1^+$ and $CD24^-CD44^+$ expression was compared between core needle biopsies (pre-treatment) and subsequent excision (post-treatment), there was a significant increase in $ALDH1^+$ cells, indicating that the proportion of stem-like cells increased following therapy[120]. It is possible that less aggressive cancers may be comprised of mostly therapy-sensitive non-CSCs, which may make early stage tumors more responsive to successful treatment with both chemotherapy and radiation. In contrast, more aggressive metastatic tumors may be mostly populated with therapy-resistant CSCs, making them extremely difficult to treat (*Figure 1.3*). In the next section, we consider potential therapeutic approaches for targeting CSCs.

1.5.1 Radiation therapy

A serious clinical problem associated with fractionated radiation therapy (RT) is accelerated re-population, or the increase in rate of growth as a result of time between treatments. During accelerated repopulation, each day of a treatment gap reduces the efficacy of radiation therapy by about 0.6 Gy, making it one of the major reasons for local failure of RT[75,114]. This may be explained by the CSC compartment within tumors. CSCs are believed to be resistant to radiation therapy by preferentially up-regulating their DNA proofreading and anti-apoptotic mechanisms in order to avoid cellular death due to DNA damage[64,75]. Studies have shown that treating a tumor with radiation can deplete the non-CSC population and increase the CSC population 3–5 fold, thus rendering the tumor even more aggressive and resistant to treatment[114].

In breast cancer model systems, $CD44^+CD24^{-/low}$ CSCs from MCF-7 and MDA-MB-231 cancer cell lines were isolated and subjected to a single dose of radiation[75]. The CSCs were observed to be more radioresistant, had fewer or no double stranded DNA breaks (or breaks were quickly repaired), and had a 50% lower dose-dependent formation of reactive oxygen

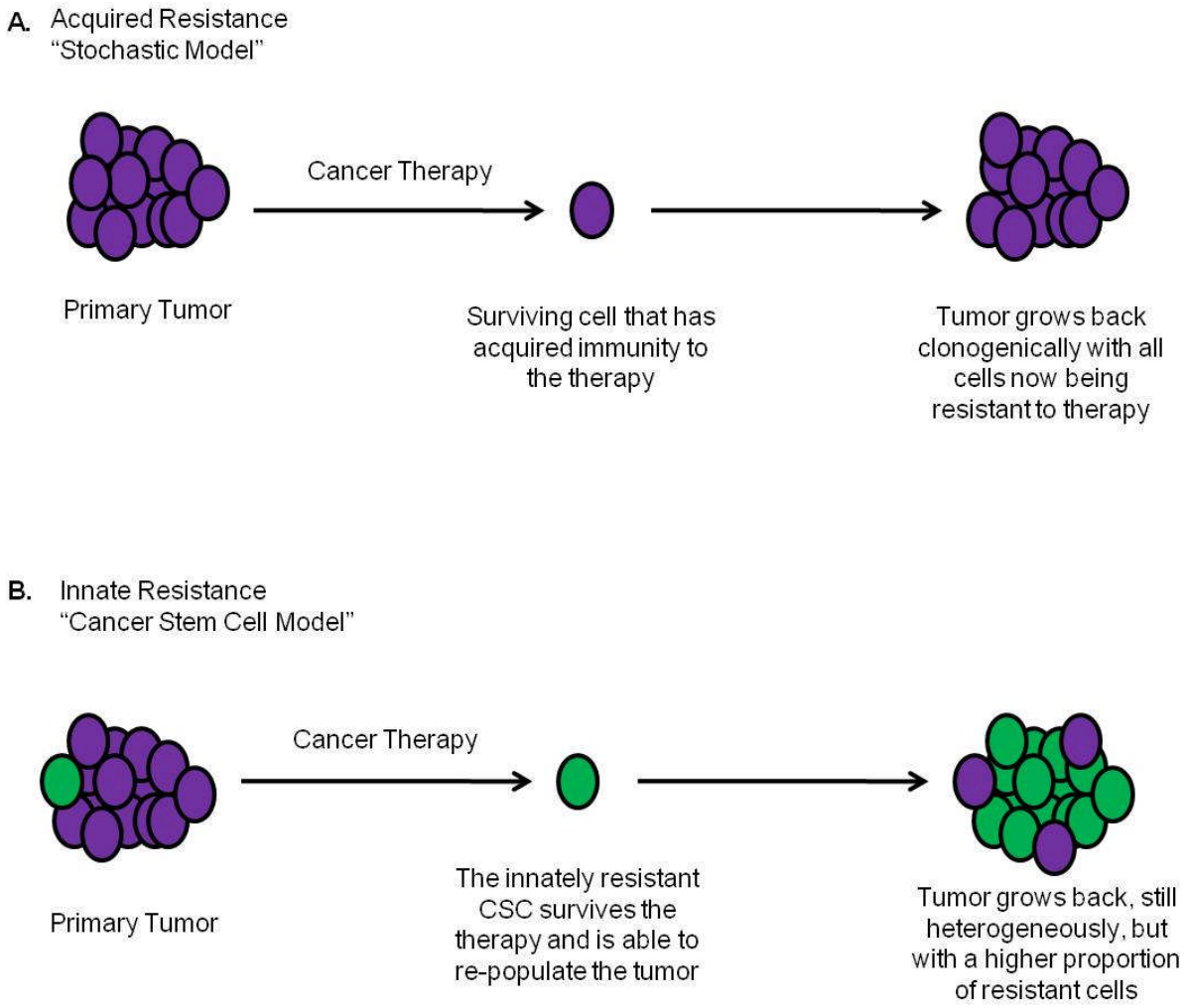


Figure 1.3. Therapeutic implications of the CSC hypothesis. A. In the traditional tumor model, when tumors are treated with chemotherapy, radiation therapy, or both, tumor relapse is explained by cells mutating and acquiring resistance to the therapy, which allows them to survive and re-populate a new, resistant tumor. **B.** The CSC hypothesis suggests that there is a small population of CSCs within tumors that are inherently resistant to cancer therapy. When tumors are treated with chemotherapy, radiation therapy, or perhaps even targeted therapy, the bulk of the tumor will shrink because the non-CSCs will die off, leaving behind the resistant CSCs that can easily repopulate the tumor, this time with a higher proportion of CSCs, rendering the tumor even more resistant to therapy.

species (ROS) in response to the radiation. In addition, the increase in the CSC population was associated with the activation of Notch-1 (important in specifying cell fate during development), so it is possible that CSCs may activate this developmental pathway in response to radiation[75,76]. Another elegant study by Bao et al. (2006) demonstrated that glioma cells expressing $CD133^+$ CSCs showed preferential survival following radiation treatment as compared to $CD133^-$ cells (non-CSCs)[64]. Interestingly, even after radiation of up to 5Gy, the $CD133^+$ CSCs retained a similar tumor formation ability and multi-lineage differentiation potential as the un-irradiated CSCs. The CSCs also demonstrated reduced apoptosis relative to non-CSCs, and this was supported by a decrease in caspase-3 activation and increased activation of the DNA checkpoint proteins Rad17, Chk1 and Chk2 in response to DNA damage by radiation[64]. Diehn et al. (2009) demonstrated that the CSC population in various tumor types contained an enhanced anti-oxidant defence system, which resulted in these cells experiencing lower levels of intracellular reactive oxygen species (ROS) and decreased DNA damage following radiation therapy[121]. Thus, in the face of radiotherapy, CSCs appear to survive better, repair their DNA more efficiently, and begin to self-renew in order to increase the CSC population within the tumor[64,75,76,114,121]. Ultimately, this may allow the tumor to become even more radioresistant.

1.5.2 Chemotherapy

Cancer cells most often become resistant to chemotherapeutic agents by inducing expression of ATP-dependent drug pumps which actively transport toxic substances out of cells, and can lead to multidrug resistance in many cancer cells[122-124]. There is evidence to suggest that the CSC compartment of many tumors inherently expresses high levels of drug resistance proteins compared to the rest of the cells in the tumor. For example, the $CD133^+$ CSCs within glioblastomas (GBM) were shown to be more resistant to chemotherapy, likely due to the high expression of breast cancer resistance protein-1 (BCRP1) and O6-methylguanine DNA methyltransferase (MGMT) that was observed in the CSC population[123]. In another GBM study,

researchers found that the CSCs within the tumor had both increased levels of multi-drug resistance-1 (MDR1) and increased resistance to chemotherapy[124]. In pancreatic tumors, it was observed that the $CD44^+$ CSCs were responsible for gemcitabine resistance[122]. Furthermore, $CD44^+$ breast cancer cells have also been shown to preferentially survive chemotherapy compared to the non-stem cancer cells[122]. Interestingly, it has been shown that the CD44 receptor actually interacts with MDR-1, indicating that CD44 itself may actively contribute to drug resistance of CSCs in various tumor types[125].

1.6 Targeting cancer stem cells

The use of gene expression arrays for identification of novel prognostic markers and/or new therapeutic targets for cancer has generally failed to account for the cellular heterogeneity present within tumors. Since the expression patterns of normal stem cells can vary significantly from their differentiated counterparts[126], it is probable that CSCs also have different expression characteristics than the bulk of the non-tumorigenic cell population. Thus, expression analysis of isolated, enriched populations of CSCs could potentially uncover better prognostic markers and more effective therapeutic targets than the current practice of analyzing mixed populations of cells present in tumor tissue. This idea is supported by a study by Liu et al. (2007), in which $CD44^+CD24^{low}$ 'tumorigenic' cells from breast tumor tissue were isolated and compared for gene expression differences relative to that of normal breast epithelium. Differentially expressed genes were used to generate a 186-gene 'invasiveness' gene signature which could be correlated with reduced overall survival and reduced metastasis-free survival in patients with breast or other types of cancer[127]. Furthermore, work done by Shipitstin et al (2007) demonstrated that compared to $CD24^+$ cells in breast tumors, $CD44^+$ cell-specific genes included many known stem-cell markers and correlated with decreased patient survival[128]. If it is indeed the CSC population that accounts for therapeutic resistance of tumors, then it is

essential to target these stem cell-like cells. Normal stem cells use various different mechanisms to protect themselves from cellular insult[108]. This next section details different stem cell pathways, the targeting of which may be of therapeutic benefit in treating tumors.

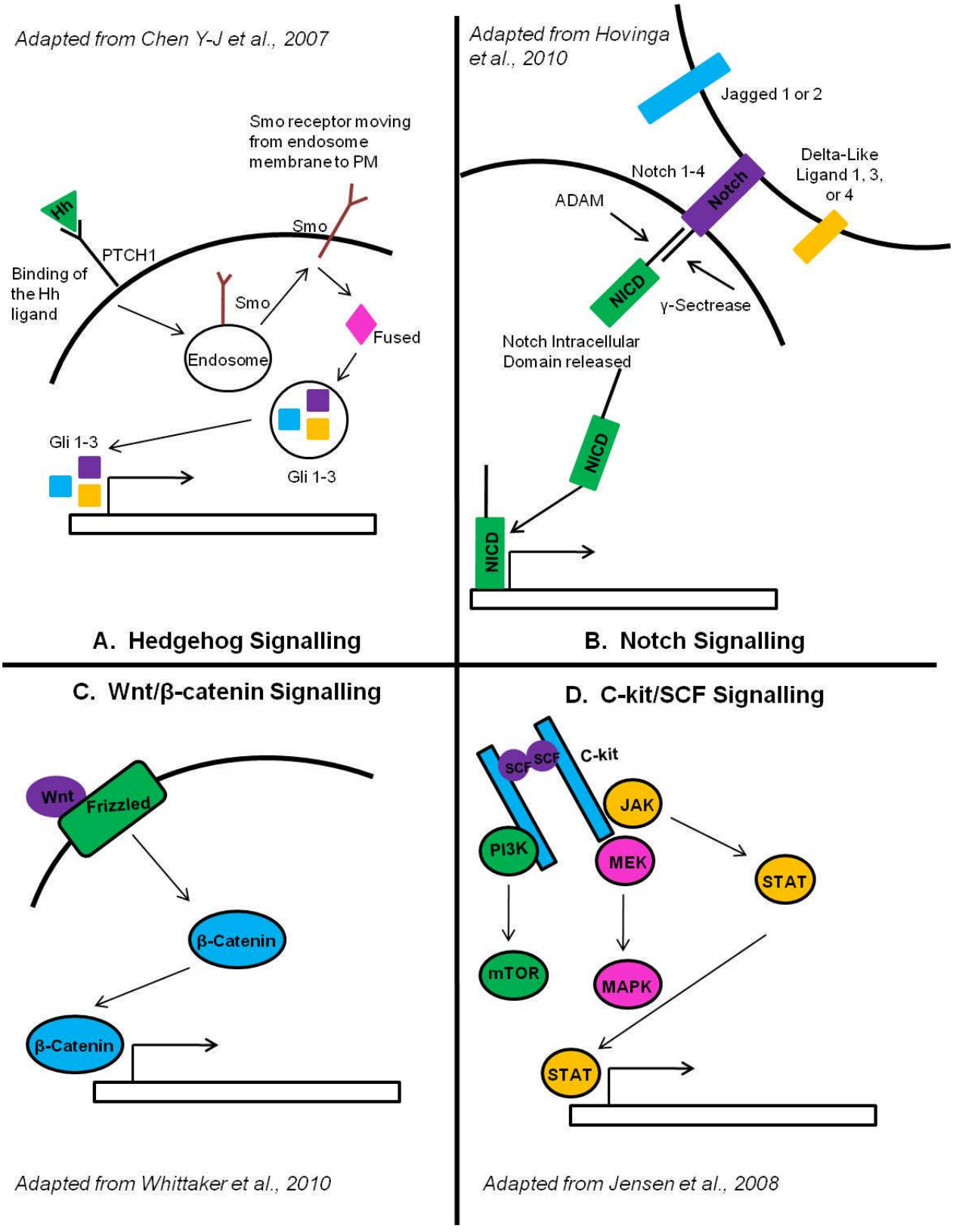
1.6.1 Targeting stem cell pathways

There is increasing evidence that stem-like cancer cells are responsible not just for tumor initiation and progression, but also for therapy resistance[64,66,129,130]. If activation of normal stem cell machinery in cancer cells contributes to or causes therapy resistance by inducing the CSC phenotype, then perhaps by inhibiting the normal stem cell pathways that are activated in cancer, we might be able to eliminate the CSC population, thereby sensitizing the cancer to conventional chemotherapy and radiation therapy. *Table 1.1* and *Figure 1.4* summarize some of the potential pathways that may be targeted for this purpose, however because these pathways are not the specific focus of this thesis, they will not be discussed in any further detail.

1.6.2 Differentiation therapy

It has been speculated that CSCs may be targeted using therapies designed to modify cell differentiation. Although differentiation therapy does not kill cancer cells, it does have the potential to restrain their self-renewal capacity and perhaps increase the efficacy of more conventional therapies (such as chemotherapy) which are often most effective on differentiated cells[131]. Furthermore, differentiation agents often have less toxicity than conventional cancer treatments[70,78]. This type of therapeutic strategy has had the greatest impact in hematologic malignancies such as leukemia, where the cancer-initiating cell and the cellular differentiation hierarchy are well-characterized[40,132,133]. The first differentiation agent found to be successful in the clinic was all-*trans*-retinoic acid (ATRA) in the treatment of acute promyelocytic leukemia (APL) (described in further detail below)[132-134]. It remains to be seen whether this type of approach would also be therapeutically advantageous for eliminating CSCs in solid cancers, either via direct targeting of CSCs or by targeting the metastatic niche in order to induce CSC

Figure 1.4. Targeting stem cell pathways. Stem cell signalling pathways identified in cancer. **A**. Hedgehog Pathway. Binding of the Hh ligand to the PTCH1 receptor causes Smo to be released from the endosome membrane so that it can be expressed at the plasma membrane (PM). Smo then activates the intracellular signalling molecule Fused, causing the release of the Gli family of transcription factors (Gli1-3) which can then translocate to the nucleus and activate gene transcription. **B**. Notch Pathway. Notch ligands bind to either Jagged or Delta-like membrane receptors, which leads to cleavage of the receptor by ADAM and γ-secretase, resulting in the release of the Notch intracellular domain (NICD). The NICD can then translocate to the nucleus where it recruits various co-activators and co-repressors leading to activation of target genes. **C**. Wnt/β-catenin Pathways. Wnt binds the Frizzled receptor, which causes a signalling cascade ending with β-catenin translocating to the nucleus and activating transcription. **D**. C-kit/SCF Pathway. When stem cell factor (SCF) binds Kit, this causes a homodimerization of two Kit molecules, which in turn, structurally changes the receptor resulting in activation of the Kit kinase domain. Phosphorylated tyrosine residues on Kit serve as binding sites for various cell signalling proteins, including members of the PI3-K/AKT, MAPK and the JAK/STAT pathways. From Croker & Allan; Chapter 23, in *Cancer Stem Cells in Solid Tumors*, 2011 and [135-138].


Adapted from Chen Y-J et al., 2007
Smo receptor moving from endosome membrane to PM
Hh
PTCH1
Smo
Binding of the Hh ligand
Smo
Endosome
Fused
Gli 1-3
Gli 1-3
A. Hedgehog Signalling
Adapted from Hovinga et al., 2010
Jagged 1 or 2
Delta-Like Ligand 1, 3, or 4
Notch 1-4
Notch
ADAM
NICD
γ-Sectrease
Notch Intracellular Domain released
NICD
NICD
B. Notch Signalling
C. Wnt/β-catenin Signalling
Wnt
Frizzled
β-Catenin
β-Catenin
Adapted from Whittaker et al., 2010
D. C-kit/SCF Signalling
SCF
SCF
C-kit
JAK
PI3K
MEK
STAT
mTOR
MAPK
STAT
Adapted from Jensen et al., 2008

differentiation.

1.6.3 Acute promyelocytic leukemia (APL): the differentiation therapy success story

Many acute myeloid leukemias (AMLs) are initiated by translocation events that fuse proteins involved in cell differentiation, apoptosis/cell survival, cell cycle control, and DNA binding[139-142]. AML-associated translocation products (AATPs) have been shown to activate Wnt signalling by increasing γ-catenin expression leading to stem cell self-renewal and accelerated cell cycle progression[142], indicating that these fusion proteins may work to give AML cells their blocked differentiation phenotype. Further support of this is found in the observation that many of the AATPs involve retinoic acid receptor α (RARα), which plays a major role in myeloid differentiation of HSCs[143-146].

The differentiation agent all-*trans* retinoic acid (ATRA) is used clinically in combination with chemotherapy to treat a specific kind of AML called acute promyelocytic leukemia (APL) because of its ability to down-regulate ALDH expression[147-149]. The increase in intracellular retinoic acid (RA) resulting from ATRA treatment suppresses levels of the ALDH isozymes ALDH1A1 and 3A1, driving differentiation of malignant promyelocytes into mature neutrophils and causing enhanced sensitivity to chemotherapy[150,151]. Cells with a PML:RARα translocation exert their pathogenic activity by recruiting histone deacetylases through nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT), activating the cyclin-dependent kinase activating kinase (CAK) complex to hyperphosphorylate RARα, and blocking terminal differentiation and apoptosis[152,153]. When cells are treated with ATRA, the RA induces ubiquitination-proteolysis of MAT-1, which results in a decrease of CAK-induced phosphorylation of RARα, promoting granulocytic differentiation[153,154]. In addition, ALDH has been shown to be involved in self-protection of normal and cancer stem cells in response to the chemotherapeutic drug cyclophosphamide, a common first-line agent for treating many cancers, including APL[128,129,147-149]. High ALDH activity renders cells exquisitely resistant to

cyclophosphamide (CP) therapy[155]. In the absence of ALDH, CP becomes phosphoramide mustard (PM) through an aldolphosphamide intermediary; the PM then acts to form DNA cross-links between and within DNA strands. In the presence of ALDH, however, aldolphosphamide gets oxidized to carboxyphosphamide, which is a harmless metabolite[152,156-159]. ATRA has therefore been used to treat these patients with great success[160-162].

1.6.4 Differentiation therapy in breast cancer: all-*trans* retinoic acid

Much less is known about the effect of differentiation therapy in solid tumors; however, there is considerable interest in ATRA treatment in breast cancer because of the way ATRA has been shown to inhibit cell growth. When MCF-7 breast cancer cells were treated with ATRA, cells accumulated in G_1 phase and by day 10, approximately 50% of the MCF-7 cells had died [163]. Interestingly, the ATRA-mediated growth inhibition in breast cancer cells correlated with the presence of functional estrogen receptors, and the action of ATRA was enhanced by the use of Tamoxifen.

Given the promising preclinical results with ATRA treatment in estrogen receptor positive (ER^+) breast cancer cell lines, breast cancer patients with measurable disease or evaluable non-measurable disease were given differing doses of ATRA (70-230mg/m^2/day) in alternating weeks during Tamoxifen treatment. Of the 7 patients with measurable disease, 2 experienced a partial response to the combination therapy of ATRA and Tamoxifen. Of the 18 patients with evaluable, non-measurable disease, 7 experienced a partial response for 6 months or more[164]. A separate Phase I/II clinical trial was initiated to investigate the use of ATRA in human metastatic breast cancer patients. In a single institution Phase II study, 17 patients with hormone refractory, metastatic breast cancer were administered 150mg/m^2 ATRA orally. Of those 17, only one patient experienced a partial response, which lasted only 4 months. Three other patients experienced stable disease for anywhere between 2-4 months[165]. These results indicate that ATRA may not be effective as a single agent, but may enhance the effects of chemotherapy or hormonal therapy in the treatment of breast cancer.

Although ATRA treatment in combination with cytotoxic therapy has been successful in APL, its success in treating other cancers has been limited, and in some cases, detrimental[166-169]. There have been a few Phase I/II studies looking at combining ATRA with Tamoxifen therapy in both estrogen receptor (ER) positive and negative breast cancers, with limited response rates, and, in fact, time to progression and survival rates were found no different than patients receiving paclitaxel alone[170]. Furthermore, although ATRA has been shown to decrease proliferation and induce apoptosis in MCF-7 ER positive breast cancer cells[171], no change in cell cycle kinetics or proliferation was seen when ATRA was added to Tamoxifen therapy in ER positive breast cancer patients[172]. Additionally, ATRA therapy was found to be difficult to tolerate due to many adverse side effects, and up to 1/3 of patients needed to be removed from the study due to these adverse effects[170]. Finally, and most disturbingly, the Phase III Beta-Carotene and Retinol Efficacy Trial (CARET) was stopped prematurely as results indicated that there was no beneficial effect of retinoid treatment, and in fact, the treatment group experienced a 28% higher incidence of lung cancers compared to the placebo group[166,167].

It is obvious that retinoic acid signaling is a complex and not yet entirely understood pathway, however, there is a great anti-cancer potential for retinoid therapies. The results of the above clinical trials have pointed out, however, that there is a desperate need to fully understand ALDH and the RA pathway before we can successfully utilize RA therapy.

1.7 Aldehyde dehydrogenase and retinoic acid signalling

1.7.1 Retinoid signaling pathways

The retinoic acid signalling pathway is a well-characterized differentiation pathway in many developmental systems, although it is best described in the hematopoietic system[173-177]. Briefly, aldehyde dehyogenase (ALDH) catalyzes the reaction of retinol to retinoic acid (RA), which then binds to a retinoic acid receptor (RAR) (*Figure 1.5*). RA binding causes a

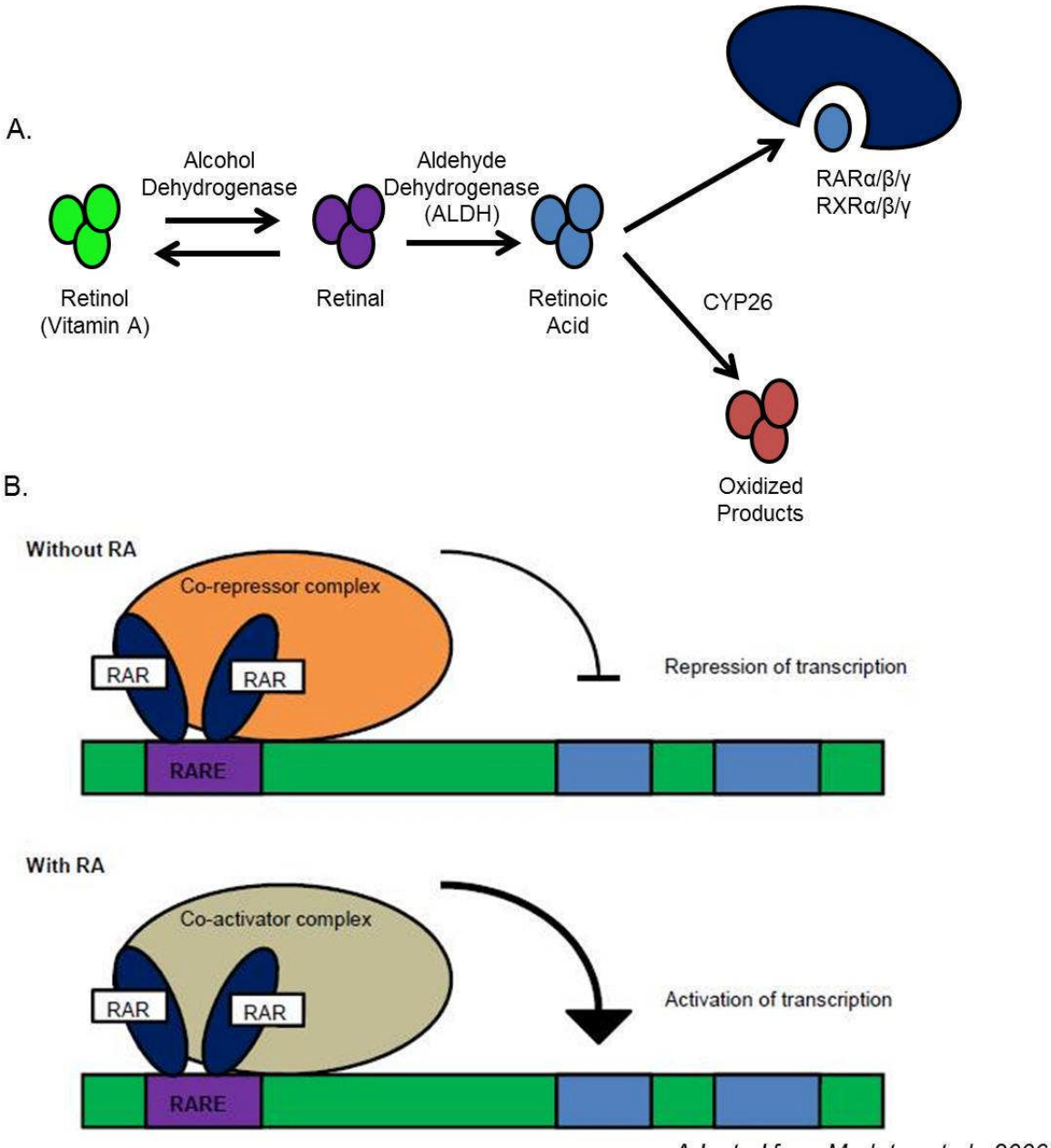
conformational change to the RAR which facilitates the release of a repressor protein and binding of an enhancer protein (i.e. C/EBPα and C/EBPƐ), which allows transcription of RARα/ß and targeting of differentiation proteins[154,178-183].

Retinoic acid has recently been found to signal through two different pathways, resulting in distinctly opposite outcomes[184] (*Figure 1.6*). Traditionally, it was thought that RA signaled through cellular retinoic acid-binding protein-2 (CRABP2), transporting RA to RA receptors (RARs) in the nucleus and causing transcription of genes resulting in cell differentiation, growth arrest, and reduced survival. More recently, an alternative pathway of RA action was identified involving binding and transport of RA into the cell nucleus by fatty acid binding protein-5 (FABP5), resulting in cell survival and proliferation via activation of the peroxisome proliferator-activated receptor (PPARδ/γ)[184].

1.7.2 The ALDH superfamily

The human ALDH superfamily currently consists of 19 known functional genes in 11 families and 4 subfamilies with distinct chromosomal locations[185-188]. The ALDH enzymes can be found in the cytosol, nucleus, mitochrondria, and endoplasmic reticulum[186,188,189]. ALDH enzymes are involved in detoxification and/or bioactivation of various intracellular aldehydes to the corresponding acids in a $NAD(P)^+$-dependent manner. Aldehydes are highly reactive electrophilic compounds that have a long lifespan, and, while they can play a vital role in physiological processes, they also have the ability to cause mutagenic, carcinogenic, and cytotoxic detrimental effects[186-188]. Of particular biological importance, the ALDH1 family of enzymes (namely ALDH1A1 and ALDH1A3) plays an important role in oxidizing vitamin A (retinal) to retinoic acid (RA) through an alcohol intermediary (*Figure 1.5*). RA functions as a ligand for nuclear retinoid receptors (RARs) and leads to transactivation and/or transrepression of target genes, and is finally degraded by CYP26 enzymes. In addition to the oxidization of aldehydes, other functions of the human ALDH superfamily of enzymes include ester hydrolysis

Figure 1.5. Aldehyde dehydrogenase and the retinoic acid signalling pathway. **A.** The metabolic pathway for synthesis and degradation of endogenous RA. ALDH synthesizes RA by oxidizing retinal. RA is then free to interact with the RARs. Endogenous RA is degraded by CYP26 enzymes. **B.** The RARs, positioned on the retinoic acid response element (RARE), mediate the effects of RA. In absence of the RA ligand, the RAR dimer is bound to DNA and co-repressors, which induces transcriptional repression of RAR-induced gene expression through histone deacetylation. Binding of RA induces a conformational change, allowing the binding of co-activators and leading to activation of transcription. From Croker & Allan; Chapter 23, in *Cancer Stem Cells in Solid Tumors*, 2011 and [190].



Adapted from Marletaz et al., 2006

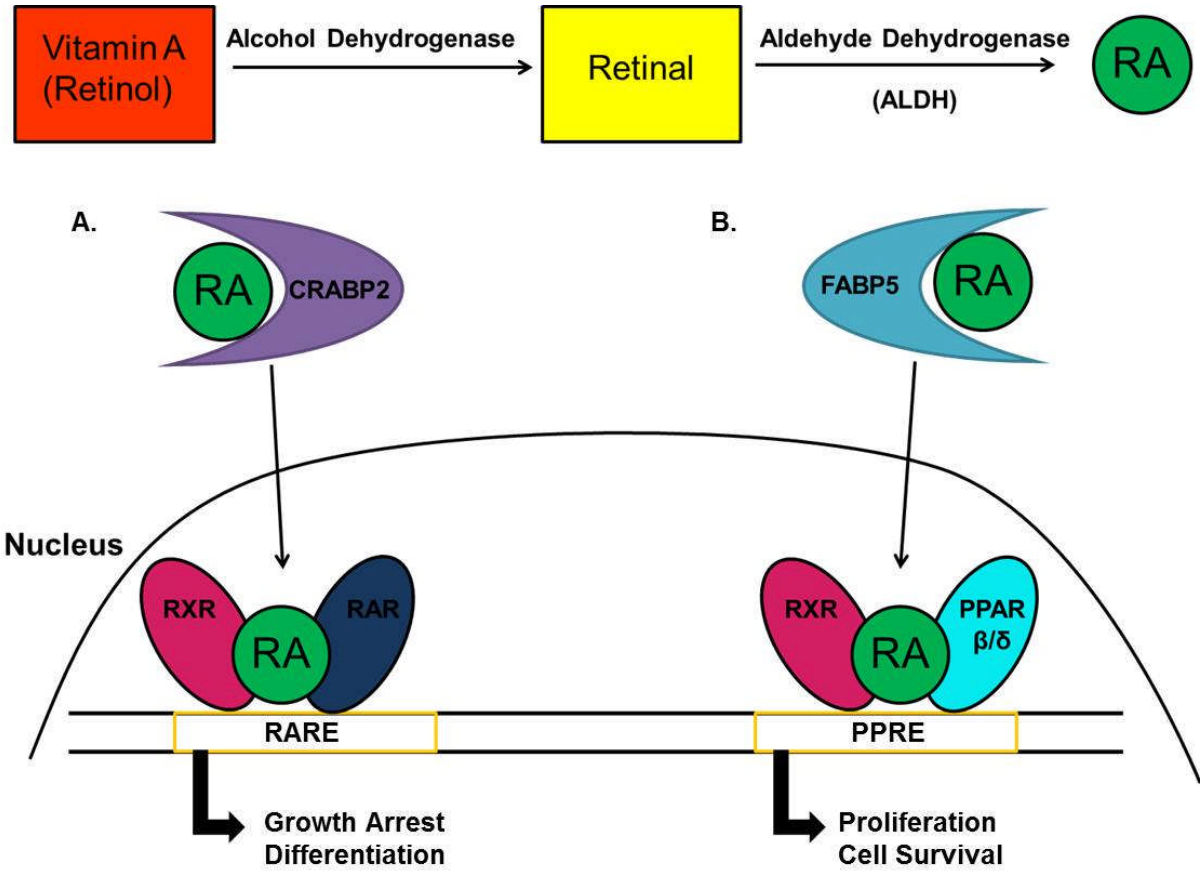


Figure 1.6. The dual effects of retinoic acid signalling. Vitamin A is oxidized to RA through an alcohol intermediate in the cytosol, and is then shuttled into the nucleus by a chaperone protein. The effects of RA signaling depend on the chaperone protein used. **A.** When RA is shuttled by CRABP2, RA binds with RARs and/or RXRs on the retinoic acid response element (RARE) and causes transcription of genes that cause differentiation. **B.** When RA is shuttled by FABP5, RA binds with RXRs and PPARβ/γ on the peroxisome proliferator-activated receptor response element (PPRE) and causes the transcription of genes involved in cell survival and proliferation. Adapted from Schug et al., 2007[184].

(ALDH1A1, ALDH2, ALDH4A1), nitrate reductase activity (ALDH2), and various antioxidant functionalities (ALDH1A1, ALDH3A1)[186-188].

1.7.3 Assessing ALDH activity and expression

In order to identify $ALDH^+$ cells, many methods have been utilized over the years. Initially, $ALDH^+$ cells were identified based on immunoblotting, or by studying the activity of ALDH using enzyme kinetics, where the rate of reduction of NAD^+ substrate to NADH by ALDH present in cell lysates can be measured at 37°C at a wavelength of 340 nm[191-194]. However, these methods require cell lysis, and in order to study $ALDH^+$ cells, it is necessary to be able to identify and recover $ALDH^+$ cells while still intact and viable. Currently, the "gold standard" for studying ALDH activity in viable cells has been the use of a flow cytometric assay using fluorescent substrates for ALDH[58,195-200]. Jones et al. (1995) first reported that intracellular ALDH1 activity could be measured in viable cells. They found that dansyl aminoacetaldehyde (DAAA), a fluorescent aldehyde, could be used in flow cytometry to isolate and enrich for viable human hematopoietic stem cells (HSCs) based on their ALDH1 activity[201]. Hydrophobic DAAA can diffuse freely across cell membranes, so cells with ALDH1 activity can oxidize DAAA into dansyl glycine. Dansyl glycine is negatively charged at physiological pH, and therefore is not able to exit the cells, thus causing the cells with dansyl glycine to become fluorescent[201]. As a negative control, cells were incubated with 4-diethylamino-benzaldehyde (DEAB), a specific ALDH1 inhibitor[201,202]. The use of this technique, however, required that the DAAA fluorescence be excited by UV emissions, which are potentially mutagenic for cells that would be isolated and used for downstream applications. In addition, the emission spectra of DAAA overlapped with other fluorochromes, making this technique difficult to combine with analysis of other stem cell markers[201,203].

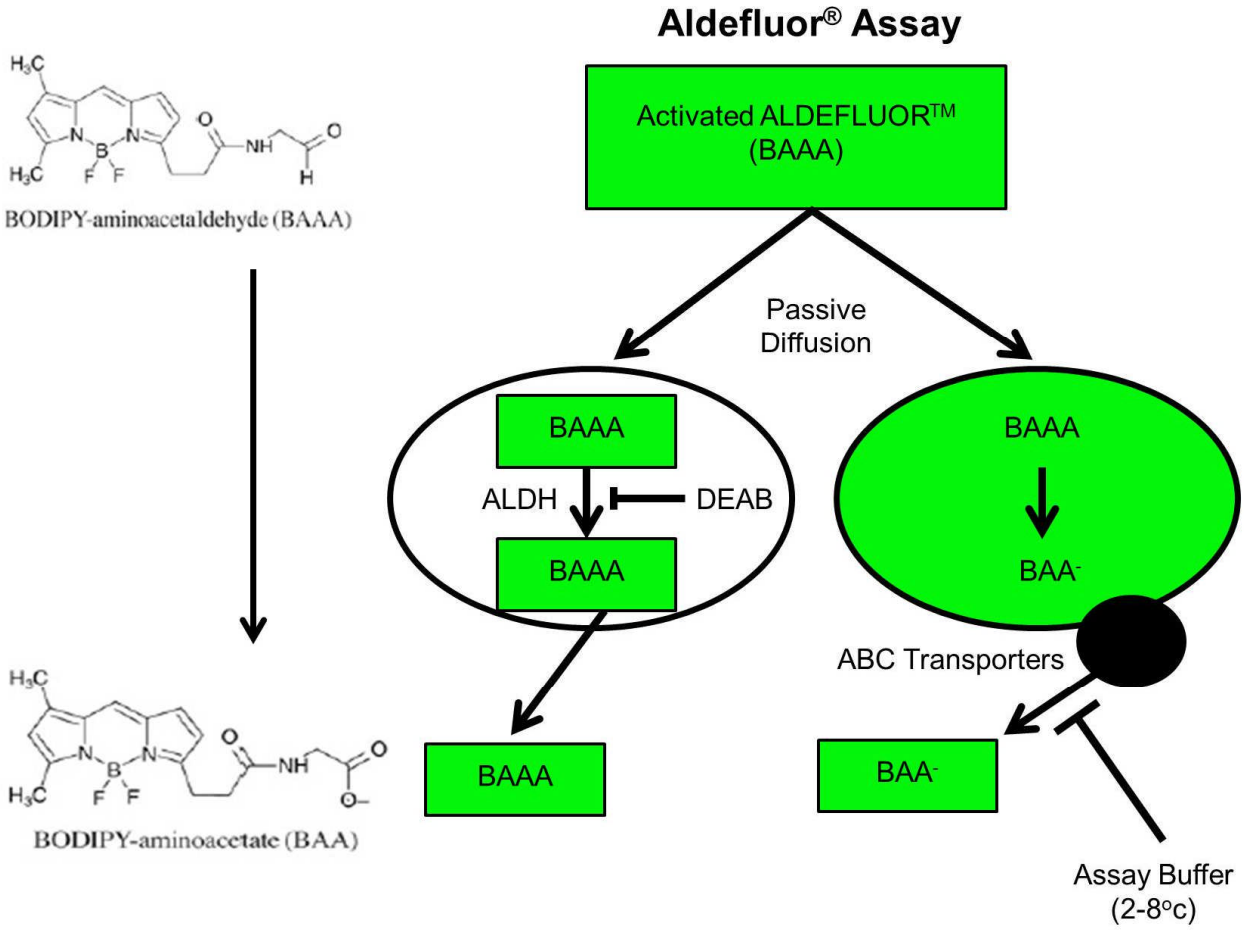
In response to this, Storms, et al. (1999) developed a more straightforward and efficient strategy for isolating primitive HSCs using the fluorescent substrate BODIPY-aminoacetaldehyde (BAAA)[203], more commonly known now as the Aldefluor® Assay (StemCell

Technologies, Vancouver, BC)[58,196-198,204] (*Figure 1.7*). The basis for this assay is that uncharged ALDH substrate (BODIPY-aminoacetaldehyde [BAAA]) is taken up by living cells via passive diffusion. Once inside the cell, BAAA is converted into negatively-charged BODIPY-aminoacetate (BAA^-) by intracellular ALDH. BAA^- is then retained inside the cell, causing the cell to become highly fluorescent. Only cells with an intact cell membrane can retain BAA^-, so only viable cells can be identified [203]. DEAB is again used as a control as it is a potent ALDH inhibitor. Cells are then analyzed using a flow cytometer, and generally populations in the top 10–20% have been considered $ALDH^{hi}$, whereas populations in the bottom 10–20% can be considered $ALDH^{low}$. These $ALDH^{hi}$ populations can be distinguished easily and specifically via comparison to the DEAB negative control[129,198,203].

1.7.4 ALDH in normal stem cells

Aldehyde dehydrogenase is highly expressed in hematopietic stem cells (HSCs)[197,203,205-207]. In fact, it has been shown that high ALDH expression correlated to more primitive HSCs than any HSC marker (i.e. $CD34^+/CD38^-$, $CD133^+$), and that most $ALDH^{hi}$ cells also co-express the CD34 and/or CD133 stem cell markers[39,206,208,209]. ALDH is an ideal candidate for identifying HSCs because it identifies stem cells on a functional level rather than relying on makers which can fluctuate depending on cell cycle and the microenvironment[206,208]. Interestingly, it was found that $ALDH^{hi}CD133^+$ cells had more efficient homing to BM, multilineage hematopoietic repopulation, and maintenance of the $CD34^+CD38^-$ stem cell phenotype compared to $ALDH^{hi}CD133^-$ cells, indicating that using both ALDH and CD133 in combination could serve to identify the most primitive HSCs with the highest engraftment potential[207]. ALDH can also be used to identify and isolate stem/progenitor cells in skeletal muscle[210,211], pancreatic cells[212,213], and prostate epithelium[195,212]. In addition, primitive neural stem cells can be isolated from adult and embryonic murine neurospheres and dissociated tissue based on the expression of high levels of ALDH activity[197]. Recently, liver progenitor cells were also shown to be isolated based on high ALDH activity. Furthermore, these $ALDH^{hi}$ cells exhibited an enrichment in the

Figure 1.7. The Aldefluor® Assay (StemCell Technologies, Vancouver, BC, Canada). The Aldefluor assay is a fluorometric assay that detects the enzymatic activity of aldehyde dehydrogenase-1 (ALDH1). The basis for this assay is that uncharged ALDH substrate (BODIPY-aminoacetaldehyde [BAAA]) is taken up by living cells via passive diffusion. Once inside the cell, BAAA is converted into negatively-charged BODIPY-aminoacetate (BAA^-) by intracellular ALDH. BAA^- is then retained inside the cell, causing the cell to become highly fluorescent. Only cells with an intact cell membrane can retain BAA^-, so only viable cells can be identified[203]. The active removal of BAA^- by ATP Binding Cassettes is quenched through the use of the assay buffer and through incubation of cells between 2-8^oC. As a negative control, the activity of ALDH is quenched by the addition of diethylaminobenzaldehyde (DEAB), and the fluorescence of these cells is read by flow cytometry. The population observed in the DEAB sample is used to create the gate for the $ALDH^+$ cells, whereby cells are only included if they demonstrate higher levels of fluorescence compared to the DEAB sample. Adapted from Ma and Allan[188], *Stem Cell Reviews and Reports*, 2011 and Aldagen's Information Sheet on the Aldefluor® Assay (www.stemcell.com).



Adapted from Ma and Allan, *Stem Cell Reviews and Reports*, 2011 and the Aldagen's Information Sheet on the Aldefluor® Assay (www.stemcell.com).

expression of liver stem cell markers such as EpCAM, CK19, CD133, and Sox9[214]. Furthermore, ALDH, potentially through its role in the formation of RA has been linked to cellular differentiation during development, and plays a key role in stem cell self-protection throughout an organisms' lifespan[205].

In the human mammary gland, high ALDH activity has been used to isolate luminal progenitor cells[215]. Additionally, work by Ginestier et al. (2007) demonstrated that in tissue from reduction mammoplasties, there was a small population (approx. 8%) of $ALDH^+$ mammary epithelial cells[58]. Furthermore, this $ALDH^+$ cell population demonstrated functional characteristics associated with adult stem cells such as a high mammosphere-forming ability and self-renewal capacity; whereas the $ALDH^-$ population did not. In an assay designed to assess the lineage differentiation potential of single cells, the $ALDH^+$ cells were shown to be enriched in bilineage progenitor cells which were able to generate uncommitted progeny, myoepithelial, and luminal epithelial cells[58]. Moreover, when $ALDH^+$, $ALDH^-$, and unsorted cells were transplanted into humanized cleared mammary fat pads of NOD/SCID mice, only $ALDH^+$ and unsorted cells had outgrowth potential, as duct formation was observed following injection of 25,000 cells[58]. Additionally, the $ALDH^+$ cell population demonstrated an enriched *in vivo* outgrowth capacity as these cells consistently generated 10-fold more ducts in the humanized area of the mammary fat pad compared to the unsorted population. Interestingly, the $ALDH^-$ population failed to repopulate the fat pads, even when 50,000 cells were injected. Taken together, these results suggest that $ALDH^+$ cells represent the cell population with the broadest differentiation potential *in vitro* and highest growth potential *in vivo*[58].

In terms of protecting normal stem cells from insult, ALDHs are generally regarded as detoxification enzymes that are critical for protecting organisms against various aldehydes that would be otherwise be harmful[186,187,216]. As previously mentioned, ALDH protects cells from cyclophosphamide therapy, and has been implicated in protecting cells from electrophillic substrates such as ROS[265,298]. Furthermore, it has been found recently that human myoblasts

with a high ALDH1 activity can confer hydrogen-peroxide mediated cytotoxicity resistance *ex vivo*, as well as enhancing cellular viability and promoting engraftment *in vivo* upon transplantation into the muscle of SCID mice[210]. Finally, deficiencies and polymorphisms of various ALDH enzymes can lead to clinical phenotypes and diseases (i.e. spina bifida (ALDH1A2)[217], ethanol-induced cancers[218] and hypertension (ALDH2)[219], Sjögren-Larsson syndrome (ALDH3A2)[220], type II hyperprolinemia (ALDH4A1)[221], γ-hydroxybutyric aciduria (ALDH5A1)[222], and pyridoxine-dependent epilepsy (ALDH7A1)[223], indicating that ALDH may play an indispensible role in stem cell self-protection[186,189,216].

1.7.5 ALDH in cancer

The activity of cytosolic ALDH1 has been shown to be a reliable marker of not only normal stem cells in various systems, but also markers of CSCs in several types of solid tumors, including tumors of the head and neck, lung, liver, pancreas, cervix, ovaries, breast, prostate, colon, and the bladder regions[58,120,195,204,224-236]. In cancer, high ALDH1 expression has been shown to correlate with poor prognosis in breast cancer patients[58], and has been associated with metastasis development and poor clinical outcome[237-239]. Furthermore, an interesting study by Marcato et al. (2011) demonstrated that ALDH1A3 expression in patient breast tumors correlates significantly with tumor grade, metastasis, and cancer stage[240], while

ALDH1A1 expression did not, indicating that even within the ALDH1 family, the isoforms may function differently. It has also been shown that high activity of ALDH1 is associated with poor prognosis in breast, bladder and prostate cancer patients[58,120,228,231,232,241]. In two independent studies analyzing 163 and 269 primary prostate cancer patient samples respectively, it was shown that patients with high ALDH1A1 expression correlated with lower overall survival[228,231], a higher Gleason score, and a worse pathologic stage[228]. It has also been shown that cancer stem cells in adenoid cystic carcinoma[233], head and neck squamous cell carcinoma[226], lung cancer[234], pancreatic adenocarcinoma[231], and cervical carcinoma[224] that highly express ALDH1 are highly tumorigenic and have enhanced stem cell characteristics *in*

vitro and *in vivo* compared to cells with low ALDH1 activity. In breast cancer , Ginestier, et al. (2007) demonstrated that when 50,000 $ALDH1^-$ breast cancer cells were transplanted into humanized cleared mammary fat pads of NOD/SCID mice, no tumor was formed, but when as few as 500 $ALDH1^+$ cells were injected, tumors were formed within 40 days, indicating that $ALDH^+$ stem-like breast cancer cells are highly tumorigenic[58].

1.7.6 ALDH as a potential therapeutic target in cancer

A study by Emmink et al (2011) demonstrated that the resistance of colorectal tumors to irinotecan (a topoisomerase I inhibitor) required the cooperative action of tumor initiating $ALDH^{hi}/ABCB1^-$ cells and their differentiated, drug-expelling, $ALDH^{low}/ABCB1^+$ daughter cells[242]. In intestinal tumors, normal intestinal stem cells have been discovered to be the cells of origin of intestinal tumors, and they too express low levels of ABC transporters. In this case, differentiated $ABCB1^{hi}$ cells are the lumen-forming cells in colon carcinomas and may keep the intratumor drug concentration low by expelling irinotecan into this lumen. In doing so, $ABCB1^{hi}/ALDH^{low}$ cells may provide protection to tumor-initiating $ABCB1^{low}/ALDH^{hi}$ cells that lack drug expelling capacity[242]. This study suggests that ALDH acts neither as a marker of therapy-resistant cells, nor as a player in therapy resistance; however, most studies suggest otherwise. For example, Canuto et al. (2001) demonstrated that compared to normal liver cells, hepatomas have increased expression of both ALDH1 and ALDH3 cytosolic enzymes. Furthermore, they demonstrated that inhibiting either ALDH1 or ALDH3 decreases cell growth in hepatomas, suggesting that ALDH activity is correlated with cancer cell growth[243]. Along those lines, Muzio et al. (2004) demonstrated that $ALDH3^{hi}$ hepatoma cells experienced a significant decrease in proliferation following ALDH3 inhibition[244]. Interestingly, the intracellular signaling transduction pathway affected by the accumulation of aldehydes due to the decrease in ALDH3 activity derived from lipid peroxidation was the MAPK cascade. More specifically, an increase in the aldehyde concentration led to a decrease in phosphorylated ERK1,2 and Raf-1[244].

In lung cancer, $ALDH^+$ lung cancer cells were more resistant to gefitinib compared to $ALDH^{low}$ cells and gefitinib resistant cell lines have a higher proportion of $ALDH^+$ cells[245]. Furthermore, $ALDH^+$ CSCs were shown to display longer telomeres than the $ALDH^{low}$ cell population[246]. Interestingly, MST312 (a novel telomerase inhibitor) demonstrated a strong anti-proliferative effect on lung $ALDH^{hi}$ cancer cells, and induced p21, p27, and apoptosis in the whole tumor population. MST312 acts both through activation of the ATM/p-H2AX DNA damage pathway (short-term effect), and also through decrease in telomere length (long-term effect). Administration of MST312 in a xenograft model resulted in significant tumor shrinkage (70% reduction compared to controls). Furthermore, treatment with MST312 significantly reduced the number of $ALDH^+$ CSCs and their telomere length *in vivo*, suggesting that MST312 may target $ALDH^{hi}$ CSCs[246].

In Ewing's sarcoma, $ALDH^{hi}$ cells were demonstrated to be more resistant to chemotherapy (doxorubicin and etoposide) than $ALDH^{low}$ cells, but in this case, it was noted that $ALDH^{hi}$ cells also expressed ABC transporters, namely ABCB1. When $ALDH^{hi}$ cells were treated with both verapamil (an ABC inhibitor) and doxorubicin, this caused a significant sensitization of $ALDH^{hi}$ cells to the doxorubicin, an 80% reduction in cell survival compared to doxorubicin alone[247].

Finally, using GSEA (gene set enrichment analysis) algorithm, Ginestier et al. (2007) were able to show that when various breast cancer cell lines were treated with ATRA, the genes that were downregulated were associated with pathways related to stem cell self-renewal programs, Wnt signaling, ATK/β-catenin, the carcinogenesis process, metastatic activity, and drug resistance[248]. The results of their study also suggest that ATRA treatment may induce breast CSC differentiation and decrease the CSC population. Conversely, genes that were overexpressed in DEAB-treated breast cancer cell lines were involved in tRNA biosynthesis, which is essential for protein synthesis and cell viability[248].

It has been reported that the difficulty in successfully eradicating CSCs originates from the expression of many CSC self-protection mechanisms, including ALDH expression[249-253]. Most of what we know about ALDH and therapy resistance is correlative, in that as cells become more resistant to therapy, there is a higher expression of ALDH1 observed in tumors, and high ALDH1 expression has been correlated with poor patient outcome[58]. This could mean that either ALDH selects for cells that contain therapy resistance mechanism(s), and/or that ALDH contributes functionally to aggressive cell behavior and therapy resistance.

1.8 Overall Rationale

Breast cancer metastasis is a leading cause of death in women. The main lethality associated with breast cancer occurs once the disease has metastasized, since current cancer therapies are non-curative in the metastatic setting. Despite being an entirely lethal process, metastasis is remarkably inefficient, with less than 1% of cells being able to successfully navigate the entire cascade in order to form clinically relevant macrometastases. However, the ability to specifically identify and target this deadly subset of cells has remained elusive. Intriguingly, the qualities of successful metastatic cells are strikingly reminiscent of normal stem cell behaviour, suggesting a possible link between metastasis, therapy resistance, and the cancer stem cell (CSC) hypothesis.

In light of several pivotal studies demonstrating the existence of CSCs in breast and other solid cancers, we believe that these “stem-like” cells may also represent the subset of tumor cells responsible for metastatic disease. However, while previous studies have demonstrated the ability of CSCs to initiate and maintain a primary tumor, the functional and mechanistic contribution of CSCs to metastasis remains poorly understood. Furthermore, while there is growing evidence to support the idea that CSCs play a role in response to breast cancer treatment, this concept requires further investigation.

ALDH is an enzyme that has been implicated in many cellular processes including cellular proliferation and therapy resistance, potentially through its ability to oxidize and detoxify various intracellular aldehydes. In a parallel role, ALDH1 has recently been shown to regulate hematopoietic stem cell differentiation, proliferation, and lineage commitment, as well as being highly expressed in undifferentiated human mammary stem/progenitor cells. In cancer, ALDH has been used as a marker to identify aggressive, stem-like cells, and its expression has been correlated with worse patient prognosis and an increased incidence of metastatic disease. However, it remains unclear whether ALDH is simply a marker of aggressive cancer cells, or whether it in fact plays a functional role in both breast cancer metastasis and response to treatment.

1.9 Overall Hypothesis and Objectives

The ***overall hypothesis*** of this thesis is that human breast cancer cells with a stem-like phenotype ($ALDH^{hi}CD44^{+}$) contribute functionally to breast cancer metastasis and therapy response; and that ALDH1 is a key player in these processes.

In order to test this hypothesis, the ***specific objectives*** of this thesis are:

1. To determine the functional role of $ALDH^{hi}CD44^{+}$ cells in breast cancer metastasis.
2. To determine the functional role of $ALDH^{hi}CD44^{+}$ cells in breast cancer therapy resistance.
3. To investigate the functional role and underlying mechanisms of ALDH1 in mediating breast cancer metastasis and therapy resistance.

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Chapter 2

High aldehyde dehydrogenase and expression of cancer stem cell markers selects for breast cancer cells with enhanced malignant and metastatic ability

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Abstract

Cancer stem cells (CSCs) have recently been identified in leukemia and solid tumors; however, the role of CSCs in metastasis remains poorly understood. This dearth of knowledge about CSCs and metastasis is due largely to technical challenges associated with the use of primary human cancer cells in preclinical models of metastasis. Therefore, the objective of this study was to develop suitable preclinical model systems for studying stem-like cells in breast cancer metastasis, and to test the hypothesis that stem-like cells play a key role in metastatic behavior. We assessed four different human breast cancer cell lines (MDA-MB-435, MDA-MB-231, MDA-MB-468, MCF-7) for expression of prospective CSC markers CD44/CD24 and CD133, and for functional activity of aldehyde dehydrogenase (ALDH), an enzyme involved in stem cell self-protection. We then used fluorescence-activated cell sorting (FACS) and functional assays to characterize differences in malignant/metastatic behavior *in vitro* (proliferation, colony-forming ability, adhesion, migration, invasion) and *in vivo* (tumorigenicity and metastasis). Sub-populations of cells demonstrating stem-cell like characteristics (high expression of CSC markers and/or high ALDH) were identified in all cell lines except MCF-7. When isolated and compared to $ALDH^{low}CD44^{low/-}$ cells, $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) and $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) cells demonstrated increased growth ($p<0.05$), colony formation ($p<0.05$), adhesion ($p<0.001$), migration ($p<0.001$), and invasion ($p<0.001$). Furthermore, following tail vein or mammary fat pad injection of NOD/SCID/IL2γ receptor null mice, $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ cells showed enhanced tumorigenicity and metastasis relative to $ALDH^{low}CD44^{low/-}$ cells ($p<0.05$). These novel results suggest that stem-like $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ cells may be important mediators of breast cancer metastasis.

2.1 Introduction

Breast cancer is a leading cause of death in women, due primarily to ineffective treatment of metastasis. Metastasis is a multi-step process that involves tumor cell escape from the primary tumor, migration through the body, adhesion and extravasation at the secondary site, initiation of micrometastatic growth, and maintenance of growth into clinically detectable macrometastases[1]. Given the onerous nature of this process, it is not surprising that metastasis is highly inefficient, with the main rate-limiting steps being initiation and maintenance of growth at secondary sites[1-3]. Taken together with the heterogeneous nature of solid tumors, this metastatic inefficiency suggests that only a small subset of cells can successfully navigate the metastatic cascade and eventually re-initiate tumor growth to form metastases. However, the ability to specifically identify and target this deadly subset of cells has remained elusive.

In light of several pivotal studies demonstrating the existence of "stem-like" cells or cancer stem cells (CSCs) in leukemia[4,5] and several types of solid cancer[6-11], we and others have hypothesized that CSCs may represent the subset of tumor cells responsible for metastatic disease[12,13]. However, while several studies have demonstrated the ability of CSCs to initiate and maintain a primary tumor[6-11], the functional contribution of CSCs to metastatic behavior remains poorly understood.

In breast cancer, stem-like cells have been prospectively isolated from primary tumors and pleural effusions based on a $CD44^+CD24^-$ phenotype[6]. Subsequent experimental studies have also isolated $CD44^+CD24^-$ breast cancer cells and demonstrated increased *in vitro* expression of stem cell markers and enhanced capacity for mammosphere formation, invasion, and resistance to radiation[14-16]. Furthermore, clinical studies indicate that $CD44^+CD24^-$ tumor-initiating cells express an invasive gene signature and may be associated with distant metastases[17-19]. Additional putative CSC phenotypes have also been identified in other solid cancers, including $CD133^+$ (brain, colon, pancreatic cancer)[8,9,11] and $CD44^+CD133^+$ (prostate

cancer)[7]. However, because of the heterogeneous nature of solid cancers, the reliability of using cell surface markers as the sole way to isolate CSCs remains controversial[20-23]. Furthermore, human stem cell sources (including tumors) may contain alternate stem/progenitor cell lineages not efficiently isolated using variably expressed cell surface markers.

A complementary strategy for identifying stem-like tumor cells involves measurement of aldehyde dehydrogenase (ALDH) activity, an enzyme involved in intracellular retinoic acid production[24]. Retinoic acid signaling is linked to cellular differentiation during development and plays a role in stem cell self-protection throughout an organism's lifespan[25]. We and others have previously used this isolation strategy alone or in combination with cell surface markers (i.e. CD133) to successfully isolate normal human hematopoietic progenitor cells[26-28], and ALDH activity has also been used to identify stem-like subsets in human hematopoietic cancers[29-31]. A recent study by Ginestier et al. (2007) elegantly demonstrated that expression of ALDH1 in breast tumors was a predictor of poor clinical outcome, and that high ALDH activity selects for both normal and tumorigenic human mammary epithelial cells with stem/progenitor properties[32]. Therefore, the use of ALDH activity as a purification strategy allows non-toxic and efficient isolation of human stem-like cells based on a developmentally conserved stem/progenitor cell function.

While there is growing evidence supporting the existence of CSCs in solid tumors, what remains less clear is the impact of CSCs on metastatic behavior. We believe that the dearth of knowledge about CSCs and metastasis is due largely to technical challenges associated with the use of primary human cancer cells in preclinical models of metastasis: even in NOD/SCID mice, it is very difficult to grow primary cells as xenograft tumors, much less as metastases. Therefore, workable alternative model systems must be developed in order to address this need. It has long been observed by tumor biologists that even with cancer cell lines, large numbers of cells need to be injected to form a tumor and/or metastasize in experimental animals[1,2,33]. This suggests that not all cells within a cell line population are equal, and only a

small subset of cells is capable of tumor initiation and progression to metastasis. Indeed, studies have shown that commonly used cell lines contain subpopulations of cells with stem-like properties[14-16]. Thus, the purification of stem-like cells from cell lines may provide a valuable model system for starting to investigate the role of such cells in breast cancer metastasis.

In the current study, we assessed four commonly used human breast cancer cell lines (including highly metastatic MDA-MB-435 [see note in Materials and Methods], moderately metastatic MDA-MB-231, weakly metastatic MDA-MB-468, and non-metastatic MCF-7) for expression of the prospective CSC markers CD44/CD24 and CD133, and for functional activity of ALDH. We then used fluorescence-activated cell sorting (FACS) and standard functional assays to characterize differences in malignant/metastatic behavior between cell populations, including cell growth, adhesion, migration, and invasion *in vitro*, and tumorigenicity and metastasis *in vivo*. The novel findings presented here suggest that high ALDH activity and CSC marker expression select for stem-like breast cancer cells with enhanced malignant and metastatic properties, and that $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ stem-like cells may be important contributors to breast cancer metastasis.

2.2 Materials and Methods

2.2.1 Cell culture

MDA-MB-231 and MCF-7 cells were obtained from ATCC (Manassas, VA). MDA-MB-435 and MDA-MB-468 cells were obtained from Dr. Janet Price, M.D. Anderson Cancer Center, Houston, TX[34]. MDA-MB-435 and MDA-MB-468 cells were grown in αMEM + 10% fetal bovine serum (FBS), MCF-7 cells were grown in DMEM + 10% FBS, and MDA-MB-231 cells were grown DMEM:F12 + 10% FBS. Media was obtained from Invitrogen (Carlsbad, CA). FBS was obtained from Sigma (St. Louis, MO). It should be noted that the MDA-MB-435 cell line was originally isolated from the pleural effusion of a woman with metastatic breast

adenocarcinoma[34]. Recently, a debate has arisen over the origins of this cell line, whether it was derived from the M14 melanoma cell or is in fact a true breast cancer cell line[35,36]. Whether melanoma or breast in origin, the MDA-MB-435 cell line does represent a highly metastatic cell line, and thus provides an opportunity to relate functional metastatic ability to expression of CSC-related markers.

2.2.2 Cell surface marker analysis

MDA-MB-435, MDA-MB-231, MDA-MB-468, and MCF-7 cells (1×10^5) were incubated with fluorescently-conjugated antibodies, including anti-CD24 (clone ML5; BD Biosciences Canada, Mississauga, ON) or anti-CD133 (clone AC133; Miltenyi Biotec, Auburn, CA) conjugated to phycoerytherin (PE); and anti-CD44 (clone IM7; BD Biosciences) conjugated to fluorescein isothiocyanate (FITC). For supplemental studies, MDA-MB-231 and MDA-MB-468 cells were also incubated with a CXCR4 antibody (clone 12G5; R&D Systems, Minneapolis, MN) conjugated to PE. Fluorescently-conjugated IgG isotype controls (BD Biosciences) were used as negative controls. Cells were analyzed using a Beckman Coulter EPICS XL-MCL flow cytometer.

2.2.3 Analysis of ALDH activity

To assess ALDH activity of the different cell lines, the ALDEFLUOR® assay kit (StemCell Technologies, Vancouver, BC) was used. The basis for this assay is that uncharged ALDH substrate (BODIPY-aminoacetaldehyde [BAAA]) is taken up by living cells via passive diffusion. Once inside the cell, BAAA is converted into negatively-charged BODIPY-aminoacetate (BAA) by intracellular ALDH. BAA^- is then retained inside the cell, causing the cell to become highly fluorescent. Only cells with an intact cell membrane can retain BAA^-, so only viable cells can be identified[24]. The ALDEFLUOR® assay was performed essentially as described previously[26-28]. Briefly, MDA-MB-435, MDA-MB-231, MDA-MB-468, and MCF-7 cells were harvested, placed in ALDEFLUOR® assay buffer (2×10^6 cells/ml), and incubated with the ALDEFLUOR® substrate

for 45 minutes @ 37^{o}C to allow substrate conversion. As a negative control for all experiments, an aliquot of ALDEFLUOR®-stained cells was immediately quenched with 1.5-mM diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. Cells were analyzed using the green fluorescence channel (FL1) on a Beckman Coulter EPICS XL-MCL flow cytometer. Human umbilical cord blood (discard product) was labeled and assayed for ALDH activity as described previously[27] in order to optimize and validate the flow cytometry protocol.

2.2.4 FACS isolation of cells

Based on cell surface marker phenotype and ALDH activity, MDA-MB-468 and MDA-MB-231 cell lines were chosen for further cell isolation by FACS and functional analysis. Cells were concurrently labeled with 7-Aminoactinomycin D (7-AAD), fluorescent antibodies (CD44-APC + CD24-PE [MDA-MB-231] or CD44-APC + CD133-PE [MDA-MB-468]) and the ALDEFLUOR™ assay kit as described above. Cell subsets were isolated using a four-color analysis protocol on a FACS Vantage/Diva cell sorter (BD Biosciences), including $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{low}CD44^{low/-}CD24^{+}$ subsets (from MDA-MB-231 cells) and $ALDH^{hi}CD44^{+}CD133^{+}$ and $ALDH^{low}CD44^{low/-}CD133^{-}$ subsets (from MDA-MB-468 cells). For both cell lines, ALDH activity was used as the primary sort criteria (top ~20%=$ALDH^{hi}$; bottom ~20%=$ALDH^{low}$) and $CD44^{+}CD24^{-}$ (MDA-MB-231 cells) or $CD44^{+}CD133^{+}$ (MDA-MB-468 cells) phenotype as the secondary sort criteria (top ~10% gated on $ALDH^{hi}$; bottom ~10% gated on $ALDH^{low}$). Cell viability was assessed by 7-AAD staining during cell sorting, and confirmed by trypan blue exclusion post-sorting. Following FACS isolation, cells were used immediately for functional *in vitro* and *in vivo* assays.

2.2.5 Cell proliferation assays

Sorted cell populations ($ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{low}CD44^{low/-}CD24^{+}$ from MDA-MB-231 cells; $ALDH^{hi}CD44^{+}CD133^{+}$ and $ALDH^{low}CD44^{low/-}CD133^{-}$ from MDA-MB-468 cells) were plated at a density of 5.0 x 10^{4} cells/60 mm plate (n=3 for each time point) and maintained in

regular growth media. Every 48 hours for 14 days, triplicate cultures were trypsinized and counted by hemacytometer. Doubling time of each cell population was estimated during the exponential growth phase according to $T_d = 0.693t/\ln(N_t/N_0)$, where t is time (in hr), N_t is the cell number at time t, and N_0 is the cell number at initial time. Lag time was taken as the time for each population to reach the exponential growth phase.

2.2.6 Colony forming assays

In preparation for the assay, 60 mm dishes were coated with 1% agarose (Bioshop; Burlington, ON) in normal growth media and allowed to set for 1 hr. Cell suspensions (1.0×10^4 cells/60 mm plate) for each sorted population ($ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{low}CD44^{low/-}CD24^{+}$ from MDA-MB-231 cell line; $ALDH^{hi}CD44^{+}CD133^{+}$ and $ALDH^{low}CD44^{low/-}CD133^{-}$ from MDA-MB-468 cell line) were prepared using 0.6% agarose in normal growth media and plated on top of the 1% agarose base layer (n=4 for each time point). Normal growth media was added on top of the cell layer and changed every 3-4 days for 4 weeks, at which time media was removed and plates were fixed in 10% neutral-buffered formalin (EM Sciences, Gladstone, NJ). Five fields of view (100x) were counted for each dish, and mean number of colonies/field and mean colony diameter was calculated.

2.2.7 Cell adhesion assays

Cells were plated onto sterile 96-well non-tissue culture plates (Titertek, Flow Laboratories Inc.; McLean, VA) treated with either 10 µg/ml of human fibronectin (Sigma) (MDA-MB-231 cells), 5 µg/ml of human vitronectin (Sigma) (MDA-MB-468 cells), or PBS (negative control), using $1x10^4$ cells/well (n=3) for each sorted cell population ($ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{low}CD44^{low/-}CD24^{+}$ from MDA-MB-231 cell line; $ALDH^{hi}CD44^{+}CD133^{+}$ and $ALDH^{low}CD44^{low/-}CD133^{-}$ from MDA-MB-468 cell line). Vitronectin and fibronectin were chosen based on previous experiments in our laboratory that have demonstrated that MDA-MB-231 and MDA-MB-468 cells differentially express integrin receptors for fibronectin and vitronectin respectively ([37] and

our unpublished data). Cells were allowed to adhere for 5 hours, after which the media was removed and non-adhered cells were rinsed away. Adhered cells were fixed with 2% gluteraldehyde and stained using Harris' hematoxylin. Five high powered fields (HPF) (400x) were counted for each well, and mean numbers of adhered cells/field were calculated.

2.2.8 Cell migration and invasion assays

Transwell plates (6.5mm, 8µm pore size; Becton Dickinson; Franklin Lakes, NJ) were coated with 6 µg/well of gelatin (Sigma) (migration assay) or 10 µg/well of Matrigel (Becton Dickinson) (invasion assay) as described previously[38,39]. Control (0.01% BSA) or chemoattractant (5% FBS) media was placed in the bottom portion of each well. 3.5×10^4 cells of each sorted population ($ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{low}CD44^{low/-}CD24^{+}$ from MDA-MB-231 cell line; $ALDH^{hi}CD44^{+}CD133^{+}$ and $ALDH^{low}CD44^{low/-}CD133^{-}$ from MDA-MB-468 cell line) were plated on top of the transwells. Cells were allowed to migrate or invade for 24 hours, after which the upper transwell was removed, inverted, fixed with 1% gluteraldehyde, and stained with Harris' hematoxylin. Non-migrated or non-invaded cells on the inner surface of the transwell were carefully removed using a cotton swab. Five HPF were counted for each well, and mean numbers of migrated or invaded cells/field were calculated.

2.2.9 *In vivo* animal experiments

All *in vivo* work was carried out using NOD/SCID-IL2γ receptor null mice, which permit increased tissue engraftment of human cells without rejection due to reduced innate immunity (NOD mutation), complete T and B cell deficiency (SCID mutation), and reduced NK-cell function (IL-2Rγ mutation)[40]. Animal procedures were conducted in accordance with the recommendations of the Canadian Council on Animal Care, under a protocol approved by the University of Western Ontario Council on Animal Care (*Appendix 1*).

Following cell sorting, sorted populations ($ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{low}CD44^{low/-}CD24^{+}$ from MDA-MB-231 cell line; $ALDH^{hi}CD44^{+}CD133^{+}$ and $ALDH^{low}CD44^{low/-}CD133^{-}$ from

MDA-MB-468 cell line) were resuspended in sterile Hank's buffered salt solution (HBSS) (MDA-MB-231) or PBS (MDA-MB-468) at 5 x 10^6 cells/ml. Using established models of experimental metastasis (tail vein injection) or spontaneous metastasis (mammary fat pad injection)[33], 100µl (5 x 10^5 cells) of each sorted cell population were injected into the lateral tail vein or right thoracic mammary fat pad of 7-10 week old female NOD/SCID-IL2Rγ null mice (n=4 mice/group). Primary tumor growth was evaluated weekly by caliper measurement in two perpendicular dimensions, and tumor volume was estimated using the following formula: [volume = 0.52 × (width)^2 × (length)] for approximating the volume (mm^3) of an ellipsoid. Metastatic growth in the lung and extrapulmonary organs was allowed to develop for 5-7 weeks (MDA-MB-231) or 12 weeks (MDA-MB-468), at which point the mice were euthanized and assessed for metastatic burden. Endpoints and cell numbers for injection were chosen based on preliminary experiments using unsorted cell populations and NOD/SCID-IL2Rγ null mice (data not shown).

Tissues and organs were examined superficially for evidence of gross macroscopic metastases at necropsy. Tissues collected at necropsy were then fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned randomly (4 µm sections; at least 100 µm apart; 5 sections/tissue), and subjected to standard hematoxylin and eosin (H&E) staining. Stained slides were evaluated by light microscopy in a blinded fashion in order to identify and characterize incidence and regions of micrometastatic involvement. Metastatic tumor burden in the lung (tumor area/total organ area) was determined quantitatively by manual outlining and analysis by ImageJ software (NIH) as described previously[38].

2.2.10 Statistical analysis

Experiments analyzing CSC marker phenotype and ALDH activity were performed a minimum of three times. *In vitro* experiments with FACS-isolated cells were performed twice (after four separate cell sorts) with at least three biological replicates included within each experiment. *In vivo* studies were carried out using multiple animals (n=4 per cell population) and

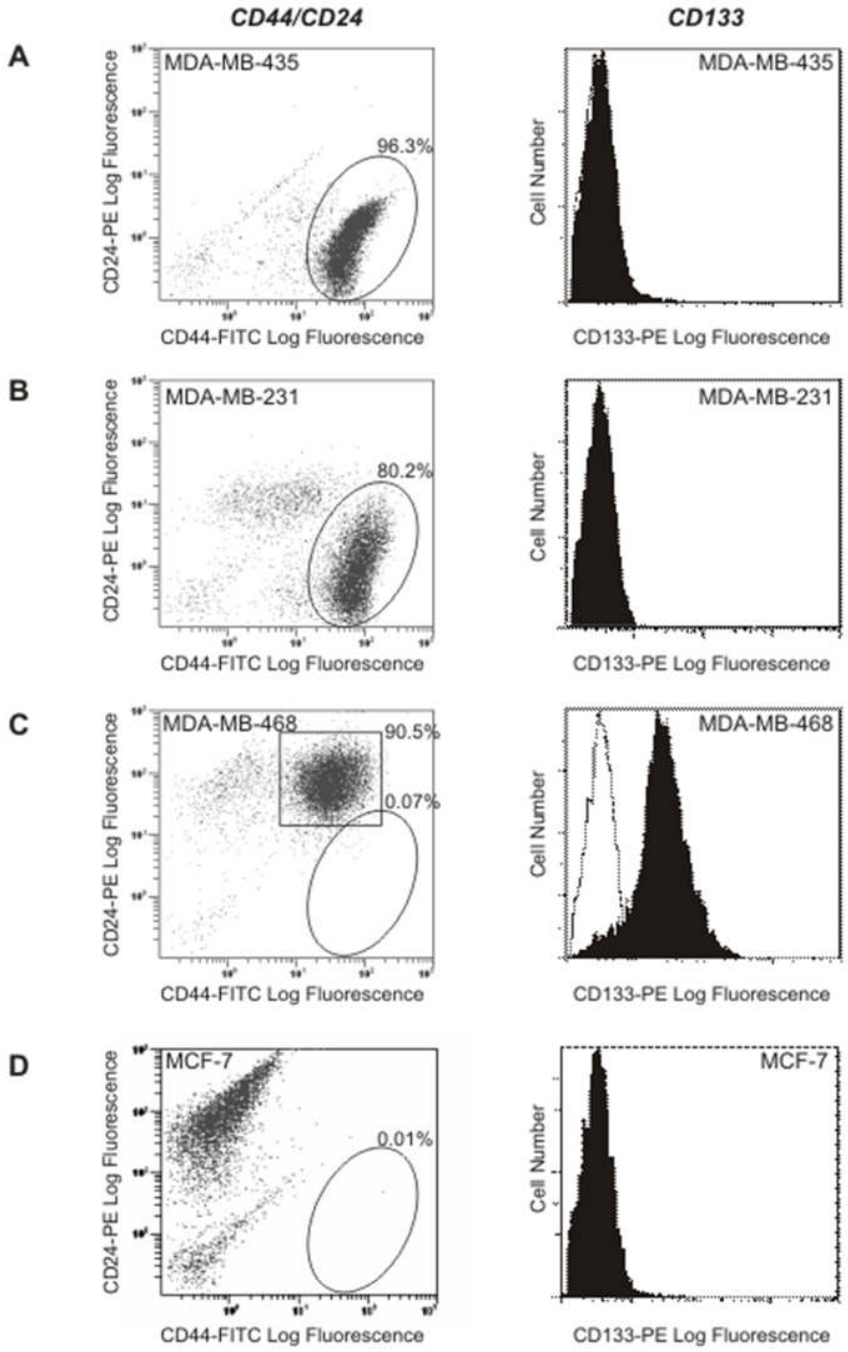
using cells obtained from 5 separate cell sorts. In all cases, quantitative data was compiled from all experiments. Statistical analysis was performed using GraphPad Prism 4.0 software© (San Diego, CA) using either t-test (for comparison between two groups) or ANOVA with Tukey post-test (for comparison between more than two groups). Differences between means were determined using the Student's t-test when groups passed both a normality test and an equal variance test. When this was not the case, the Mann-Whitney Rank-Sum test was used. Linear regression was used to assess the relationship between primary tumor size and metastatic burden in the lung. Unless otherwise noted, data is presented as the mean ± SEM. Values of $P \leq 0.05$ were regarded as being statistically significant.

2.3 Results

2.3.1 Human breast cancer cell lines contain sub-populations of cells expressing prospective CSC markers

Flow cytometry was used to assess the expression of prospective CSC markers, including CD44/CD24 and CD133. Interestingly, the most aggressive cell line (MDA-MB-435) had the highest proportion of $CD44^+CD24^-$ cells (96.6 ± 1.2%) (*Figure 2.1A*), while the least aggressive cell line (MCF-7) had the lowest proportion of $CD44^+CD24^-$ cells (0.05 ± 0.01%) (*Figure 2.1D*). The moderately metastatic MDA-MB-231 cell line contained 79.5 ± 2.7% $CD44^+CD24^-$ cells (*Figure 2.1B*). In contrast, the weakly metastatic MDA-MB-468 cell line contained very few cells (0.09 ± 0.05%) with a $CD44^+CD24^-$ phenotype, however the majority of cells (92.9 ± 4.4%) did express both CD44 and CD24 ($CD44^+CD24^+$) (*Figure 2.1C*). Quantitative analysis demonstrated that the MDA-MB-435 cell line contained significantly more cells with a $CD44^+CD24^-$ phenotype than any of the other cell lines ($p<0.01$), and that the MDA-MB-231 cell line had significantly more cells with a $CD44^+CD24^-$ phenotype than either the MDA-MB-468 or MCF-7 cell lines ($p<0.01$) (*Figure 2.2*). MDA-MB-435, MDA-MB-231, and MCF-7 cell lines did

Figure 2.1. Human breast cancer cell lines contain sub-populations of cells expressing prospective CSC markers. ***(A-D)*** Flow cytometry analysis of CD44/CD24 and CD133. Antibodies used included an anti-CD44 antibody (clone IM7) conjugated to fluorescein isothiocyanate (FITC), an anti-CD24 antibody (clone ML5) conjugated to phycoerytherin (PE), an anti-CD133 antibody (clone AC133) conjugated to PE, or appropriate FITC and PE-conjugated IgG isotype controls. ***(A)*** MDA-MB-435 cells; ***(B)*** MDA-MB-231 cells; ***(C)*** MDA-MB-468 cells; and ***(D)*** MCF-7 cells. Left hand panels show representative dot plots of CD44-FITC versus CD24-PE expression. Cells which fell within the circled regions were considered to express the phenotype of interest ($CD44^+CD24^-$). Compiled quantitative analysis of $CD44^+CD24^-$ phenotype expression from 3 separate experiments is shown in *Figure 2.2*. In ***(C)***, cells which fell within the square region were considered to express the phenotype $CD44^+CD24^+$. Right hand panels show representative histograms of CD133 expression (*black profiles*) relative to the IgG isotype control (*white profiles*). A minimum of 10,000 events were collected per sample.


CD44/CD24
CD133
A
MDA-MB-435
96.3%
CD24-PE Log Fluorescence
CD44-FITC Log Fluorescence
MDA-MB-435
Cell Number
CD133-PE Log Fluorescence
B
MDA-MB-231
80.2%
CD24-PE Log Fluorescence
CD44-FITC Log Fluorescence
MDA-MB-231
Cell Number
CD133-PE Log Fluorescence
C
MDA-MB-468
90.5%
0.07%
CD24-PE Log Fluorescence
CD44-FITC Log Fluorescence
MDA-MB-468
Cell Number
CD133-PE Log Fluorescence
D
MCF-7
0.01%
CD24-PE Log Fluorescence
CD44-FITC Log Fluorescence
MCF-7
Cell Number
CD133-PE Log Fluorescence

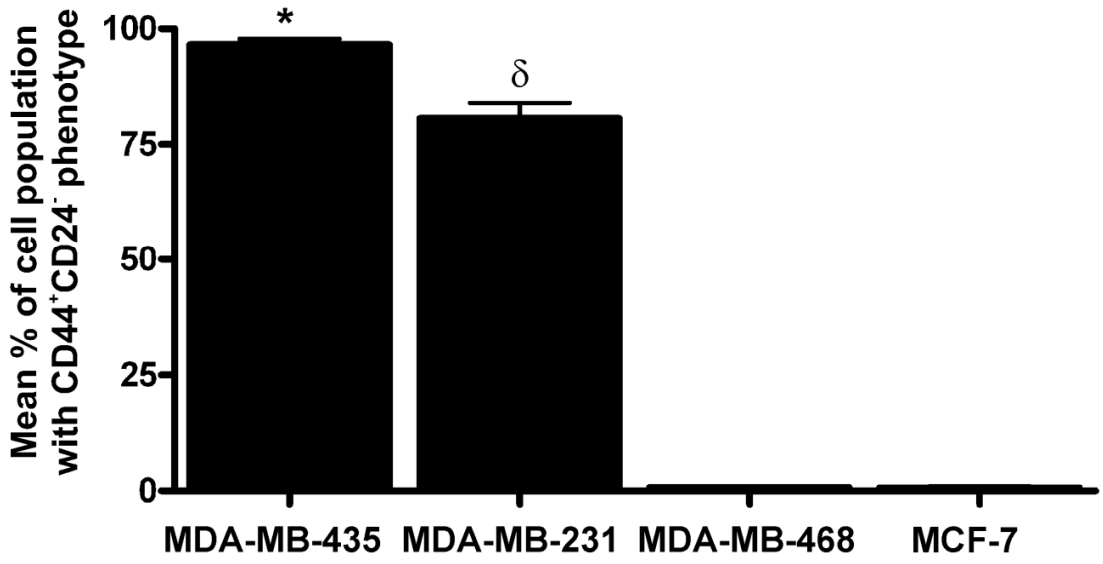


Figure 2.2 Compiled quantitative analysis of $CD44^{+}CD24^{-}$ surface marker expression in human breast cancer cell lines. Human breast cancer cells (MDA-MB-435, MDA-MB-231, MDA-MB-468, MCF-7) were incubated with an anti-CD44 antibody (clone IM7) conjugated to fluorescein isothiocyanate (FITC) and an anti-CD24 antibody (clone ML5) conjugated to phycoerytherin (PE), or appropriate FITC and PE-conjugated IgG isotype controls. Representative dot plots from the flow cytometry analysis can be observed in *Figure 2.1*. The percentage of cells with a $CD44^{+}CD24^{-}$ phenotype was used to assess CD44/CD24 cell surface expression in each cell line. Data are presented as the mean ± SEM. * = significantly larger $CD44^{+}CD24^{-}$ population relative to all other cell lines ($p<0.01$). δ= significantly larger $CD44^{+}CD24^{-}$ population relative to MDA-MB-468 and MCF-7 cell lines ($p<0.01$).

not demonstrate expression of CD133 (*Figure 2.1A,B,D*). However, MDA-MB-468 cells did consistently express CD133 on their surface, at a level significantly higher than the other cell lines (*Figure 2.1C*).

2.3.2 Human breast cancer cell lines contain sub-populations of cells with enhanced ALDH activity

A complementary strategy for identifying cells with a stem/progenitor phenotype involves measurement of ALDH activity [24]. Human umbilical cord blood was assayed for ALDH activity in order to provide a control for setting up the flow cytometry protocol and for confirming that the assay was working appropriately (*Figure 2.3*). The MDA-MB-435 cell line did not demonstrate any significant increase in ALDH activity (*Figure 2.4A*). Interestingly, both the MDA-MB-231 and MDA-MB-468 cell lines showed two definitive subpopulations of cells: one that had ALDH activity at the level of the DEAB control, and one that had increased ALDH activity (*Figure 2.4B,C*). The MCF-7 cell line did not demonstrate any increased ALDH activity (*Figure 2.4D*).

2.3.3 Strategy for isolation of stem-like human breast cancer cells

MDA-MB-231 and MDA-MB-468 cell lines were chosen for further characterization and functional analysis because they had the most distinct subpopulations of cells with stem-like characteristics (CSC marker expression, ALDH activity). Subsets of cells were isolated from MDA-MB-231 and MDA-MB-468 cell lines by FACS, using ALDH activity as the primary sort criteria and $CD44^{+}CD24^{-}$ (MDA-MB-231 cells) or $CD44^{+}CD133^{+}$ (MDA-MB-468 cells) phenotype as the secondary sort criteria. The resulting cell subsets were designated as $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) or $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) ("stem-like") and $ALDH^{low}CD44^{low/-}CD24^{+}$ (MDA-MB-231) or $ALDH^{low}CD44^{low/-}CD133^{-}$ (MDA-MB-468) (non "stem-like"). Examples of the cell sorting strategy are represented in *Figure 2.5* and *Figure 2.6*. From this point forward in the study, the non-"stem-like" cells from both cell lines will be collectively referred to as respective $ALDH^{low}CD44^{low/-}$ subsets.

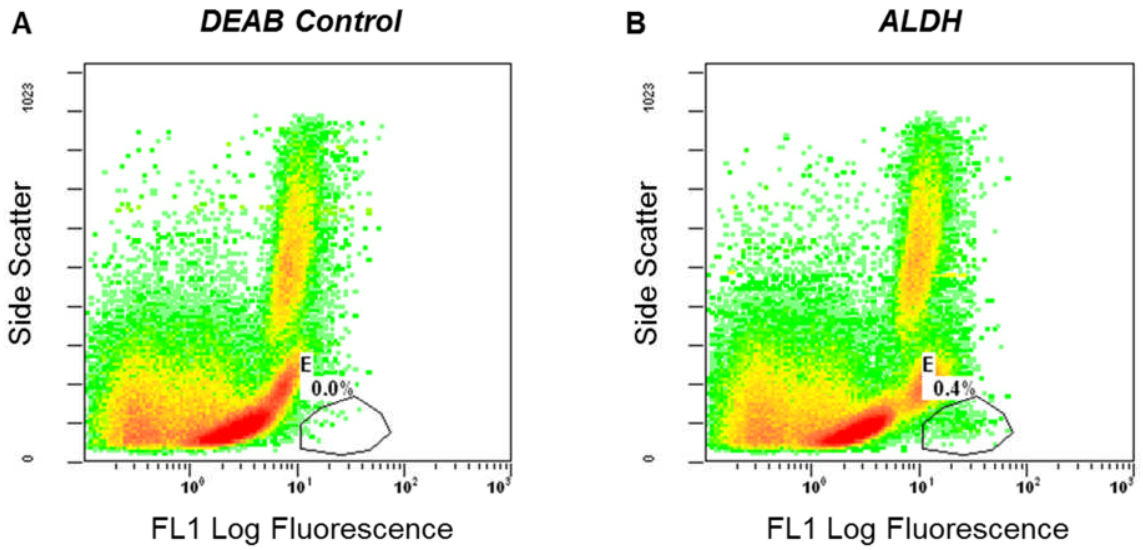


Figure 2.3 Optimization and validation of the ALDEFLUOR® assay kit and flow cytometry protocol using human umbilical cord blood. Cord blood was assayed with ALDEFLUOR® assay kit (StemCell Technologies) as per the manufacturer's guidelines. As a negative control for all experiments, an aliquot of ALDEFLUOR®-stained cord blood was immediately quenched with 1.5-mM diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. Samples were analyzed using the green fluorescence channel (FL1) on a Beckman Coulter EPICS XL-MCL flow cytometer, using the protocol set-up and data acquisition template recommended by the manufacturer. ***(A)*** Representative dot plots of cord blood samples treated with the ALDH-specific inhibitor DEAB (negative control). ***(B)*** Representative dot plots of ALDH activity in cord blood. Cells which fell within the circled regions represent subpopulations of cells with enhanced ALDH activity relative to the rest of the cell population.

Figure 2.4 Human breast cancer cell lines contain sub-populations of cells with enhanced ALDH activity. ***(A-D)*** Flow cytometry analysis of ALDH activity. Cells were assayed with the ALDEFLUOR® assay kit (StemCell Technologies) as per the manufacturer's guidelines. As a negative control for all experiments, an aliquot of ALDEFLUOR®-stained cells was immediately quenched with 1.5-mM diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. Cells were analyzed using the green fluorescence channel (FL1) on a Beckman Coulter EPICS XL-MCL flow cytometer. ***(A)*** MDA-MB-435 cells; ***(B)*** MDA-MB-231 cells; ***(C)*** MDA-MB-468 cells; and ***(D)*** MCF-7 cells. Left hand panels show representative dot plots of cells treated with the ALDH-specific inhibitor DEAB (*negative controls*). Right hand panels show representative dot plots of ALDH activity. A minimum of 10,000 events were collected per sample. Cells which fell within the circled regions were considered to represent subpopulations of cells with enhanced ALDH activity relative to the rest of the cell population. The flow cytometry protocol was optimized and validated using human umbilical cord blood, which has a known population of stem/progenitor cells with enhanced ALDH activity[51] (*Figure 2.3*).

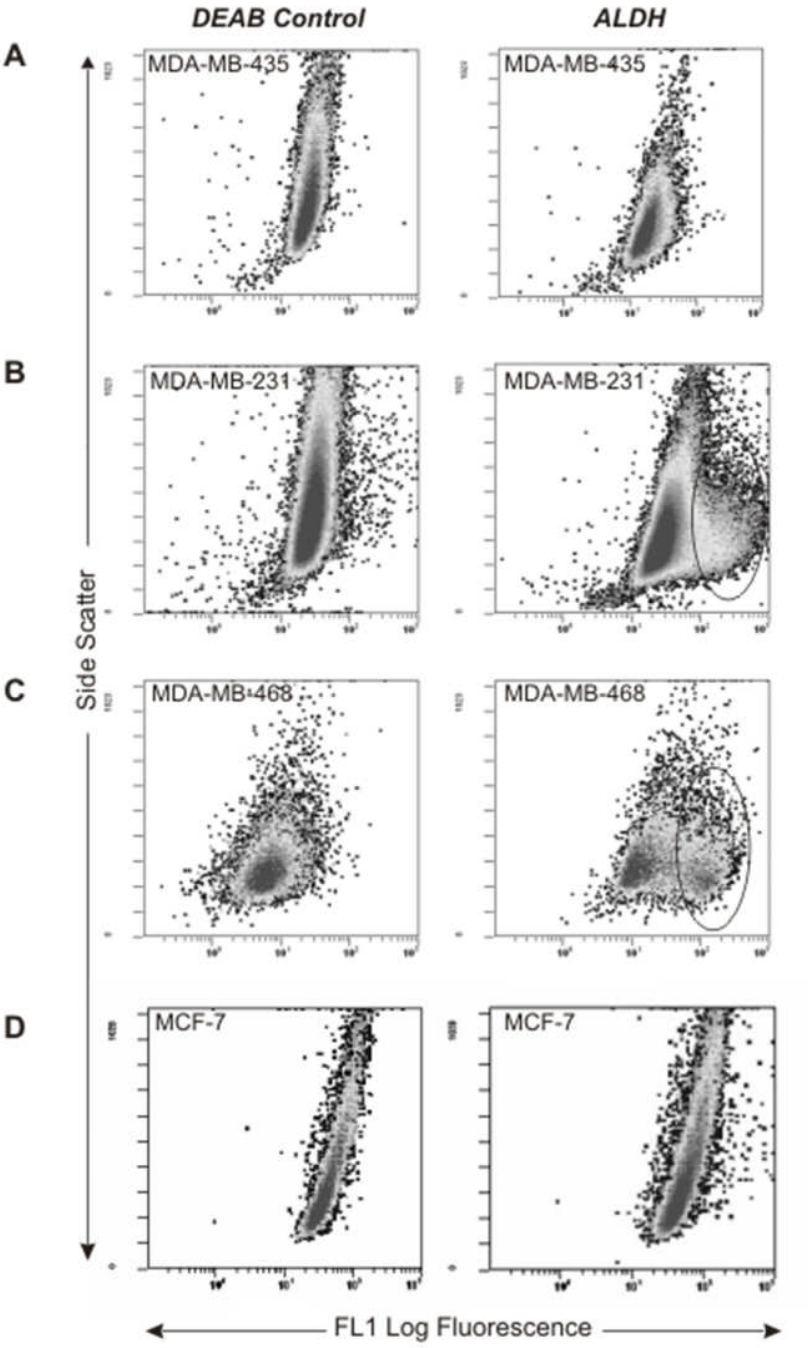

DEAB Control
ALDH
A
MDA-MB-435
MDA-MB-435
B
MDA-MB-231
MDA-MB-231
C
MDA-MB-468
MDA-MB-468
D
MCF-7
MCF-7
Side Scatter
FL1 Log Fluorescence

Figure 2.5 Strategy for isolation of stem-like human breast cancer cells from the MDA-MB-231 cell line. Fluorescence-activated cell sorting (FACS) was used to isolate $ALDH^{hi}CD44^{+}$ and $ALDH^{low}CD44^{low/-}$ human breast cancer cells for functional assays. MDA-MB-231 cells were concurrently labelled with 7-AAD, fluorescent antibodies (CD44-APC + CD24-PE) and the ALDEFLUOR™ assay kit. Cell subsets were isolated using a four-colour protocol on a FACS Vantage/Diva cell sorter, including $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{low}CD44^{low/-}CD24^{+}$ subsets ***(A–C)***: Representative schematic of a sequentially gated MDA-MB-231 cell line sort. ***(A)*** Cells were first selected for viability based on 7-AAD negativity (R1, left panel) and for singlets (R2, right panel). ***(B)*** Cells were then selected based on light scatter (R3, left panel) and divided into $ALDH^{low}$ (R4, bottom ~20% of parent population) and $ALDH^{hi}$ (R5, top ~20% of parent population) based on ALDH activity (right panel). ***(C)*** Finally, $ALDH^{low}$ cells were further selected based on a $CD44^{low/-}CD24^{+}$ phenotype (R6, bottom 10% of parent population, gated on R1 + R2 + R3 + R4) (left panel), whereas $ALDH^{hi}$ cells were further selected based on expression of a $CD44^{+}CD24^{-}$ phenotype (R7, top ~10% of parent population, gated on R1 + R2 + R3 + R5) (right panel). The resulting cell subsets were designated as $ALDH^{hi}CD44^{+}CD24^{-}$ (R7, 'stem-like') and $ALDH^{low}CD44^{low/-}CD24^{+}$ (R6, non 'stem-like'), and were collected for functional analysis of differences in malignant and metastatic behaviour *in vitro* and *in vivo*.

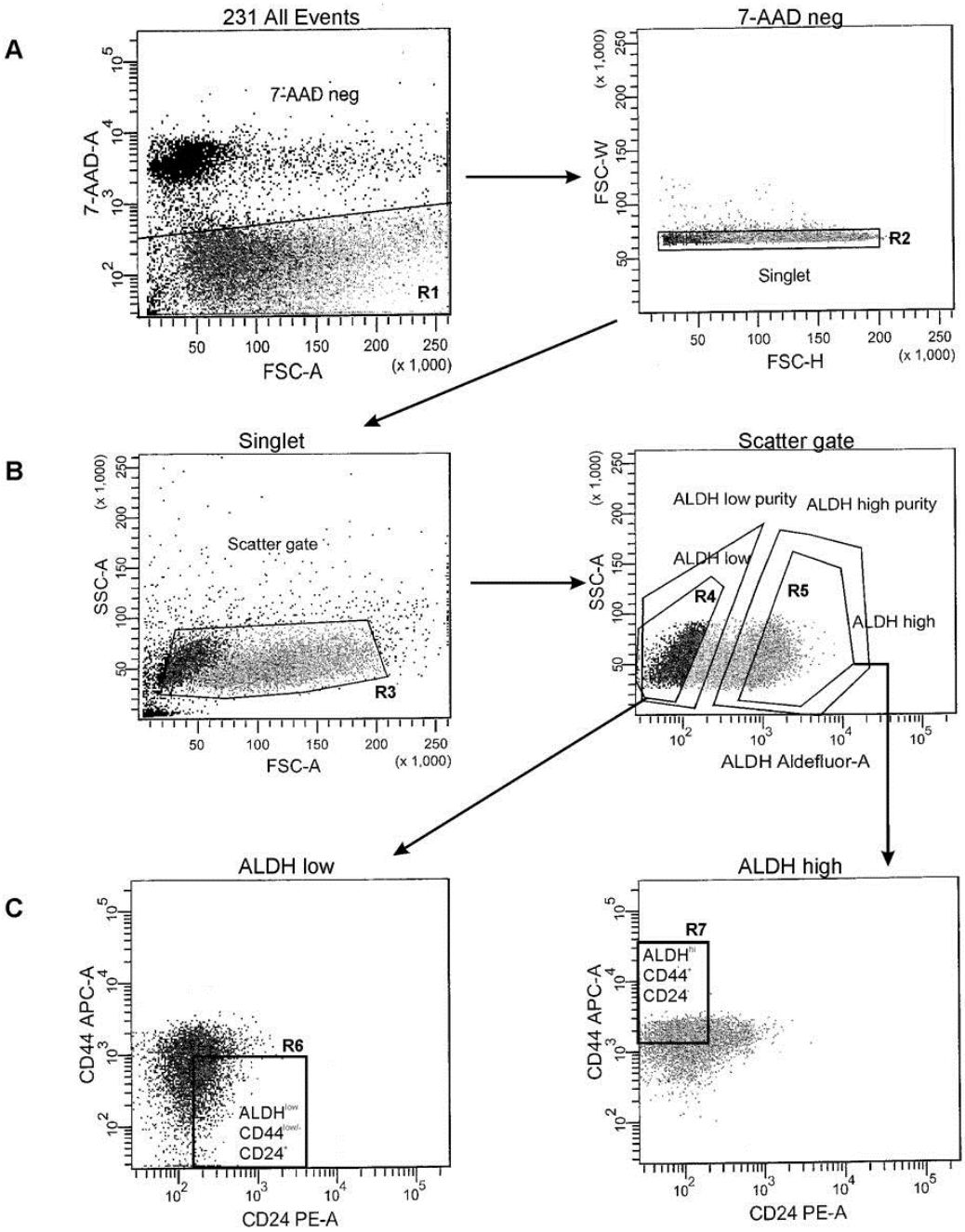
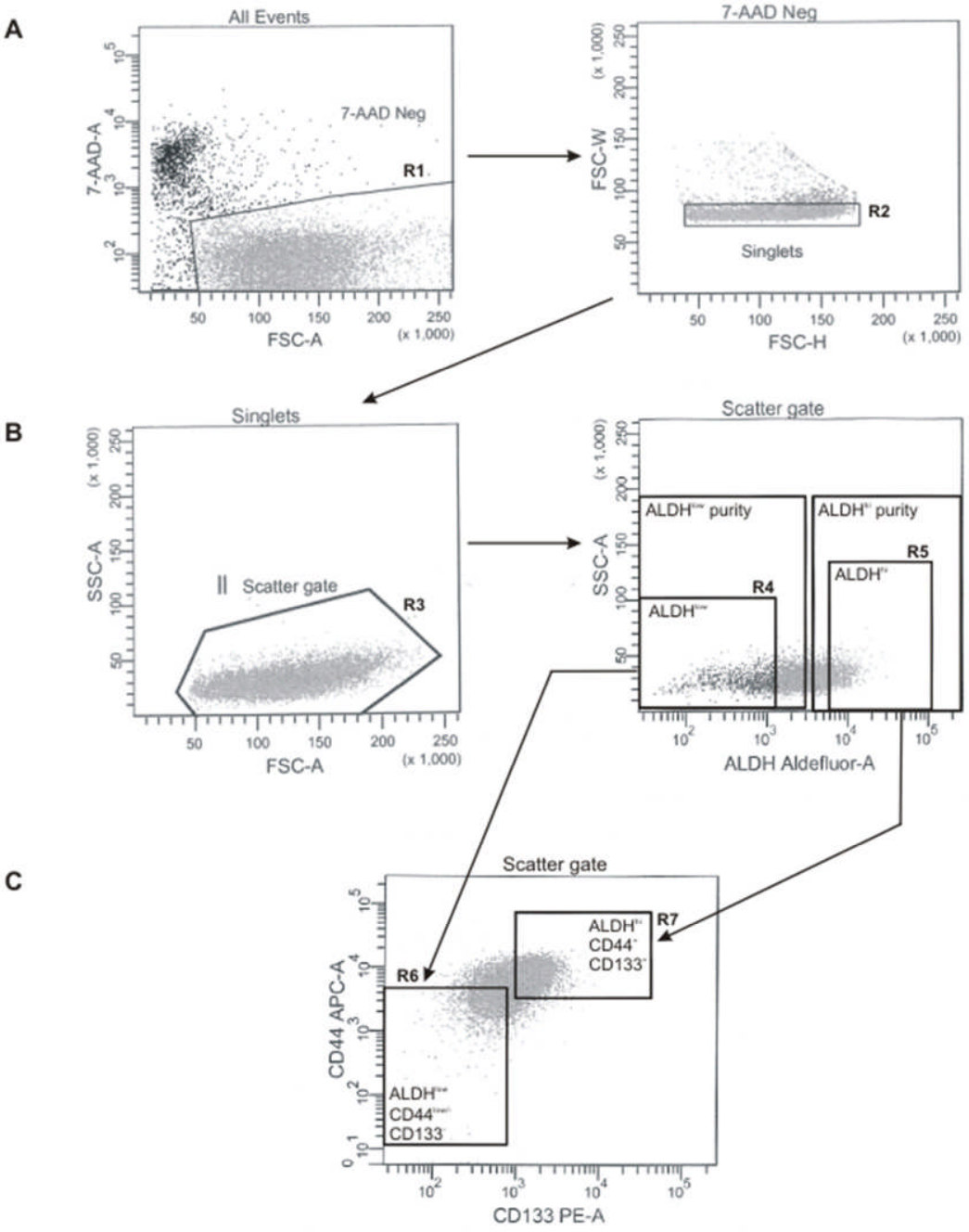

A
231 All Events
7-AAD neg
R1
7-AAD-A
FSC-A
(x 1,000)
7-AAD neg
R2
Singlet
FSC-W
FSC-H
(x 1,000)
B
Singlet
Scatter gate
R3
SSC-A
FSC-A
(x 1,000)
Scatter gate
ALDH low purity
ALDH high purity
ALDH low
R4
R5
ALDH high
SSC-A
ALDH Aldefluor-A
C
ALDH low
R6
ALDH low CD44 low/- CD24+
CD44 APC-A
CD24 PE-A
ALDH high
R7
ALDH hi CD44+ CD24-
CD44 APC-A
CD24 PE-A

Figure 2.6 Strategy for isolation of stem-like human breast cancer cells from the MDA-MB-468 cell line. Fluorescence-activated cell sorting (FACS) was used to isolate $ALDH^{hi}CD44^{+}$ and $ALDH^{low}CD44^{low/-}$ human breast cancer cells for functional assays. MDA-MB-468 cells were concurrently labelled with 7-AAD, fluorescent antibodies (CD44-APC + CD133-PE) and the ALDEFLUOR™ assay kit. Cell subsets were isolated using a four-colour protocol on a FACS Vantage/Diva cell sorter, including $ALDH^{hi}CD44^{+}CD133^{+}$ and $ALDH^{low}CD44^{low/-}CD133^{-}$ subsets ***(A–C)***: Representative schematic of a sequentially gated MDA-MB-468 cell line sort. ***(A)*** Cells were first selected for viability based on 7-AAD negativity (R1, left panel) and for singlets (R2, right panel). ***(B)*** Cells were then selected based on light scatter (R3, left panel) and divided into $ALDH^{low}$ (R4, bottom ~20% of parent population) and $ALDH^{hi}$ (R5, top ~20% of parent population) based on ALDH activity (right panel). ***(C)*** Finally, $ALDH^{low}$ cells were further selected based on a $CD44^{low/-}CD133^{-}$ phenotype (R6, bottom 10% of parent population, gated on R1 + R2 + R3 + R4) (left panel), whereas $ALDH^{hi}$ cells were further selected based on expression of a $CD44^{+}CD133^{+}$ phenotype (R7, top ~10% of parent population, gated on R1 + R2 + R3 + R5) (right panel). The resulting cell subsets were designated as $ALDH^{hi}CD44^{+}CD133^{+}$ (R7, 'stem-like') and $ALDH^{low}CD44^{low/-}CD133^{-}$ (R6, non 'stem-like'), and were collected for functional analysis of differences in malignant and metastatic behaviour *in vitro* and *in vivo*.


A
All Events
7-AAD-A
7-AAD Neg
R1
FSC-A
(x 1,000)
7-AAD Neg
FSC-W
R2
Singlets
FSC-H
(x 1,000)
B
Singlets
SSC-A
Scatter gate
R3
FSC-A
(x 1,000)
Scatter gate
ALDH^{low} purity
ALDH^{hi} purity
R5
ALDH^{hi}
R4
ALDH^{low}
SSC-A
ALDH Aldefluor-A
C
Scatter gate
ALDH^{hi}
CD44^{+}
CD133^{+}
R7
R6
ALDH^{low}
CD44^{low}
CD133^{-}
CD44 APC-A
CD133 PE-A

2.3.4 ALDH^{hi}CD44^{+}CD24^{-} and ALDH^{hi}CD44^{+}CD133^{+} breast cancer cells demonstrate enhanced cell growth and colony formation *in vitro*

Differences in cell growth characteristics *in vitro* between sorted subpopulations for both cell lines were assessed (*Figure 2.7*). ALDH^{hi}CD44^{+}CD24^{-} (MDA-MB-231) and ALDH^{hi}CD44^{+}CD133^{+} (MDA-MB-468) cells demonstrated increased growth in normal culture relative to respective ALDH^{low}CD44$^{low/-}$ cell subsets. Lag times (time to reach exponential growth phase) were also observed to be shorter for ALDH^{hi}CD44^{+}CD24^{-} (MDA-MB-231 = 1 day) and ALDH^{hi}CD44^{+}CD133^{+} (MDA-MB-468=2 days) cells versus respective ALDH^{low}CD44$^{low/-}$ cell subsets (7 days for both cell lines) (*Figure 2.7A*). Differences in colony-forming ability and anchorage-independent growth between sorted subpopulations was assessed using a soft agar assay (*Figure 2.7B,C*). Both ALDH^{hi}CD44^{+}CD24^{-} (MDA-MB-231) and ALDH^{hi}CD44^{+}CD133^{+} (MDA-MB-468) cell subsets formed significantly more colonies ($p<0.001$) (*Figure 2.7B*) and larger colonies ($p<0.05$) (*Figure 2.7C*) than respective ALDH^{low}CD44$^{low/-}$ cell subsets.

2.3.5 ALDH^{hi}CD44^{+}CD24^{-} and ALDH^{hi}CD44^{+}CD133^{+} breast cancer cells demonstrate enhanced adhesion, migration, and invasion *in vitro*

In vitro assays were used to compare sorted subpopulations from the perspective of differences in cell adhesion, migration, and invasion (*Figure 2.8*). ALDH^{hi}CD44^{+}CD24^{-} (MDA-MB-231) and ALDH^{hi}CD44^{+}CD133^{+} (MDA-MB-468) cells were observed to be significantly more adherent to fibronectin (MDA-MB-231) or vitronectin (MDA-MB-468) (*Figure 2.8A*), significantly more migratory towards serum (*Figure 2.8B*), and significantly more invasive through Matrigel (*Figure 2.8C*) than respective ALDH^{low}CD44$^{low/-}$ cell subsets ($p<0.001$).

2.3.6 ALDH^{hi}CD44^{+}CD24^{-} and ALDH^{hi}CD44^{+}CD133^{+} breast cancer cells demonstrate enhanced tumorigenicity and metastasis *in vivo*

Standard experimental or spontaneous metastasis assays were used to compare the ability of sorted subpopulations to establish themselves and grow *in vivo* following cell injection into the tail vein (*Figure 2.9*) or mammary fat pad (*Figure 2.10*) of NOD/SCID-IL2Rγ null mice.

Figure 2.7 $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ breast cancer cells demonstrate enhanced cell growth and colony formation *in vitro*. Cells were isolated by FACS as described in *Figure 2.5* and *Figure 2.6* and subjected to *in vitro* assays for growth and colony formation. ***(A)*** Cell growth kinetics in normal (anchorage-dependent) culture over time of $ALDH^{hi}CD44^{+}CD24^{-}$ (■) versus $ALDH^{low}CD44^{low/-}CD24^{+}$ (□) cells isolated from the MDA-MB-231 cell line (*left panel*); and $ALDH^{hi}CD44^{+}CD133^{+}$ (●) versus $ALDH^{low}CD44^{low/-}CD133^{-}$ (○) cells isolated from the MDA-MB-468 cell line (*right panel*) (5.0×10^{4} cells/60 mm plate; n=3 plates/time point). Data are presented as the mean ± SEM. * = significantly different cell number than respective $ALDH^{low}CD44^{low/-}$ cell subsets ($p<0.05$). ***(B,C)*** Colony forming ability of $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) and $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) cells (*black bars*) versus respective $ALDH^{low}CD44^{low/-}$ cell subsets (*white bars*) isolated from MDA-MB-231 (*left panels*) or MDA-MB-468 (*right panels*) cell lines. Cells (1.0×10^{4} cells/60 mm plate, n=4 plates/cell population) were grown in soft agar (0.6%) for 4 weeks. Five high powered fields (HPF) of view (100x) were counted for each dish. ***(B)*** Mean number of colonies per plate and ***(C)*** Mean colony diameter (μm^{2}). Data are presented as the mean ± SEM. δ = significantly different colony number than respective $ALDH^{low}CD44^{low/-}$ cell subsets ($p<0.001$). ϕ= significantly different colony size than respective $ALDH^{low}CD44^{low/-}$ cell subsets ($p<0.05$).

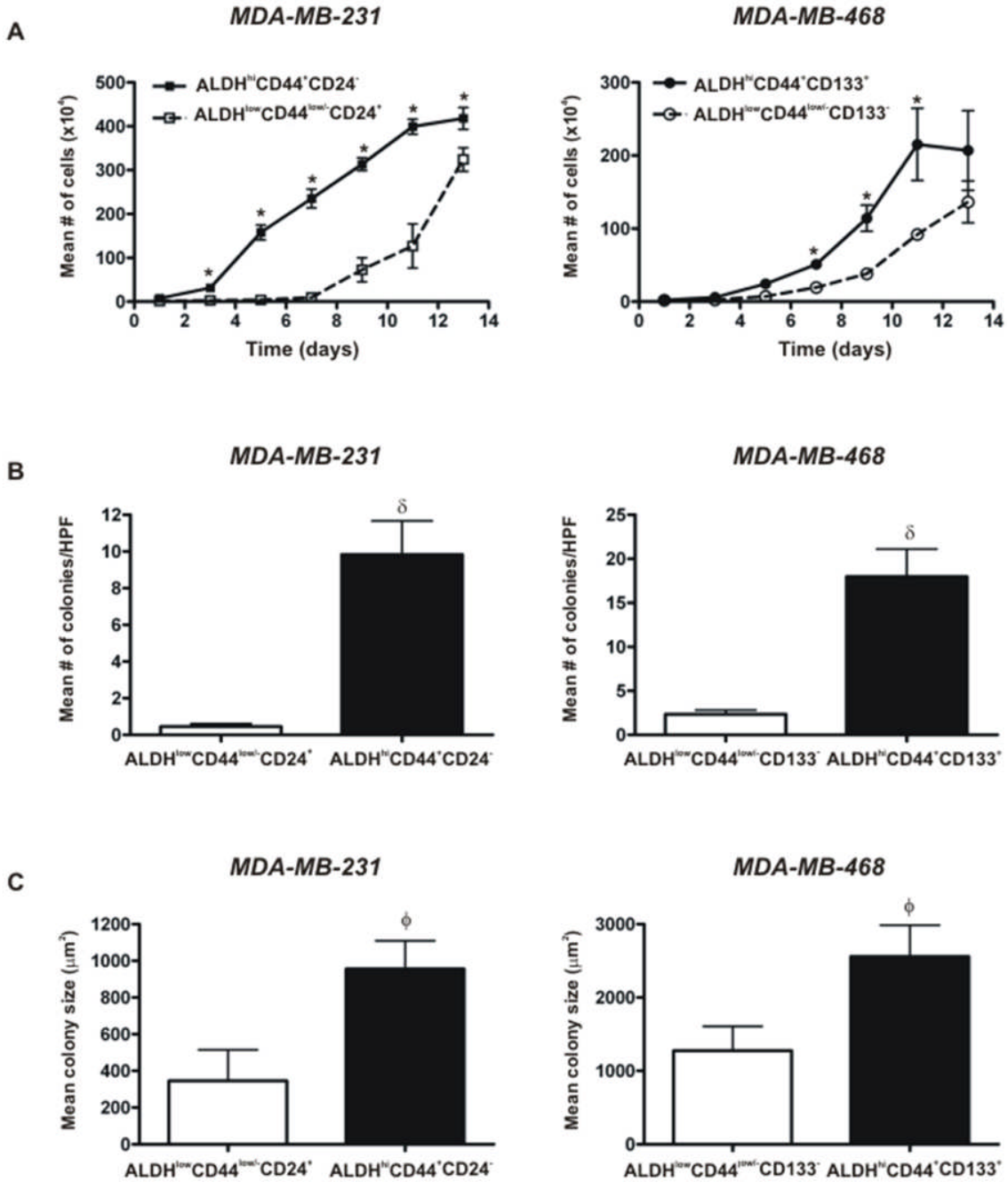

A
MDA-MB-231
MDA-MB-468
ALDHhiCD44+CD24-
ALDHlowCD44low/-CD24+
ALDHhiCD44+CD133+
ALDHlowCD44low/-CD133-
Mean # of cells (x10⁴)
Time (days)
B
MDA-MB-231
MDA-MB-468
Mean # of colonies/HPF
δ
ALDHlowCD44low/-CD24+
ALDHhiCD44+CD24-
ALDHlowCD44low/-CD133-
ALDHhiCD44+CD133+
C
MDA-MB-231
MDA-MB-468
Mean colony size (μm²)
ϕ
ALDHlowCD44low/-CD24+
ALDHhiCD44+CD24-
ALDHlowCD44low/-CD133-
ALDHhiCD44+CD133+

Figure 2.8 $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ breast cancer cells demonstrate enhanced adhesion, migration, and invasion *in vitro.* *(A)* Cell adhesion of $ALDH^{hi}CD44^{+}CD24^{-}$ versus $ALDH^{low}CD44^{low/-}CD24^{+}$ cells isolated from the MDA-MB-231 cell line (*left panel*); and $ALDH^{hi}CD44^{+}CD133^{+}$ versus $ALDH^{low}CD44^{low/-}CD133^{-}$ cells isolated from the MDA-MB-468 cell line (*right panel*). Cells were isolated by FACS as described in *Figure 2.5* and *Figure 2.6* and plated onto sterile 96-well non-tissue culture plates treated with either 10 µg/ml of fibronectin (MDA-MB-231 cells), 5 µg/ml of vitronectin (MDA-MB-468 cells) (*black bars*), or PBS (*negative control*) (*white bars*), using $1x10^4$ cells/well in triplicate wells for each sorted cell population. Cells were allowed to adhere for 5 hours and adhered cells were quantified by manual counting of 5 high-powered fields (HPF) per well. Data are presented as the mean ± SEM. * = significantly different than respective population of $ALDH^{low}CD44^{low/-}$ cells adhered to vitronectin or fibronectin ($p<0.001$). ***(B)*** Cell migration and ***(C)*** Cell invasion of $ALDH^{hi}CD44^{+}$ $CD24^{-}$ versus $ALDH^{low}CD44^{low/-}CD24^{+}$ cells isolated from the MDA-MB-231 cell line (*left panels*); and $ALDH^{hi}CD44^{+}CD133^{+}$ versus $ALDH^{low}CD44^{low/-}CD133^{-}$ cells isolated from the MDA-MB-468 cell line (*right panels*). Transwells (8 µm) were pre-coated with either gelatin (migration assays; 6 µg/well) or Matrigel (invasion assays; 10 µg/well) and FACS-isolated cells ($3.5x10^4$ cells/well; n=3 for each cell population) were allowed to migrate or invade for 24 hrs towards chemo-attractant media (5% fetal bovine serum) (*black bars*) or control media (0.01% bovine serum albumin) (*white bars*). Migrated or invaded cells were quantified by manual counting of 5 HPF per well. Data are presented as the mean ± SEM. δ = significantly different than respective population of $ALDH^{low}CD44^{low/-}$ cells migrating towards FBS ($p<0.001$). ϕ = significantly different than respective population of $ALDH^{low}CD44^{low/-}$ cells invading through Matrigel towards FBS ($p<0.001$).

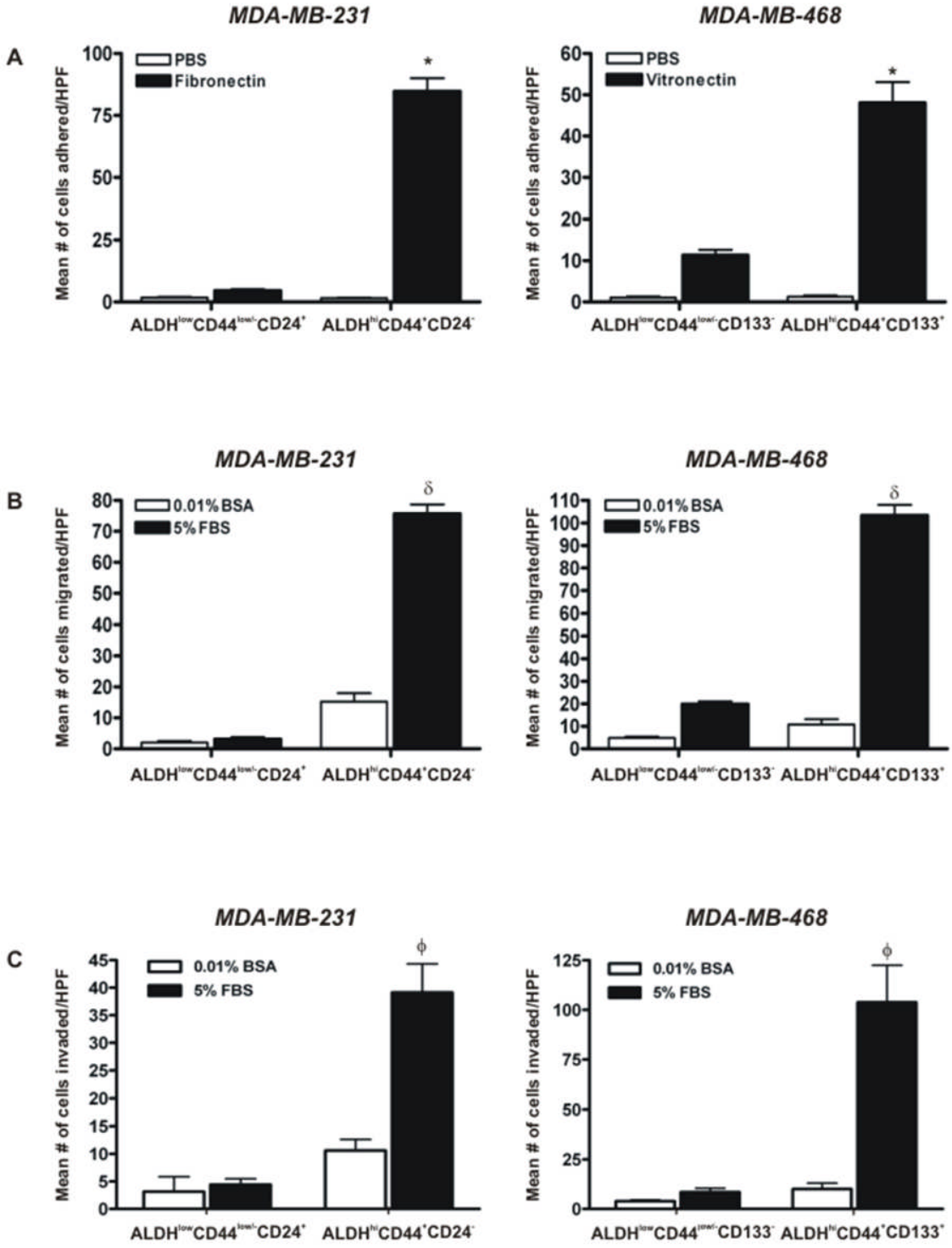

A
MDA-MB-231
MDA-MB-468
PBS
Fibronectin
Vitronectin
Mean # of cells adhered/HPF
ALDH^low CD44^low/- CD24^+
ALDH^hi CD44^+ CD24^-
ALDH^low CD44^low/- CD133^-
ALDH^hi CD44^+ CD133^+
B
0.01% BSA
5% FBS
Mean # of cells migrated/HPF
C
Mean # of cells invaded/HPF

Figure 2.9 $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ breast cancer cells demonstrate enhanced metastatic growth *in vivo* following tail vein injection. Cells were isolated by FACS as described in *Figure 2.5* and *Figure 2.6* and injected into the lateral tail vein of female NOD/SCID-IL2Rγ null mice using an established model of experimental metastasis ($5x10^5$ cells/mouse; 4 mice/cell population). At 5 weeks (MDA-MB-231) or 12 weeks (MDA-MB-468) post-injection, mice were sacrificed and assessed for metastatic burden in the lung and elsewhere. Tissue sections were subjected to H&E staining (5 random sections/tissue/mouse), and the incidence and extent of metastasis was determined in a blinded fashion. ***(A,B)*** Quantitative analysis of tumor burden (mean % of lung occupied by tumor) (*left panels*) and representative H&E stained lung sections (*right panels*) following tail vein injection of ***(A)*** $ALDH^{hi}CD44^{+}CD24^{-}$ versus $ALDH^{low}CD44^{low/-}CD24^{+}$ cells isolated from the MDA-MB-231 cell line; and ***(B)*** $ALDH^{hi}CD44^{+}CD133^{+}$ versus $ALDH^{low}CD44^{low/-}CD133^{-}$ cells isolated from the MDA-MB-468 cell line. Arrowheads on H&E images indicate regions of tumor within the lung. Data are presented as the mean ± SEM. * = significantly different than respective $ALDH^{low}CD44^{low/-}$ subsets ($p<0.05$). ***(C)*** Incidence of metastatic growth in lung and extra-pulmonary tissues following tail vein injection of $ALDH^{hi}CD44^{+}CD24^{-}$ versus $ALDH^{low}CD44^{low/-}CD24^{+}$ cells isolated from the MDA-MB-231 cell line *(left panels)*; and $ALDH^{hi}CD44^{+}CD133^{+}$ versus $ALDH^{low}CD44^{low/-}CD133^{-}$ cells isolated from the MDA-MB-468 cell line (*right panels*).

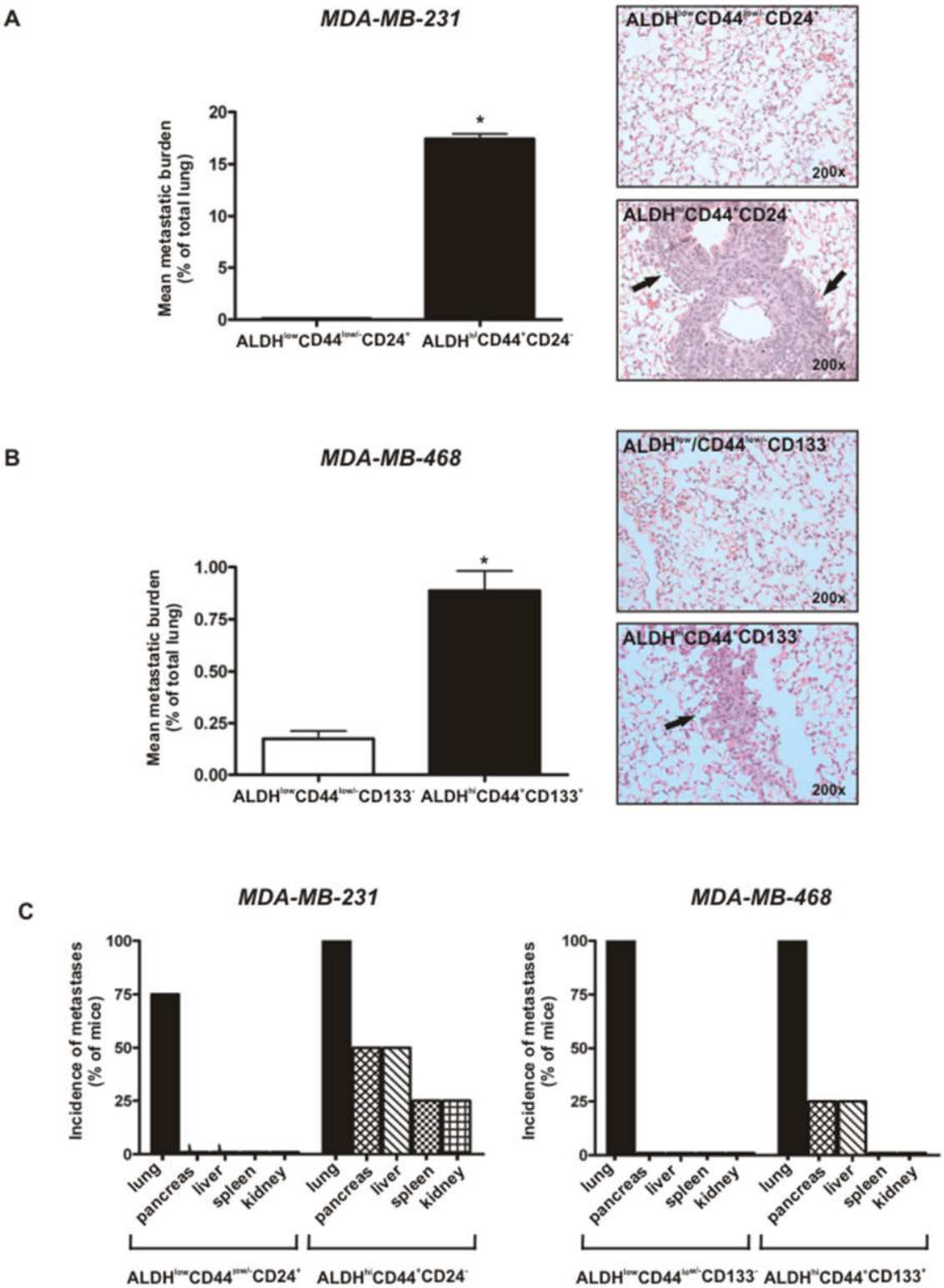
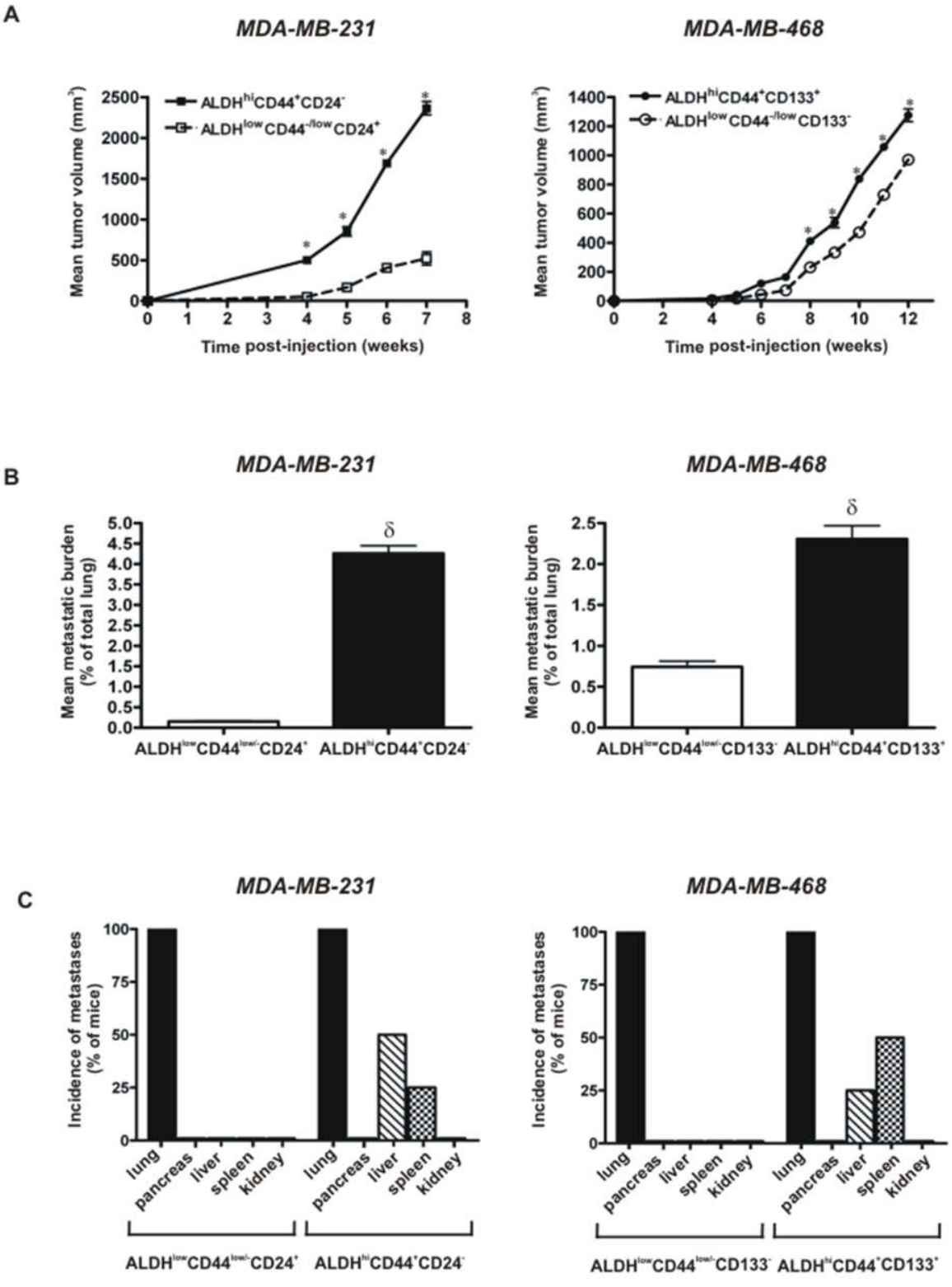

A
MDA-MB-231
Mean metastatic burden (% of total lung)
20
15
10
5
0
*
ALDHlowCD44low/-CD24+
ALDHhiCD44+CD24-
ALDHlowCD44low/-CD24+
200x
ALDHhiCD44+CD24-
200x
B
MDA-MB-468
Mean metastatic burden (% of total lung)
1.00
0.75
0.50
0.25
0.00
*
ALDHlowCD44low/-CD133-
ALDHhiCD44+CD133+
ALDHlow/CD44low/-CD133-
200x
ALDHhiCD44+CD133+
200x
C
MDA-MB-231
Incidence of metastases (% of mice)
100
75
50
25
0
lung
pancreas
liver
spleen
kidney
ALDHlowCD44low/-CD24+
ALDHhiCD44+CD24-
MDA-MB-468
Incidence of metastases (% of mice)
100
75
50
25
0
lung
pancreas
liver
spleen
kidney
ALDHlowCD44low/-CD133-
ALDHhiCD44+CD133+

Figure 2.10 $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ breast cancer cells demonstrate enhanced tumorigenicity and metastatic growth *in vivo* following mammary fat pad injection. Cells were isolated by FACS as described in *Figure 2.5* and *Figure 2.6* and injected into the right thoracic mammary fat pad of female NOD/SCID-IL2Rγ null mice using an established model of spontaneous metastasis ($5x10^5$ cells/mouse; 4 mice/cell population). At 7 weeks (MDA-MB-231) or 12 weeks (MDA-MB-468) post-injection, mice were sacrificed and assessed for metastatic burden in the lung and elsewhere. Tissue sections were subjected to H&E staining (5 random sections/tissue/mouse), and the incidence and extent of metastasis was determined in a blinded fashion. ***(A)*** Primary tumor growth kinetics following mammary fat pad injection of $ALDH^{hi}CD44^{+}CD24^{-}$ (■) versus $ALDH^{low}CD44^{low/-}CD24^{+}$ (□) cells isolated from the MDA-MB-231 cell line (*left panel*); and $ALDH^{hi}CD44^{+}CD133^{+}$ (●) versus $ALDH^{low}CD44^{low/-}CD133^{-}$ (○) cells isolated from the MDA-MB-468 cell line (*right panel*). Data are presented as the mean ± SEM. * = significantly different tumor size than respective $ALDH^{low}CD44^{low/-}$ subsets at the same time point ($p<0.05$). ***(B)*** Quantitative analysis of spontaneous lung metastasis tumor burden (mean % of lung occupied by tumor) following mammary fat pad injection of $ALDH^{hi}CD44^{+}CD24^{-}$ versus $ALDH^{low}CD44^{low/-}CD24^{+}$ cells isolated from the MDA-MB-231 cell line (*left panel*); and $ALDH^{hi}CD44^{+}CD133^{+}$ versus $ALDH^{low}CD44^{low/-}CD133^{-}$ cells isolated from the MDA-MB-468 cell line (*right panel*). Data are presented as the mean ± SEM. δ = significantly different than respective $ALDH^{low}CD44^{low/-}$ subsets ($p<0.05$). ***(C)*** Incidence of spontaneous metastatic growth in lung and extrapulmonary tissues following mammary fat pad injection of $ALDH^{hi}CD44^{+}CD24^{-}$ versus $ALDH^{low}CD44^{low/-}CD24^{+}$ cells isolated from the MDA-MB-231 cell line *(left panel)*; and $ALDH^{hi}CD44^{+}CD133^{+}$ versus $ALDH^{low}CD44^{low/-}CD133^{-}$ cells isolated from the MDA-MB-468 cell line (*right panel*).


A
MDA-MB-231
MDA-MB-468
Mean tumor volume (mm³)
Time post-injection (weeks)
ALDHhiCD44+CD24-
ALDHlowCD44-/lowCD24+
ALDHhiCD44+CD133+
ALDHlowCD44-/lowCD133-
B
MDA-MB-231
MDA-MB-468
Mean metastatic burden (% of total lung)
δ
ALDHlowCD44low/-CD24+
ALDHhiCD44+CD24-
ALDHlowCD44low/-CD133-
ALDHhiCD44+CD133+
C
MDA-MB-231
MDA-MB-468
Incidence of metastases (% of mice)
lung
pancreas
liver
spleen
kidney
ALDHlowCD44low/-CD24+
ALDHhiCD44+CD24-
ALDHlowCD44low/-CD133-
ALDHhiCD44+CD133+

Following tail vein injection, metastatic tumor burden in the lung (% of lung occupied by tumor) was significantly higher in mice injected with $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) or $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) cells versus respective $ALDH^{low}CD44^{low/-}$ cell subsets (*Figure 2.9A,B*) ($p<0.05$). Analysis of incidence of metastases (% of mice demonstrating metastatic growth) in the lung and extrapulmonary organs revealed that, although all subpopulations of cells were able to establish themselves in the lung (*Figure 2.9C*), only $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) and $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) cells were able to maintain that growth into larger metastases (*Figure 2.9A,B*). Furthermore, metastases in extrapulmonary organs such as the pancreas, liver, spleen, and/or kidney were only observed in mice injected with $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) or $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) cells, and not in mice injected with $ALDH^{low}CD44^{low/-}$ cell subsets (*Figure 2.9C*).

In order to compare tumorigenicity and metastatic ability in a more clinically relevant model system, we next used a spontaneous model of metastasis involving orthotopic injection of breast cancer cells into the mammary fat pad. We observed that $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) and $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) cells demonstrated enhanced primary tumor growth relative to respective $ALDH^{low}CD44^{low/-}$ cell subsets (*Figure 2.10A*). Spontaneous metastatic tumor burden in the lung was significantly higher in mice injected with $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) and $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) cells versus respective $ALDH^{low}CD44^{low/-}$ cell subsets (*Figure 2.10B*) ($p<0.05$). Interestingly, we did not observe a significant correlation between primary tumor size and metastatic burden in the lungs of individual mice, either in the “stem-like” populations ($ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$) ($R^2=0.365$) or in the respective $ALDH^{low}CD44^{low/-}$ populations ($R^2=0.530$). Similar to the observations following tail vein injection, although all subpopulations of cells were able to establish themselves in the lung (*Figure 2.10C*), only $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) and $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) had an enhanced capacity to maintain that

growth into larger metastases (*Figure 2.10B*), and to spontaneously metastasize to extrapulmonary organs such as the pancreas and spleen (*Figure 2.10C*).

2.4 Discussion

The majority of breast cancer deaths occur as a result of metastatic disease rather than from the effects of the primary tumor. The inefficiency of the metastatic process, the inherently heterogeneous nature of solid tumors, and the influence of the tumor microenvironment dictate that only a small subset of cells can successfully navigate the metastatic cascade and eventually re-initiate tumor growth to form life-threatening metastases[1]. While it has been speculated that this subset of cells may be cancer stem cells (CSCs), most studies to date have focused on the role of CSCs in primary tumor growth rather than on their potential contribution to metastatic behavior. Furthermore, the challenges of obtaining large numbers of primary cells from patient samples combined with the technical complexity of studying metastasis of primary cells *in vivo* suggests that suitable alternative model systems need to be developed and validated in order to gain a greater insight into the role of stem-like cells in metastasis.

In the present study, we demonstrated that commonly studied human breast cancer cell lines contain subpopulations of stem-like cells based on both putative CSC marker expression (CD44/CD24 and CD133), and functional stem cell properties (enhanced ALDH activity). Furthermore, the "stem-like" cell content of the cell lines seemed to be associated with their aggressiveness (i.e. the more aggressive/metastatic the cell line, the greater the $CD44^+$ and/or $ALDH^{hi}$ cell content). Our novel findings also demonstrate that $ALDH^{hi}CD44^+CD24^-$ (MDA-MB-231) and $ALDH^{hi}CD44^+CD133^+$ (MDA-MB-468) cells isolated from human breast cancer cell lines display significantly enhanced malignant/metastatic behavior compared to respective $ALDH^{low}CD44^{low/-}$ subsets, including increased *in vitro* growth and colony forming ability in an anchorage-independent environment and increased *in vitro* adhesion, migration, and invasion.

Moreover, when the different cell populations were isolated and injected into the tail vein or mammary fat pad of NOD/SCID-IL2Rγ null mice, the $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) and $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) populations showed increased tumorigenicity, increased metastatic growth in the lungs, and extrapulmonary metastases, while the respective $ALDH^{low}CD44^{low/-}$ populations showed minimal metastatic growth *in vivo*.

Our findings are supported by two recently published studies that also demonstrate a potential link between stem-like cancer cells and successful metastatic behavior[8,41]. During the course of carrying out the present study, Yu et al. (2007) published findings demonstrating that stem-like cells isolated from the SKBR3 human breast cancer cell line based on their mammosphere forming ability were more metastatic *in vivo* than unsorted parental SKBR3 cells[41]. In pancreatic cancer, Hermann et al. (2007) identified a subpopulation of cells within a $CD133^{+}$ cell population that expressed the chemokine receptor CXCR4[8]. In concert with its chemokine ligand SDF-1, CXCR4 has been shown to play a key role in both normal stem cell migration and homing, as well as cancer cell migration and metastasis[42]. When the $CXCR4^{+}$ pancreatic CSC subpopulation was eliminated in various highly metastatic pancreatic cancer cell lines, the cells could still efficiently form primary tumors but were no longer able to metastasize[8]**.** This suggests that the CSC population, at least in pancreatic cancer, is responsible for metastasis, but only because of a small subgroup of $CD133^{+}CXCR4^{+}$ CSCs. Interestingly, both MDA-MB-231 and MDA-MB-468 cell lines demonstrate moderate and uniform expression of CXCR4 (*Figure 2.11*), suggesting that CXCR4 may also be important for metastasis of stem-like cells in breast cancer.

Conventionally, CSC populations have been isolated from solid human tumors based on cell surface expression of markers such as CD133 and CD44[6-11]. CD133 is a marker expressed by many types of normal stem cells, including neural and hematopoietic stem cells[9,11], and has

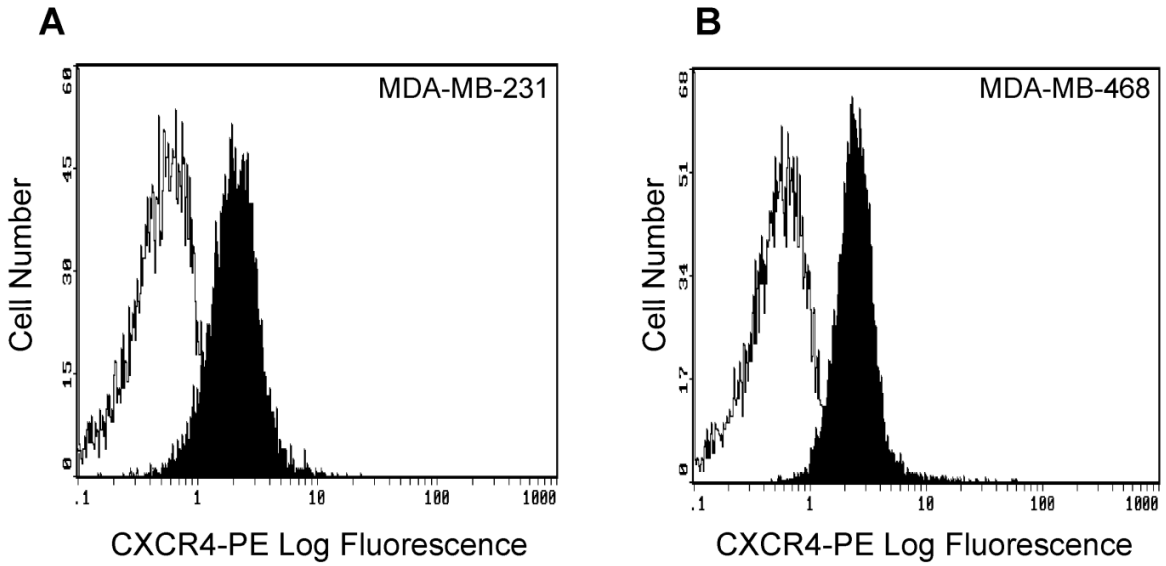


Figure 2.11 MDA-MB-231 and MDA-MB-468 human breast cancer cell lines express CXCR4*. (A-B)* Representative flow cytometry histograms of ***(A)*** MDA-MB-231 cells and ***(B)*** MDA-MB-468 cells. Cells were incubated with an anti-CXCR4 antibody (clone 12G5) conjugated to phycoerytherin (PE) (*black profiles*) or a PE-conjugated IgG isotype control (*white profiles*). A minimum of 10,000 events were collected per sample.

been shown to play a role in stem cell migration and asymmetric division[43,44]. CD44 is a cell surface receptor for hyaluronic acid, and is involved in cell adhesion, migration, and metastasis of cancer cells[23]. From a functional perspective, it is therefore not surprising that these markers would select for highly aggressive tumor cells. However, although defined cell surface markers have long been used to reliably isolate normal stem cells of various lineages, the inherent genetic instability of solid cancers suggests that it may be problematic to rely on cell surface marker expression alone to prospectively identify and isolate CSCs[20-23]. Self-protection is a key property of normal stem cells, and is of vital importance for protecting and maintaining the stem cell pool throughout the lifespan of an organism. Aldehyde dehydrogenase (ALDH) activity is important for this self-protection through its role in retinoic acid signaling and oxidation of intracellular aldehydes[24,45], and thus provides a means of identifying and isolating stem-like cancer cells based on a conserved stem/progenitor cell function.

When we analyzed ALDH activity in human breast cancer cell lines, the pattern of ALDH activity in different cell lines often corresponded to their observed pattern of CSC marker expression. For example, in the case of the moderately metastatic MDA-MB-231 and weakly metastatic MDA-MB-468 cell lines (where subpopulations were evident in the marker expression analysis), two distinct subpopulations were also observed with respect to ALDH activity. Similarly, non-metastatic MCF-7 cells did not contain a detectable $CD44^+CD24^-$ or $CD133^+$ subpopulation and showed minimal increase in ALDH activity. There is evidence to support the idea that isolating CSC-like cells by both marker expression and ALDH activity is most accurate. When Ginestier et al (2007) isolated stem-like cells from primary human breast tumors using both ALDH activity and $CD44^+CD24^-$, the $ALDH^{hi}CD44^+CD24^-$ population was more tumorigenic than populations identified by either marker expression or ALDH activity alone. Interestingly, the overlap between CSC marker expression ($CD44^+CD24^-$) and high ALDH activity in primary tumors was observed to be quite small (~1%)[32]. In the present study, we investigated two distinct subpopulations of “stem-like” cells; $ALDH^{hi}CD44^+CD24^-$ cells isolated from the

moderately metastatic MDA-MB-231 cell line and $ALDH^{hi}CD44^{+}CD133^{+}$ cells isolated from the weakly metastatic MDA-MB-468 cell line. Although both populations behaved in a similar manner from the point of view of having significantly enhanced malignant and metastatic ability relative to their respective $ALDH^{low}CD44^{low/-}$ subsets, the *in vitro* and *in vivo* differences in growth-related functional behavior of $ALDH^{hi}CD44^{+}CD133^{+}$ and $ALDH^{low}CD44^{low/-}CD133^{-}$ subsets were more subtle than those between $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{low}CD44^{low/-}CD24^{+}$ subsets. These may simply be cell-line specific differences, or they may be related to the differential use of CD133 versus CD24 to refine the ALDH isolation procedure. This is supported by a recent study by Shmelkov et al (2008) which demonstrated that CD133 may not be restricted to stem cells and that both $CD133^{+}$ and $CD133^{-}$ metastatic colon cancer cells can initiate tumors *in vivo*[46]. This, combined with the findings of our study and those of Ginestier et al (2007)[32] suggests that there is heterogeneity even within the CSC population of breast tumors, and that $ALDH^{hi}CD44^{+}$ cells (and $ALDH^{hi}CD44^{+}CD24^{-}$ cells in particular) may represent the most aggressive and malignant population within the CSC pool.

When the *in vitro* proliferative capacity of $ALDH^{hi}CD44^{+}CD24^{-}$ (MDA-MB-231) and $ALDH^{hi}CD44^{+}CD133^{+}$ (MDA-MB-468) versus $ALDH^{low}CD44^{low/-}$ populations was compared in this study, it was observed that the $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ populations demonstrated enhanced growth. Similarly, *in vivo* studies demonstrated that $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ showed enhanced primary tumor growth in the mammary fat pad relative to respective $ALDH^{low}CD44^{low/}$ populations. Interestingly, although all cell subsets could establish themselves in the lung as very small micrometastases, again only the $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ cells had a significantly enhanced capacity for metastatic growth resulting in increased tumor burden in the lung and metastases to extrapulmonary organs such as spleen and pancreas. In the context of stem cell properties, these results were a bit surprising, since it is well established that the proliferation rate of primitive stem cells is lower than that of committed cells[43,47-49]. However, ALDH has been shown

to identify rapidly dividing cells that represent a progenitor cell population in human umbilical cord blood and bone marrow ([50] and our unpublished data), and this may also be true in the context of breast cancer. The studies described here suggest that both $ALDH^{hi}CD44^{+}$ $CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ breast cancer cells may in fact demonstrate a more proliferative progenitor-like phenotype (rather than a quiescent stem cell phenotype), although further studies are required to investigate this idea in more detail.

In summary, the novel findings presented here indicate that $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ stem-like cancer cells isolated from MDA-MB-231 and MDA-MB-468 human breast cancer cell lines demonstrate enhanced malignant/metastatic behavior *in vitro* and *in vivo,* and suggest that breast cancer cell lines may provide a suitable model system for starting to investigate the role of stem-like cells in metastasis. Furthermore, our study represents the first report that selection and isolation of stem-like breast cancer cells on the basis of ALDH activity can enhance for functional cell properties that contribute to metastasis, including *in vitro* adhesion, migration, and invasion, and *in vivo* growth in primary and secondary organ sites. Further elucidation of the mechanisms by which $ALDH^{hi}CD44^{+}CD24^{-}$ and $ALDH^{hi}CD44^{+}CD133^{+}$ cell populations successfully metastasize and the translation of this knowledge into the clinic could have potentially important implications for the management and treatment of breast cancer.

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Chapter 3

Inhibition of aldehyde dehydrogenase (ALDH) activity reduces chemotherapy and radiation resistance of stem-like $ALDH^{hi}CD44^{+}$ human breast cancer cells

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Abstract

The majority of breast cancer deaths are due to ineffective treatment of metastatic disease. We previously identified a subpopulation of cells in human breast cancer cell lines that demonstrate high activity of aldehyde dehydrogenase (ALDH) and high expression of CD44. These $ALDH^{hi}CD44^{+}$ cells displayed enhanced metastatic behavior *in vitro* and *in vivo* relative to $ALDH^{low}CD44^{-}$ cells. The goal of the current study was to test the hypothesis that $ALDH^{hi}CD44^{+}$ breast cancer cells are more resistant to standard cancer therapy, and that inhibiting ALDH activity via all-*trans* retinoic acid (ATRA) or the specific ALDH inhibitor diethylaminobenz-aldehyde (DEAB) sensitizes these cells to treatment. $ALDH^{hi}CD44^{+}$ and $ALDH^{low}CD44^{-}$ populations were isolated from MDA-MB-231 and MDA-MB-468 cells lines and exposed to chemotherapy (doxorubicin/paclitaxel) or radiotherapy ± ATRA or DEAB. Cell populations were assessed for differences in survival, colony-formation, and protein expression related to therapy resistance and differentiation. Significantly more $ALDH^{hi}CD44^{+}$ cells survived chemotherapy/ radiotherapy relative to $ALDH^{low}CD44^{-}$ cells ($P<0.001$). Glutathione-s-transferase pi, p-glycoprotein, and/or CHK1 were overexpressed in $ALDH^{hi}CD44^{+}$ populations compared to $ALDH^{low}CD44^{-}$ populations ($P<0.05$). Pre-treatment of cell populations with DEAB or ATRA had no effect on $ALDH^{low}CD44^{-}$ cells, but resulted in significant initial sensitization of $ALDH^{hi}CD44^{+}$ cells to chemotherapy/radiotherapy. However, only DEAB had a long-term effect, resulting in reduced colony-formation ($P<0.01$). ATRA also significantly increased expression of CK8/18/19 in MDA-MB-468 $ALDH^{hi}CD44^{+}$ cells compared to control ($P<0.05$). Our novel findings indicate that $ALDH^{hi}CD44^{+}$ breast cancer cells contribute to both chemotherapy and radiation resistance and suggest a much broader role for ALDH in treatment response than previously reported.

3.1 Introduction

Breast cancer is a leading cause of death in women. At its initial stages, breast cancer is highly treatable using surgery and/or a combination of hormonal therapy, radiation, and chemotherapy[1,2]. However, once the disease has metastasized, most patients become incurable due to the fact that the majority of current cancer therapies fail in the metastatic setting[3]. In order to reduce mortality from breast cancer, it is therefore essential to learn more about the biology of the metastatic process, specifically by identifying the particular subpopulation of cells within a heterogeneous primary tumor that are responsible for metastasis and what makes these cells so resistant to therapy.

In breast cancer, "stem-like" tumor-initiating cells have been prospectively isolated from primary tumors, pleural effusions, and breast cancer cell lines based on a $CD44^+CD24^-$ phenotype and/or high aldehyde dehydrogenase (ALDH) activity ($ALDH^{hi}$ phenotype)[4-8]. The ALDH superfamily of enzymes is involved in detoxification of intracellular aldehydes. Certain isoenzymes such as ALDH1A1 and ALDH3A1 have been shown to play important functional roles in normal stem cells with regards to self-protection, and may also play a role in early differentiation of stem cells through their role in oxidizing retinol to retinoic acid[9]. In addition, high ALDH1 expression has been shown to correlate with poor prognosis in breast cancer patients[8], and has been associated with the development of metastases[5-7] and poor clinical outcome[5].

While there is accumulating evidence supporting the existence of stem-like cancer cells in primary tumors, what remains less clear is the functional and mechanistic impact of these cells in mediating metastatic behaviour and resistance to therapy. Our research group recently identified subpopulations of stem-like $ALDH^{hi}CD44^+$ cells in several human breast cancer cell lines[7]. Intriguingly, highly metastatic cell lines such as MDA-MB-435 contained the highest proportion of $ALDH^{hi}CD44^+$ cells, whereas non-metastatic cell lines such as MCF-7 contained very few $ALDH^{hi}CD44^+$ cells. When these cells were isolated and compared to $ALDH^{low}CD44^-$

cells, we observed that $ALDH^{hi}CD44^{+}$ breast cancer cells demonstrated enhanced metastatic behaviour *in vitro* (adhesion, migration, invasion, proliferation), as well as enhanced metastatic capacity *in vivo*[7]. Subsequent studies by Charafe-Jauffret et al. (2009, 2010) demonstrated that stem-like $ALDH^{hi}$ cells isolated from breast cancer cell lines also had increased metastatic potential *in vivo*[5,6] supporting the idea that $ALDH^{hi}CD44^{+}$ cells may have a role as metastasis-initiating cells.

Clinically, the lethality associated with metastatic disease in breast cancer arises mainly from the inability of current therapies to successfully treat resistant metastatic tumors[10]. It is well documented that stem-like cells play an important role in therapy resistance in a variety of different cancer types via activation of DNA repair[11-13], up-regulation of drug resistance proteins[14-16], and/or through specific pathways such as Notch[17], Snail/Slug[18], Hedgehog[19], or Wnt/ß-catenin[20]. While the therapy resistance properties of $CD44^{+}CD24^{-}$ breast cancer tumor-initiating cells have been previously described[13,21] it is less clear whether metastasis-initiating $ALDH^{hi}CD44^{+}$ breast cancer cells also possess such resistance properties. However, the functional role of ALDH activity in self-protection and differentiation of normal stem cells and in resistance to the chemotherapeutic drug cyclophosphamide (CP)[22] suggests that this enzyme may be an important contributor to the therapy resistance of stem-like cancer cells.

The differentiation agent all-*trans* retinoic acid (ATRA) is used clinically in combination with CP to treat acute promyelocytic leukemia (APL)[23-25]. The increase in intracellular retinoic acid (RA) resulting from ATRA treatment suppresses levels of ALDH1A1 and 3A1, driving differentiation of promyelocytes into neutrophils and causing enhanced sensitivity to CP therapy[26,27]. ATRA has been shown to modulate cell growth and induce apoptosis in breast cancer cells, as well as induce properties of differentiation in stem-like breast cancer cells[8,28]. However, while the role that ALDH activity plays in CP resistance is well understood, the function of this enzyme in conferring resistance to other commonly used chemotherapeutic agents and to radiation therapy requires further elucidation.

The goal of the current study was therefore to test the hypothesis that $ALDH^{hi}CD44^{+}$ breast cancer cells are more resistant to standard cancer therapy, and that inhibiting ALDH activity by treating $ALDH^{hi}CD44^{+}$ cells with ATRA or the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) sensitizes these cells to treatment. $ALDH^{hi}CD44^{+}$ and $ALDH^{low}CD44^{-}$ populations were isolated from MDA-MB-231 and MDA-MB-468 breast cancer cells lines and exposed to chemotherapy (doxorubicin, paclitaxel) or radiotherapy in the presence or absence of ATRA or DEAB. Cell populations were assessed for differences in survival, colony formation, and protein expression related to therapy resistance and differentiation. The novel findings presented here indicate that $ALDH^{hi}CD44^{+}$ breast cancer cells not only have the capacity to be metastasis-initiating cells[7], but that they also contribute to both chemotherapy and radiation resistance, suggesting a much more widespread role for ALDH in therapeutic resistance than has previously been reported.

3.2 Materials and Methods

3.2.1 Cell culture, reagents, and therapy conditions

MDA-MB-231 cells were obtained from ATCC (Manassas, VA) and were maintained in DMEM:F12 + 10% fetal bovine serum (FBS). MDA-MB-468 cells were a kind gift from Dr. Janet Price, M.D. Anderson Cancer Center, Houston, TX[29], and were maintained in αMEM + 10% fetal bovine serum (FBS). Both MDA-MB-231 and MDA-MB-468 cell lines were authenticated via third party testing of 9 short tandem repeat (STR) loci on June 14, 2010 (CellCheck, RADIL, Columbia, MO). Media was obtained from Invitrogen (Carlsbad, CA). FBS was obtained from Sigma (St. Louis, MO). All tissue culture plastic was obtained from NUNC (Roskilde, Denmark). ATRA and DEAB (Sigma) were constituted in 100% ethanol and diluted in either DMEM:F12 or α-MEM media at 5μM (ATRA) or 100μM (DEAB). Doxorubicin (Novopharm Limited, Toronto, ON) and paclitaxel (Biolyse Pharma Corporation, St. Catharines, ON) were obtained from the London Regional Cancer Program pharmacy and diluted in either DMEM:F12 or α-MEM to a

concentration of 0.4μM (doxorubicin) or 0.2μM (paclitaxel). Radiation was administered to cells using a Cobalt-60 irradiator (Theratron 60, Atomic Energy of Canada Limited). MDA-MB-231 cells received 2x5Gy and MDA-MB-468 cells received 2x3Gy. All chemotherapy and radiation dose levels were selected based on LC_{50} values determined in preliminary experiments (*data not shown*).

In order to assess ALDH activity following ATRA or DEAB treatment, the ALDEFLUOR® assay kit was used (StemCell Technologies, Vancouver, BC) in conjunction with flow cytometry analysis as per the manufacturers' guidelines.

3.2.2 Identification and fluorescence activated cell sorting (FACS) isolation of cell populations

Stem-like ($ALDH^{hi}CD44^{+}$) and non-stem-like ($ALDH^{low}CD44^{-}$) cell populations were identified and isolated from MDA-MB-231 and MDA-MB-468 cell lines using the ALDEFLUOR® assay kit (StemCell Technologies) and fluorescently conjugated antibodies against CD44, CD24 (BD Biosciences, Mississauga, ON), and CD133 (Miltenyi Biotec, Auburn, CA) as described previously[7]. Antibodies used included anti-CD44 (clone IM7) conjugated to allophycocyanin (APC), anti-CD24 (clone ML5), or anti-CD133 (clone AC133) conjugated to phycoerytherin (PE). Appropriate fluorescently-conjugated IgG isotype controls (BD Biosciences) were used as negative controls. Cells were concurrently labelled with 7-Amino-actinomycin (7-AAD), fluorescent antibodies (CD44-APC + CD24-PE [MDA-MB-231] or CD44-APC + CD133-PE [MDA-MB-468]) and the ALDEFLUOR™ assay kit. $ALDH^{hi}CD44^{+}$ and $ALDH^{low}CD44^{low/-}$ subsets were isolated from MDA-MB-231 and MDA-MB-468 cell lines using a 4-color analysis protocol on a FACS Vantage/Diva cell sorter (BD Biosciences) as described previously[7]. ALDH activity was used as the primary sort criteria (top ~20%=$ALDH^{hi}$; bottom ~20%=$ALDH^{low}$) and $CD44^{+}CD24^{low/-}$ (MDA-MB-231 cells) or $CD44^{+}CD133^{+}$ (MDA-MB-468 cells) phenotype as the secondary sort criteria (top ~10% gated on $ALDH^{hi}$; bottom ~10% gated on $ALDH^{low}$). Cell purity for each population was confirmed to be 98-99% pure for each individual sort. Cell viability was

assessed by 7-AAD staining during cell sorting, and confirmed by trypan blue exclusion post-sorting. Following FACS isolation, the resulting cell populations from both cell lines were designated as $ALDH^{hi}CD44^{+}$ and $ALDH^{low}CD44^{-}$ cells and were placed into culture for no more than 24 hours prior to use in functional *in vitro* assays as described below.

3.2.3 Cell survival assays

Isolated $ALDH^{hi}CD44^{+}$ and $ALDH^{low}CD44^{-}$ cell populations were plated at a density of $5x10^{4}$ cells in 12-well plates (n=3/treatment group) and maintained in normal growth medium for 48 hours. Cells were treated with normal media alone, ethanol (EtOH) vehicle control, chemotherapy (paclitaxel [0.2μM]; doxorubicin [0.4μM]), or radiation (2x3Gy, MDA-MB-468; or 2x5Gy, MDA-MB-231), and cells were cultured for a further 72 hours. For experiments involving ATRA and DEAB, cells were pre-treated with ATRA (5μM) or DEAB (100μM) in normal media for 48 hours prior to initiating treatment with chemotherapy or radiation. Cells were then harvested and viable cells were quantified using trypan blue exclusion and manual counting on a hemacytometer using light microscopy.

3.2.4 Colony formation assays

Isolated $ALDH^{hi}CD44^{+}$ and $ALDH^{low}CD44^{-}$ cell populations were plated at a density of $1x10^{3}$ cells per well of 12-well plates (n=3/treatment group) and maintained in normal growth medium for 48 hours. Cells were treated with normal media alone, EtOH vehicle control, chemotherapy (paclitaxel [0.2μM]; doxorubicin [0.4μM]), or radiation (2x3Gy, MDA-MB-468; or 2x5Gy, MDA-MB-231), and cells were cultured for a further 72 hours. For experiments involving ATRA (5μM) and DEAB (100μM), cells were pre-treated for 48 hours prior to initiating treatment with chemotherapy or radiation. Cells were then harvested and viable cells were quantified using trypan blue exclusion and manual counting. One thousand viable cells were plated on 6-well plates (n=3/treatment group) and maintained in culture for 2 weeks in order to test their ability to re-grow. Resulting cell colonies were fixed with 1% gluteraldehyde, stained with Harris'

hematoxylin, and quantified using ImageJ software.

3.2.5 Western blotting

Cell lysates were extracted from FACS-sorted $ALDH^{hi}CD44^{+}$ cells and $ALDH^{low}CD44^{-}$ cell populations (+/-ATRA or DEAB) using lysis buffer containing PMSF (100mM), benzamidine (100mM), leupeptin (1mg/ml), pepstatin A (1mg/ml), and aprotinin (1mg/ml). Ten μg of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10%) and transferred onto polyvinylidene difluoride membranes (PVDF; Immobilon™, Millipore; Bedford, MA, USA). Blocking and antibody dilution was done using 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST). Anti-human primary antibodies against glutathione-S-transferase pi (GSTpi), p-glycoprotein (Pgp), CHK1 checkpoint homolog (CHK1), phospho-CHK1 checkpoint homolog (P-CHK1), CHK2 checkpoint homolog (CHK2), phospho-CHK2 checkpoint homolog (P-CHK2), N-cadherin (CDH2), vimentin, E-cadherin (CDH1), cytokeratins 8, 18, 19 (CK8/18/19), retinoic acid receptor-α (RAR-α), Y box binding protein (YB1), phospho-Y box binding 1 (P-YB1), and β-actin loading control are described in *Table 3.1*. Secondary antibodies used include goat anti-mouse, mouse anti-rabbit (Calbiochem, Gibbstown, NJ), and rabbit anti-chicken (Sigma-Aldrich, St. Louis, MO) antibodies conjugated to horseradish peroxidase (1:2000 dilution for all proteins except E-cadherin; 1:10,000). Protein expression was visualized using the Amersham ECL Plus Western Blot Detection System (GE Healthcare, Baie d'Urfe, QC) and BIOMAX MS scientific imaging film (Kodak, Rochester, NY), and developed in the Kodak M35A X-OMAT Processor (Rochester, NY). Expression of each protein was normalized to the β-actin loading control and signal intensity was quantified (n=3 blots per protein) using the computer-assisted densitometry program AlphaEase FC™ Software version 3.1.2 (Alpha Innotech Corporation, Miami FL).

Table 3.1 Details of anti-human antibodies used for western blot analysis of proteins related to therapy resistance and/or differentiation

Protein/Antibody	Clone	Commercial Source	Lot	Dilution
Glutathione S Tranferase pi	USal-hGST-Pi-McAb-1	Alexis Biochemicals	L23236	1:1000
P-glycoprotein	C219	Calbiochem	D00089430	1:100
Chk1	polyclonal	Cell Signaling Technology	3	1:2000
Phospho-Chk1	polyclonal	Cell Signaling Technology	8	1:1000
Chk2	polyclonal	Cell Signaling Technology	4	1:1000
Phospho-Chk2	polyclonal	Cell Signaling Technology	7	1:1000
N-Cadherin	EPR1791-4	Abcam	812783	1:1000
Vimentin	V9	Millipore	LV1700725	1:1000
E-Cadherin	36/E-Cadherin	BD Biosciences	09705	1:20000
Cytokeratins 8/18/19	2A4	Abcam	794363	1:1000
Retinoic Acid Receptor α	H1920	R&D Systems Inc	A-1	1:1000
Y-Box Binding Protein	EP2708Y	Abcam	795739	1:2000
Phospho-Y-Box Binding Protein	polyclonal	Abcam	791762	1:1000
β-actin	polyclonal	Sigma	083K4834	1:5000

3.2.6 *In vivo* animal experiments

All *in vivo* work was carried out using NOD/SCID mice, which permit increased tissue engraftment of human cells without rejection due to reduced innate immunity (NOD mutation) and complete T- and B-cell deficiency (SCID mutation). Animal procedures were conducted in accordance with the recommendations of the Canadian Council on Animal Care, under a protocol approved by the University of Western Ontario Council on Animal Care (*Appendix 2*).

One x 10^6 MDA-MB-231 cancer cells suspended in sterile Hank's buffered salt solution were injected into the right thoracic mammary fat pad of 7-10 week old female NOD/SCID mice (n=8mice/group). Tumors were allowed to grow to a mean tumor volume of 500 mm^3, and then the mice were randomized into groups and treated with either placebo pellets, ATRA (10mg pellets that release 160mg/kg/day of ATRA from Innovative Research of America), placebo + paclitaxel (i.p., 5mg/kg), or ATRA + paclitaxel once weekly for 6 weeks. Dexamethasone, obtained from the London Regional Cancer Program pharmacy and diluted in PBS (i.p, 2mg/kg daily), was used to help the mice survive the ATRA therapy. Primary tumor growth was evaluated weekly by caliper measurement in two perpendicular dimensions, and tumor volume was estimated using the following formula: (volume= 0.52 x $[width]^2$ x [length]) for approximating the volume (mm^3) of an ellipsoid. After 6 weeks of therapy, the mice were sacrificed and primary tumors were measured and then flash-frozen in OCT for cryosectioning. Tissues from distant organs were collected and assessed for differences in metastatic involvement using histological staining. Lung tissue was sectioned and stained with hematoxylin and eosin staining (H&E) in order to determine mean metastatic burden. End-points and cell numbers for injection were chosen based on preliminary experiments (*data not shown*).

Tissues and organs were examined superficially for evidence of gross macroscopic metastases at necropsy. Tissues collected at necropsy were then fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned randomly (4µm sections; at least 100µm apart; five sections/tissue), and subjected to standard H&E staining. Stained slides were evaluated by light

microscopy in a blinded fashion in order to identify and characterize incidence and regions of micrometastatic involvement. Metastatic tumor burden in the lung (tumor area/total organ area) was determined quantitatively by manual outlining and analysis by ImageJ software (NIH, Bethesda, MD, USA) as described previously[7].

3.2.7 Statistical analysis

All experiments with FACS-isolated cells were performed following at least 2 separate cell sorts with at least 3 biological replicates included within each experiment. In all cases, quantitative data was compiled from all experiments. Statistical analysis was performed using GraphPad Prism 4.0 software© (San Diego, CA) using either t-test (for comparison between 2 groups) or ANOVA with Tukey post-test (for comparison between more than 2 groups). Differences between means were determined using the Student's t-test when groups passed both a normality test and an equal variance test. When this was not the case, the Mann-Whitney Rank-Sum test was used. Unless otherwise noted, data is presented as the mean ± SEM. In all cases, *P* values of ≤0.05 were regarded as being statistically significant.

3.3 Results

3.3.1 $ALDH^{hi}CD44^{+}$ human breast cancer cells demonstrate enhanced resistance to chemotherapy and radiation treatment

In order to determine whether metastasis-initiating $ALDH^{hi}CD44^{+}$ human breast cancer cells[7] were resistant to standard cancer therapies, we assessed their response to standard chemotherapy and radiation. $ALDH^{hi}CD44^{+}$ and $ALDH^{low}CD44^{-}$ cell populations were isolated via FACS and exposed to the common breast cancer chemotherapy drugs doxorubicin or paclitaxel[10] (*Figure 3.1*), or to radiation treatment (*Figure 3.2*). We observed that, relative to $ALDH^{low}CD44^{-}$ cells, $ALDH^{hi}CD44^{+}$ cells from both the MDA-MB-231 and MDA-MB-468 cell lines demonstrated a significantly enhanced ability to survive doxorubicin or paclitaxel chemotherapy

(*Figure 3.1A,B*) and a significantly increased ability to subsequently re-grow and form colonies following treatment (*Figure 3.1C,D*) ($P<0.001$). Similarly, relative to $ALDH^{low}CD44^{-}$ cells, $ALDH^{hi}CD44^{+}$ cells also showed significantly enhanced survival (*Figure 3.2A,B*) and re-growth/colony-forming ability in response to radiation therapy (*Figure 3.2B,D*) ($P<0.05$).

3.3.2 $ALDH^{hi}CD44^{+}$ human breast cancer cells demonstrate enhanced expression of drug resistance proteins

In order to investigate why $ALDH^{hi}CD44^{+}$ breast cancer cells were more resistant to both chemotherapy (paclitaxel and doxorubicin) and radiation therapy, cell lysates were collected from FACS-sorted $ALDH^{hi}CD44^{+}$ and $ALDH^{low}CD44^{-}$ cell populations, and western blots were performed to analyze the expression of several drug resistance proteins. P-glycoprotein (Pgp) is a well characterized ABC-efflux pump, which efficiently pumps chemotherapeutic agents out of the cell[30]. Glutathione-S-transferase pi (GSTpi) catalyzes the reaction of glutathione with electrophilic substances before they can interact with DNA[30]. Members of the ataxia telangiectasia mutated (ATM) family (i.e. CHK1 and CHK2) are involved in the activation of a DNA repair response[11]. We also investigated the expression of phosphorylated CHK1 and CHK2 following radiation therapy in order to determine if these proteins were activated in response to the radiation damage. In the MDA-MB-231 cell line, $ALDH^{hi}CD44^{+}$ cells were found to express significantly higher levels of GSTpi and Pgp relative to $ALDH^{low}CD44^{-}$ cells. In $ALDH^{hi}CD44^{+}$ cells from the MDA-MB-468 cell line, significantly higher expression levels of GSTpi, CHK1, and phosphorylated CHK1 (P-CHK1) were observed relative to $ALDH^{low}CD44^{-}$ cells ($P<0.05$) (*Figure 3.3* and *Figure 3.4*).

Figure 3.1 $ALDH^{hi}CD44^{+}$ human breast cancer cells show increased resistance to doxorubicin and paclitaxel therapy *in vitro*. MDA-MB-231 ***(a,c)*** and MDA-MB-468 ***(b,d)*** breast cancer cells were isolated by FACS into $ALDH^{hi}CD44^{+}$ (*black bars*) and $ALDH^{low}CD44^{-}$ (*white bars*) cell populations. Cells were plated on 12-well dishes, maintained in normal growth medium for 48 hours, and subjected to treatment with either vehicle (EtOH), doxorubicin (0.4µM), or paclitaxel (0.2µM). After 72 hours, cells were harvested and viable cells were quantified using trypan blue exclusion (n=6/cell line) ***(a,b)***, or 1000 viable cells were re-plated into 6-well dishes to test their ability to re-grow/form colonies over 2 weeks (n=6/cell line) ***(c,d)***. Resulting cell colonies were fixed, stained, and quantified as described in the Materials and Methods. Data are presented as the mean +/- SEM. * = significantly different relative to $ALDH^{low}CD44^{-}$ cells exposed to doxorubicin ($P<0.001$). # = significantly different relative to $ALDH^{low}CD44^{-}$ cells exposed to paclitaxel ($P<0.001$)

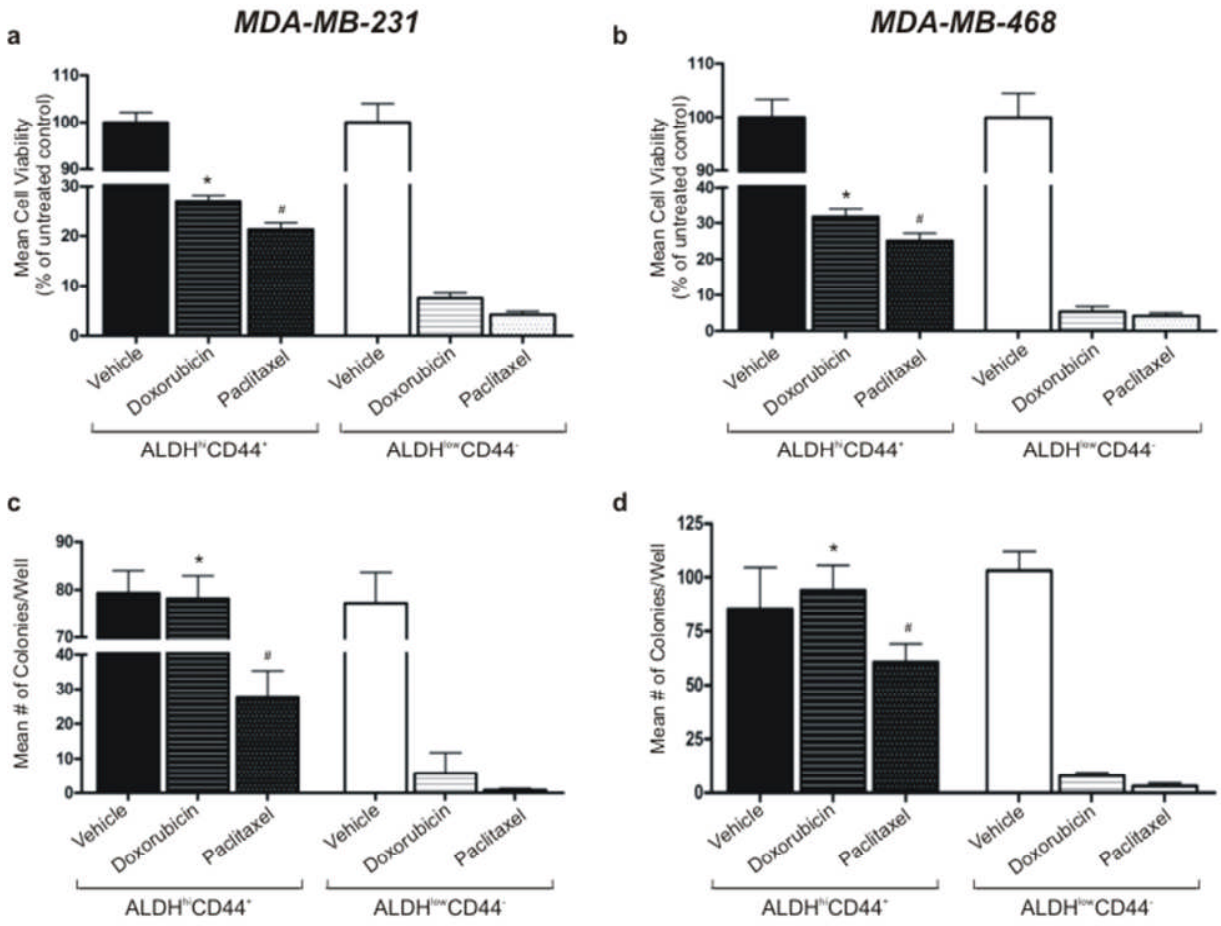
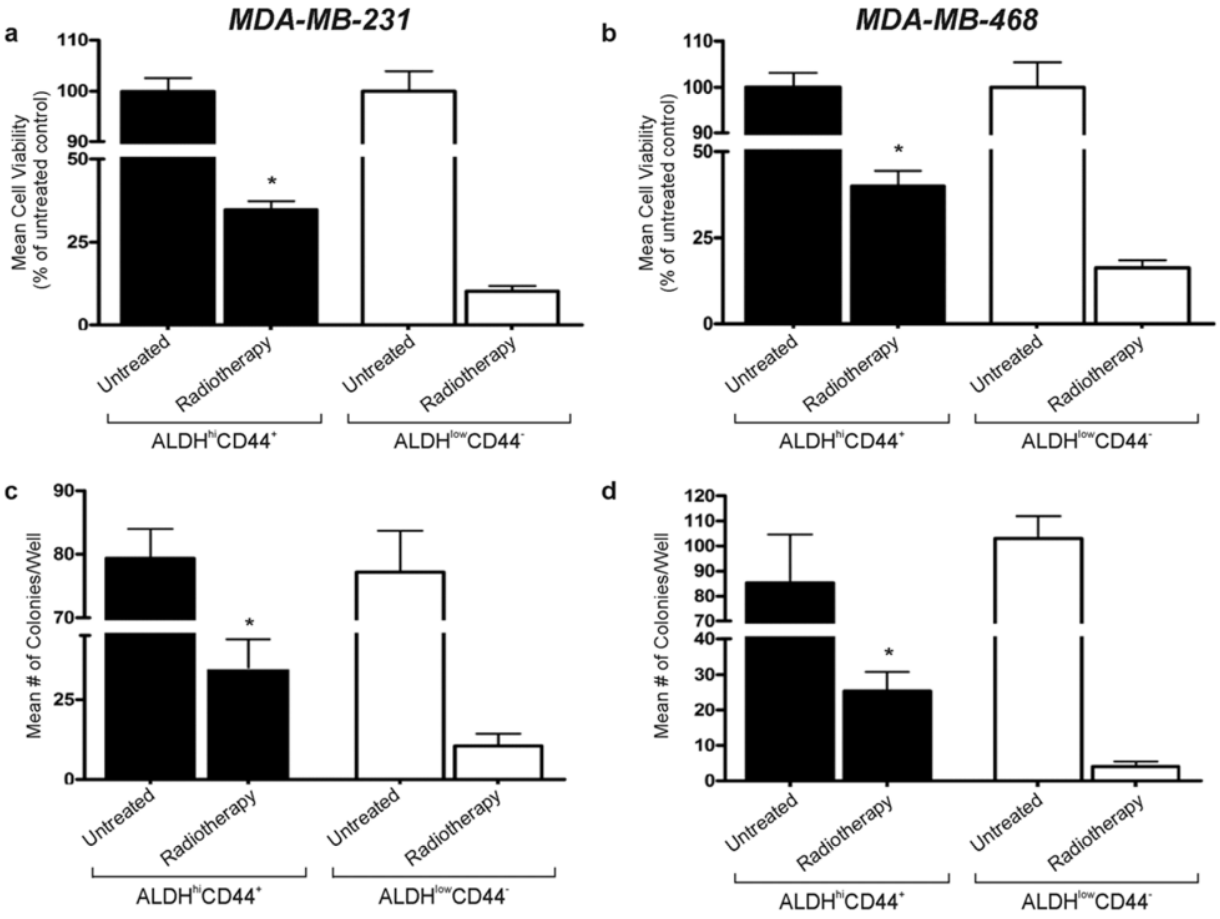

a
MDA-MB-231
Mean Cell Viability (% of untreated control)
110
100
90
30
20
10
0
*
#
Vehicle
Doxorubicin
Paclitaxel
Vehicle
Doxorubicin
Paclitaxel
ALDHhiCD44+
ALDHlowCD44-
b
MDA-MB-468
Mean Cell Viability (% of untreated control)
110
100
90
40
30
20
10
0
*
#
Vehicle
Doxorubicin
Paclitaxel
Vehicle
Doxorubicin
Paclitaxel
ALDHhiCD44+
ALDHlowCD44-
c
Mean # of Colonies/Well
90
80
70
40
30
20
10
0
*
#
Vehicle
Doxorubicin
Paclitaxel
Vehicle
Doxorubicin
Paclitaxel
ALDHhiCD44+
ALDHlowCD44-
d
Mean # of Colonies/Well
125
100
75
50
25
0
*
#
Vehicle
Doxorubicin
Paclitaxel
Vehicle
Doxorubicin
Paclitaxel
ALDHhiCD44+
ALDHlowCD44-

Figure 3.2 $ALDH^{hi}CD44^{+}$ human breast cancer cells show increased resistance to radiation therapy *in vitro*. MDA-MB-231 ***(a,c)*** and MDA-MB-468 ***(b,d)*** breast cancer cells were isolated by FACS into stem-like $ALDH^{hi}CD44^{+}$ (*black bars*) and non-stem-like $ALDH^{low}CD44^{-}$ (*white bars*) cell populations. Cells were then plated on 12-well dishes, maintained in normal growth medium for 48 hours, and subjected to radiation (2x5Gy; MDA-MB-231, and 2x3Gy; MDA-MB-468). Non-irradiated cells ("untreated") served as the control. After 72 hours, cells were harvested and viable cells were quantified using trypan blue exclusion (n=6/cell line) ***(a,b)***, or 1000 viable cells were re-plated into 6-well dishes to test their ability to re-grow over 2 weeks (n=6/cell line) ***(c,d)***. Resulting cell colonies were fixed, stained, and quantified as described in the Materials and Methods. Data are presented as the mean +/- SEM. * = significantly different relative to $ALDH^{low}CD44^{-}$ cells exposed to radiation therapy ($P < 0.05$).


a
MDA-MB-231
Mean Cell Viability
(% of untreated control)
110
100
90
50
25
0
*
Untreated
Radiotherapy
Untreated
Radiotherapy
ALDHhiCD44+
ALDHlowCD44-
b
MDA-MB-468
Mean Cell Viability
(% of untreated control)
110
100
90
50
25
0
*
Untreated
Radiotherapy
Untreated
Radiotherapy
ALDHhiCD44+
ALDHlowCD44-
c
Mean # of Colonies/Well
90
80
70
25
0
*
Untreated
Radiotherapy
Untreated
Radiotherapy
ALDHhiCD44+
ALDHlowCD44-
d
Mean # of Colonies/Well
120
110
100
90
80
70
40
30
20
10
0
*
Untreated
Radiotherapy
Untreated
Radiotherapy
ALDHhiCD44+
ALDHlowCD44-

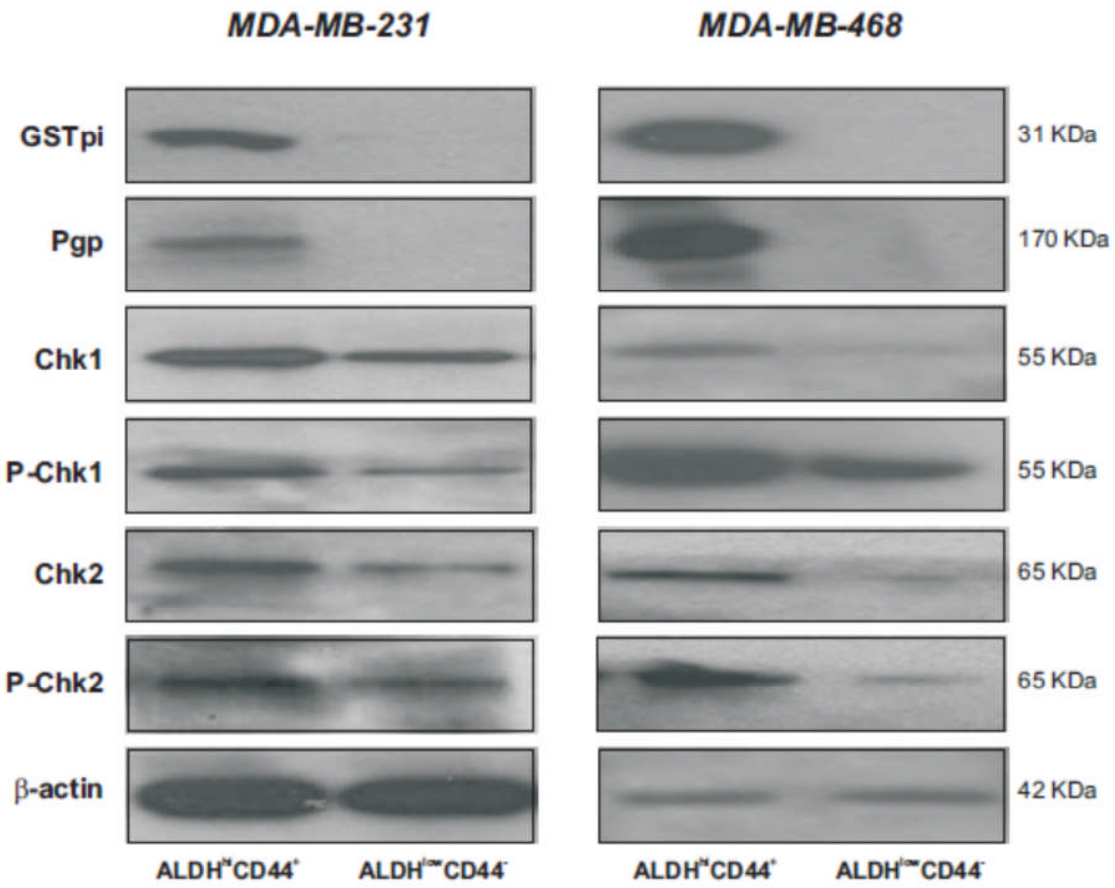
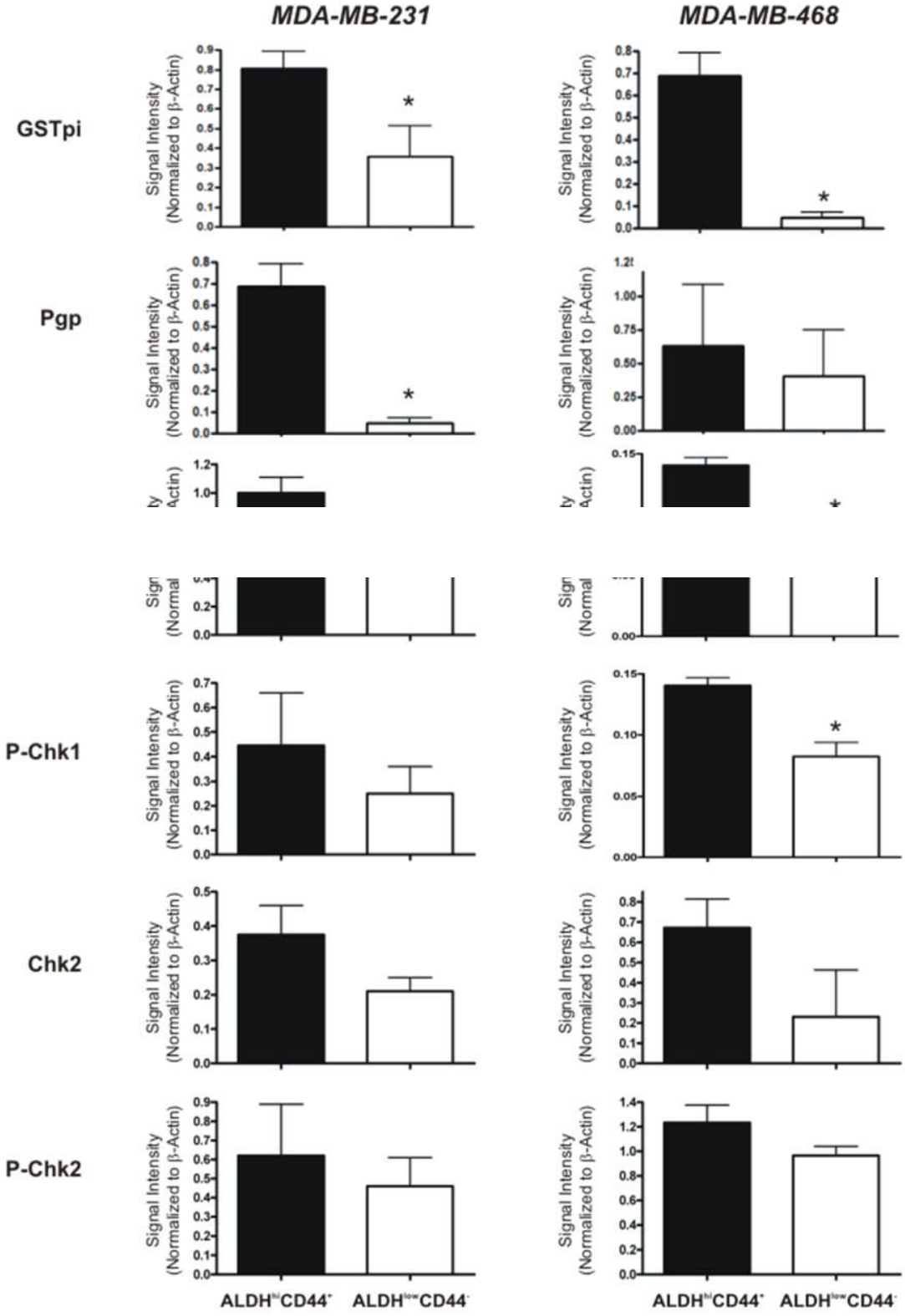


Figure 3.3 $ALDH^{hi}CD44^{+}$ breast cancer cells have high expression of therapy resistance proteins. MDA-MB-231 (*left panels*) and MDA-MB-468 (*right panels*) breast cancer cells were isolated by FACS into stem-like $ALDH^{hi}CD44^{+}$ and non-stem-like $ALDH^{low}CD44^{-}$ cell populations. Cell lysates were isolated from each population and protein expression of the therapy-resistance proteins GSTPi, Pgp, CHK1, P-CHK1, CHK2, and P-CHK2 was analyzed by western blot analysis as described in the Materials and Methods and *Table 3.1*. Representative western blots (of n=3 per protein) are shown as cropped gel images. Quantitative densitometric analysis of expression levels is provided in *Figure 3.4.*

Figure 3.4 Quantitative densitometric analysis of *Figure 3.3* western blots. MDA-MB-231 (*left panels*) and MDA-MB-468 (*right panels*) breast cancer cells were isolated by FACS into stem-like $ALDH^{hi}CD44^{+}$ *(black bars)* and non-stem-like $ALDH^{low}CD44^{-}$ *(white bars)* cell populations. Cell lysates were isolated from each population and protein expression of the therapy-resistance proteins GSTPi, Pgp, CHK1, P-CHK1, CHK2, and P-CHK2 was analyzed by Western blot analysis as described in the Materials and Methods and shown as representative images in *Figure 3.3*. Expression of each protein was normalized to the β-actin loading control and signal intensity was quantified using the computer-assisted densitometry program AlphaEase FCTM Software version 3.1.2 (Alpha Innotech Corporation, Miami FL). Data are compiled from n=3 blots per protein and are presented as the mean ± SEM. * = significantly different relative to $ALDH^{hi}CD44^{+}$ cells ($P < 0.05$).


MDA-MB-231
MDA-MB-468
GSTpi
Pgp
P-Chk1
Chk2
P-Chk2
Signal Intensity (Normalized to β-Actin)
*
ALDH^hiCD44^+
ALDH^low^CD44^-

3.3.3 Targeting of ALDH activity via ATRA or DEAB sensitizes ALDH^{hi}CD44^{+} human breast cancer cells to chemotherapy and radiation treatment

Given the known role of ALDH activity in stem cell self-protection and drug detoxification[9], we hypothesized that targeting ALDH activity might sensitize ALDH^{hi}CD44^{+} cells to treatment and reduce the observed resistance to chemotherapy and radiation. To test this, cells were pre-treated with all-*trans* retinoic acid (ATRA) or the specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) to reduce ALDH activity. We observed that treatment of unsorted MDA-MB-231 or MDA-MB-468 cells with either DEAB or ATRA resulted in a significant down-regulation of ALDH activity for 24 and 48 hours (respectively) (*Figure 3.5A,B*) without altering CD44 expression (*Figure 3.5C,D)*. We also observed that treatment with DEAB or ATRA alone (i.e. in the absence of chemotherapy or radiation) did not significantly influence cell viability or proliferation of either MDA-MD-231 or MDA-MB-468 cells (P>0.05) (*Figure 3.5A-D).* Therefore, in subsequent experiments we only tested the influence of DEAB or ATRA in combination with chemotherapy and radiation, and not as single agents.

For combination therapy experiments, FACS-sorted ALDH^{hi}CD44^{+} and ALDH^{low}CD44^{-} cell populations were isolated from MDA-MB-231 and MDA-MB-468 cells and subjected to treatment with doxorubicin, paclitaxel, or radiation in the presence or absence of pre-treatment with DEAB or ATRA. In doxorubicin- or paclitaxel-treated ALDH^{hi}CD44^{+} cells from the MDA-MB-231 cell line, we observed that pre-treatment with DEAB or ATRA resulted in significantly reduced cell viability relative to ALDH^{hi}CD44^{+} cells treated with doxorubicin or paclitaxel chemotherapy alone (P<0.05) (*Figure 3.6A*). However, only DEAB pre-treatment was able to reduce the re-growth/colony-forming ability of doxorubicin- or paclitaxel-treated ALDH^{hi}CD44^{+} cells relative to treatment with doxorubicin or paclitaxel alone (P<0.05) (*Figure 3.6C*). Pre-treatment with DEAB or ATRA did not significantly influence the viability or colony-forming ability of doxorubicin- or paclitaxel-treated MDA-MB-231 ALDH^{low}CD44^{-} cells relative to treatment with doxorubicin or paclitaxel alone (P>0.05) (*Figure 3.6A,C*).

Figure 3.5 Treatment of human breast cancer cells with DEAB or ATRA down-regulates ALDH activity but does not influence CD44 expression or cell proliferation/viability. MDA-MB-231 (*left panels*) and MDA-MB-468 (*right panels*) breast cancer cells were cultured in the presence of either vehicle (EtOH), DEAB (100μM), or ATRA (5μM). ***(a-d)*** At 24 and 48 hours post-treatment, cells were harvested and assessed by flow cytometry for ALDH activity using the ALDEFLUOR® assay kit ***(a)*** or for CD44 expression ***(b)*** as described in the Materials and Methods. ***(c)*** At 48 hours post-treatment, cells were subjected to staining with AlamarBlue® solution as per the manufacturer's instructions and analyzed on a UV spectrophotometer at 570 nm. Absorbance is proportional to the number of living cells and corresponds to cells' metabolic activity/proliferation. ***(d)*** At 48 hours post-treatment, cells were harvested and viable cells were quantified using Trypan blue exclusion and manual counting on a hemocytometer using light microscopy. Data are presented as the mean ± SEM. * = significantly different relative to vehicle-treated control ($P < 0.05$).

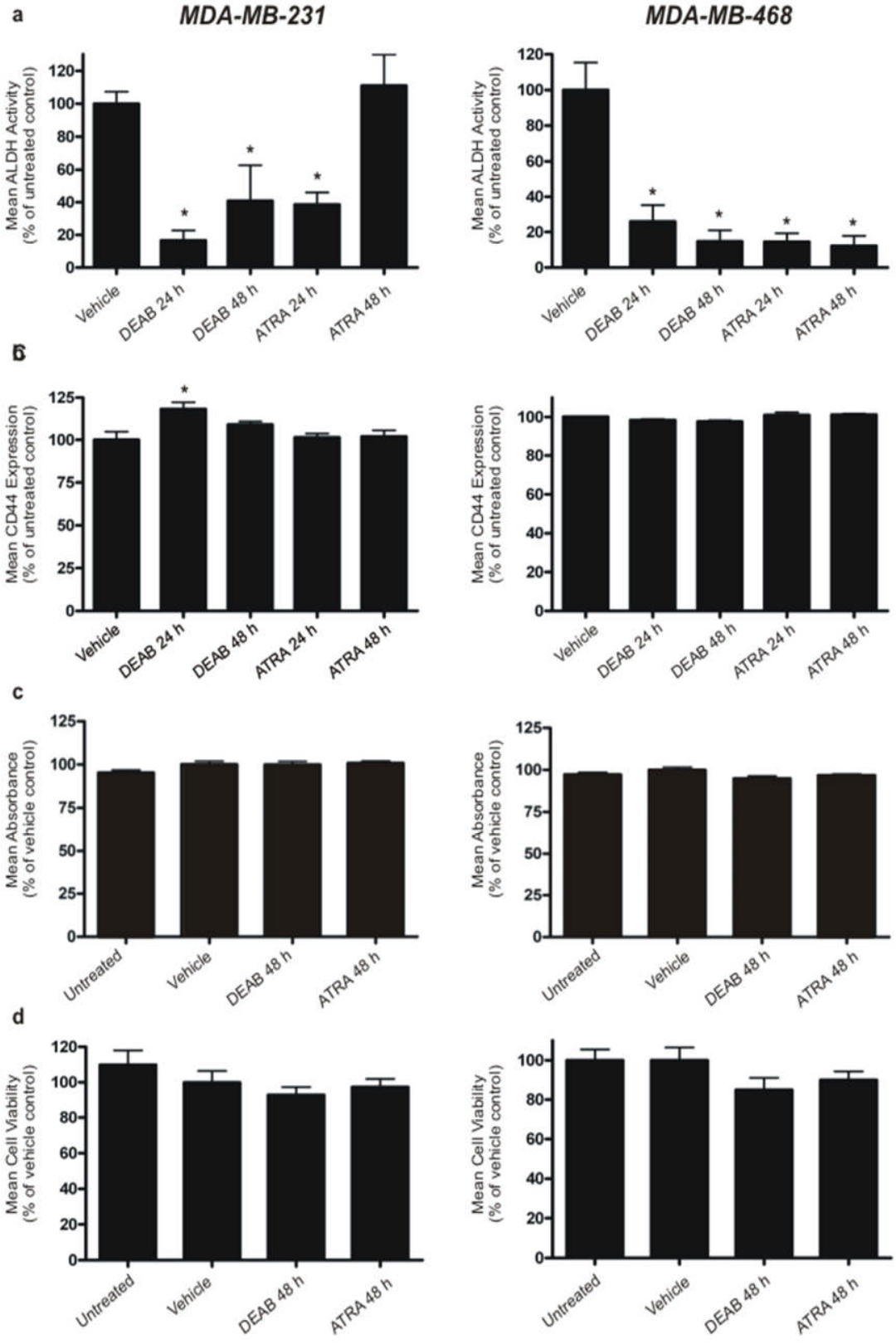
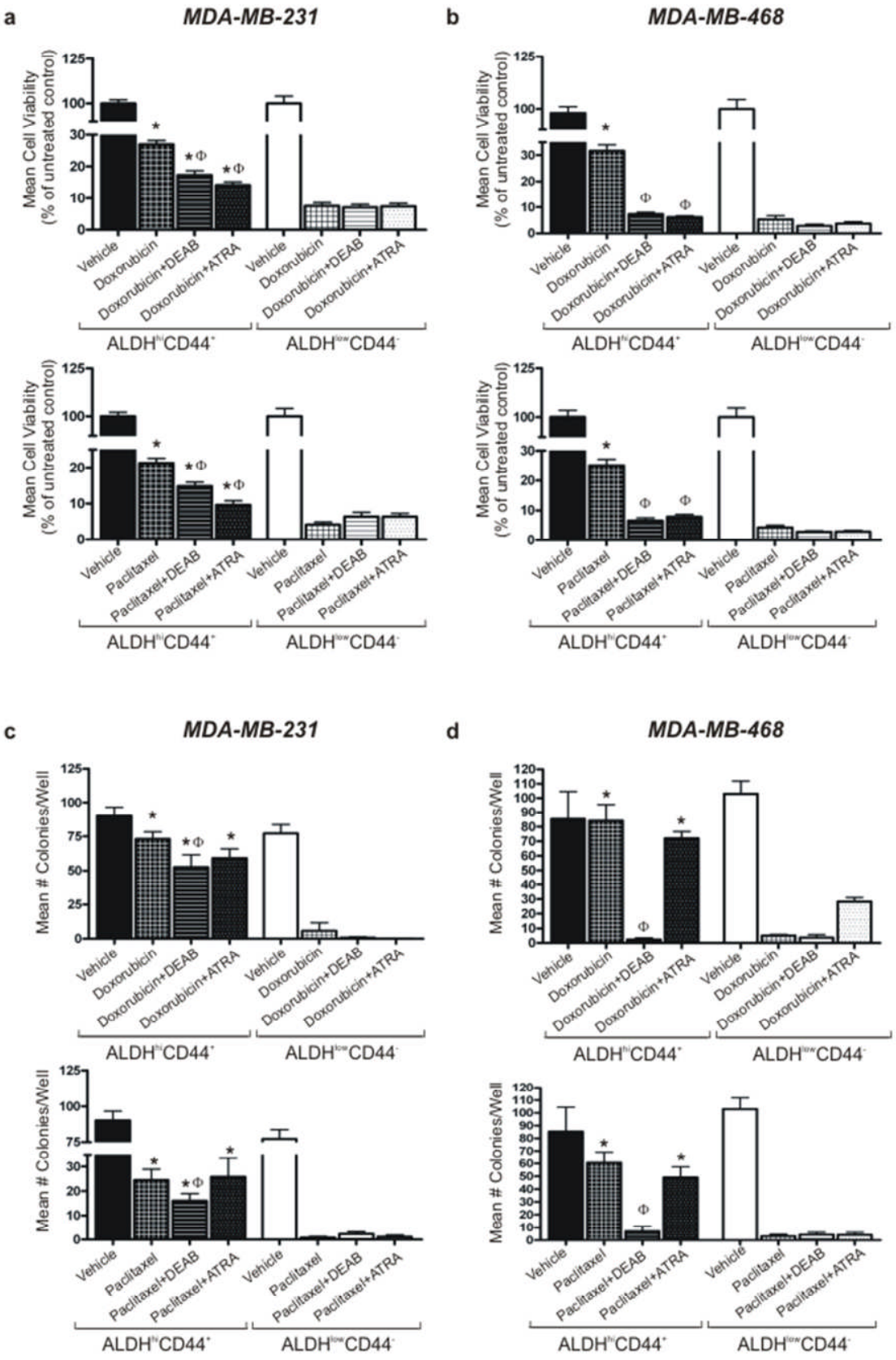

a
MDA-MB-231
MDA-MB-468
Mean ALDH Activity (% of untreated control)
Vehicle
DEAB 24 h
DEAB 48 h
ATRA 24 h
ATRA 48 h
b
Mean CD44 Expression (% of untreated control)
c
Mean Absorbance (% of vehicle control)
Untreated
d
Mean Cell Viability (% of vehicle control)

Figure 3.6 DEAB and ATRA sensitizes $ALDH^{hi}CD44^{+}$ human breast cancer cells to doxorubicin and paclitaxel therapy *in vitro*. MDA-MB-231 ***(a,c)*** and MDA-MB-468 ***(b,d)*** breast cancer cells were isolated by FACS into stem-like $ALDH^{hi}CD44^{+}$ (*black bars*) and non-stem-like $ALDH^{low}CD44^{-}$ (*white bars*) cell populations. Cells were plated on 12-well dishes and subjected to treatment with either vehicle (EtOH), DEAB (100µM) or ATRA (5µM) for 48 hours, after which cells were also exposed to treatment with either doxorubicin (0.4µM) or paclitaxel (0.2µM) chemotherapy. After 72 hours, cells were harvested and viable cells were quantified using trypan blue exclusion (n=6/cell line) ***(a,b)*** or 1000 viable cells were re-plated onto 6-well dishes to test their ability to re-grow over 2 weeks (n=6/cell line) ***(c,d)***. Resulting cell colonies were fixed using 1% gluteraldehyde, stained with Harris' haematoxylin, and the number of colonies was quantified. Data are presented as the mean +/- SEM. * = significantly different relative to $ALDH^{low}CD44^{-}$ cells treated with doxorubicin or paclitaxel alone ($P < 0.05$). Φ = significantly different relative to $ALDH^{hi}CD44^{+}$ cells treated with doxorubicin or paclitaxel alone ($P < 0.05$).


a
MDA-MB-231
b
MDA-MB-468
Mean Cell Viability (% of untreated control)
Vehicle
Doxorubicin
Doxorubicin+DEAB
Doxorubicin+ATRA
Paclitaxel
Paclitaxel+DEAB
Paclitaxel+ATRA
ALDHhiCD44+
ALDHlowCD44-
c
MDA-MB-231
d
MDA-MB-468
Mean # Colonies/Well

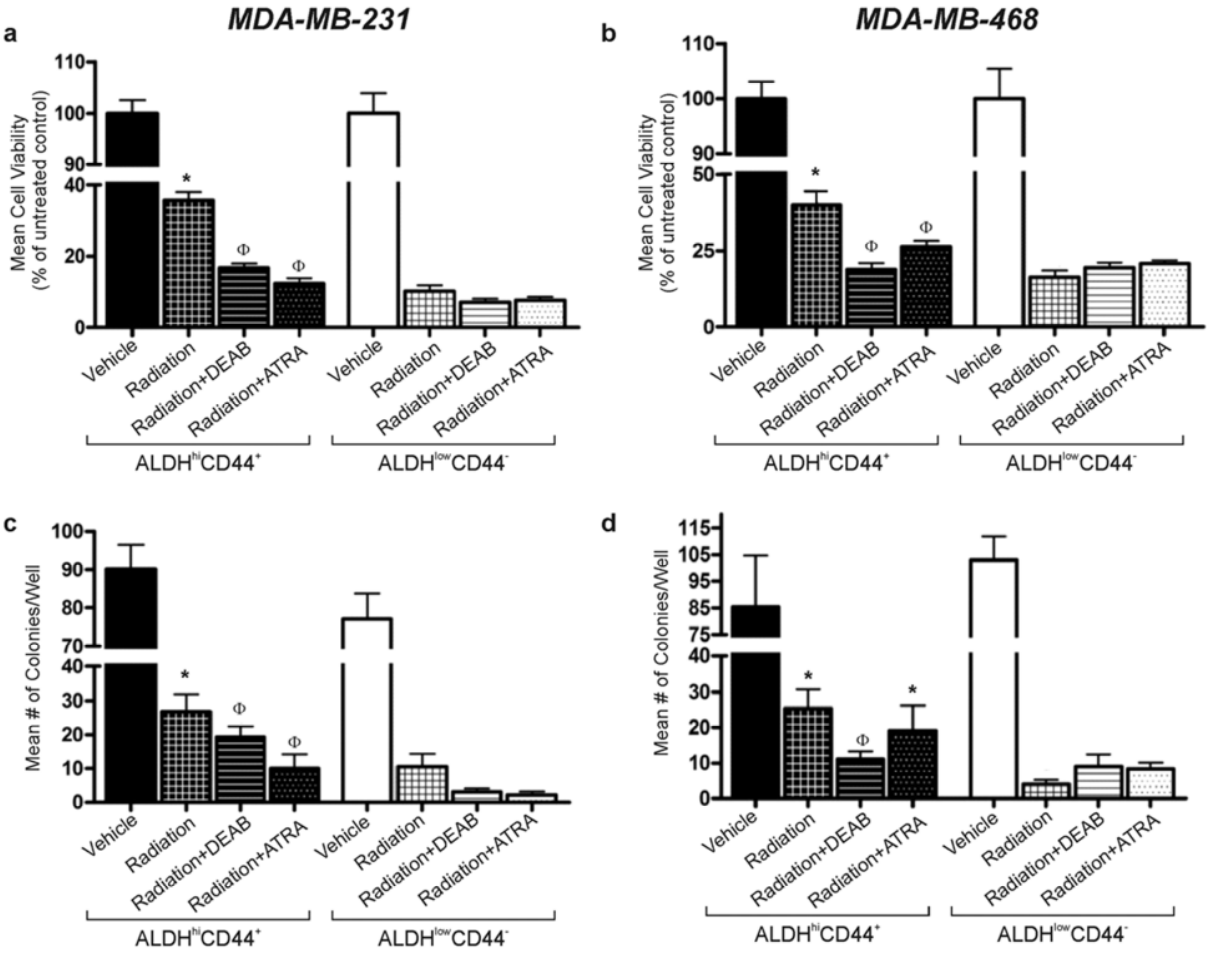
In ALDH^{hi}CD44^{+} cells from the MDA-MB-468 cell line, pre-treatment with DEAB or ATRA resulted in significant initial sensitization to doxorubicin or paclitaxel chemotherapy such that there was a reduction in viable cells down to the level of the non-resistant ALDH^{low}CD44^{-} cells ($P<0.05$) (*Figure 3.6B*). However, again only DEAB pre-treatment was able to reduce the re-growth/colony-forming ability of chemotherapy-treated MDA-MB-468 ALDH^{hi}CD44^{+} cells ($P<0.05$) (Figure *3.6D*). Pre-treatment with DEAB or ATRA did not significantly influence the viability of doxorubicin- or paclitaxel-treated MDA-MB-468 ALDH^{low}CD44^{-} cells relative to cells treated with doxorubicin or paclitaxel alone ($P>0.05$) (*Figure 3.6B*). Furthermore, pre-treatment with DEAB or ATRA did not significantly influence the colony-forming ability of paclitaxel-treated MDA-MB-468 ALDH^{low}CD44^{-} cells relative to cells treated with paclitaxel alone ($P>0.05$) (*Figure 3.6D, bottom panel*), although pre-treatment with ATRA did enhance the colony-forming ability of doxorubicin-treated MDA-MB-468 ALDH^{low}CD44^{-} cells relative to cells treated with doxorubicin alone ($P<0.05$) (*Figure 3.6D, top panel*).

Similarly, DEAB or ATRA pre-treatment of ALDH^{hi}CD44^{+} cells from both the MDA-MB-231 and MDA-MB-468 cell lines resulted in significant initial sensitization to radiation therapy such that there was a reduction in viable cells down to the level of the non-resistant ALDH^{low}CD44^{-} cells ($P<0.05$) (*Figure 3.7A,B*). Interestingly, although both DEAB and ATRA also significantly reduced the subsequent re-growth/colony-forming ability of MDA-MB-231 ALDH^{hi}CD44^{+} cells following radiation treatment (*Figure 3.7C*, $P<0.001$), only DEAB pre-treatment was able to reduce the re-growth/colony-forming ability of radiation-treated MDA-MB-468 ALDH^{hi}CD44^{+} cells relative to cells treated with radiation alone (*Figure 3.7D*, $P<0.05$).

3.3.4 Expression of drug resistance and differentiation proteins in response to DEAB or ATRA treatment

In order to examine potential mechanisms underlying the observed response to DEAB or ATRA, ALDH^{hi}CD44^{+} cells from MDA-MB-231 or MDA-MB-468 cell lines were exposed to DEAB (100µM), ATRA (5µM), or EtOH vehicle in culture for one week. Cell lysates were collected and

Figure 3.7 DEAB and ATRA treatment sensitizes $ALDH^{hi}CD44^{+}$ human breast cancer cells to radiation therapy *in vitro* . MDA-MB-231 ***(a,c)*** and MDA-MB-468 ***(b,d)*** breast cancer cells were isolated by FACS into stem-like $ALDH^{hi}CD44^{+}$ (*black bars*) and non-stem-like $ALDH^{low}CD44^{-}$ (*white bars*) cell populations. Cells were plated on 12-well dishes, maintained in normal growth medium for 48 hours, and pre-treated with either vehicle (EtOH), DEAB (100µM), or ATRA (5µM) for 48 hours. Cells were subjected to radiation (2x5Gy; MDA-MB-231 cells, and 2x3Gy; MDA-MB-468 cells). Non-irradiated cells served as the control. After 72 hours, cells were harvested and viable cells were quantified using trypan blue exclusion (n=6/cell line) ***(a,b)*** or 1000 viable cells were re-plated into 6-well dishes to test their ability to re-grow over 2 weeks (n=6/cell line) ***(c,d)***. Resulting cell colonies were fixed, stained, and quantified as described in the Materials and Methods. Data are presented as the mean +/- SEM. * = significantly different relative to $ALDH^{low}CD44^{-}$ cells exposed to radiation therapy alone ($P < 0.05$). Φ = significantly different relative to $ALDH^{hi}CD44^{+}$ cells treated with radiation alone ($P < 0.05$).


a
MDA-MB-231
Mean Cell Viability (% of untreated control)
110
100
90
40
20
0
*
Φ
Φ
Vehicle
Radiation
Radiation+DEAB
Radiation+ATRA
Vehicle
Radiation
Radiation+DEAB
Radiation+ATRA
ALDHhiCD44+
ALDHlowCD44-
b
MDA-MB-468
Mean Cell Viability (% of untreated control)
110
100
90
50
25
0
*
Φ
Φ
Vehicle
Radiation
Radiation+DEAB
Radiation+ATRA
Vehicle
Radiation
Radiation+DEAB
Radiation+ATRA
ALDHhiCD44+
ALDHlowCD44-
c
Mean # of Colonies/Well
100
90
80
70
40
30
20
10
0
*
Φ
Φ
Vehicle
Radiation
Radiation+DEAB
Radiation+ATRA
Vehicle
Radiation
Radiation+DEAB
Radiation+ATRA
ALDHhiCD44+
ALDHlowCD44-
d
Mean # of Colonies/Well
115
105
95
85
75
40
30
20
10
0
*
Φ
*
Vehicle
Radiation
Radiation+DEAB
Radiation+ATRA
Vehicle
Radiation
Radiation+DEAB
Radiation+ATRA
ALDHhiCD44+
ALDHlowCD44-

analyzed by western blot to examine the expression of proteins associated with drug resistance (GSTpi, Pgp, CHK1, CHK2)[11,30] or the epithelial-to-mesenchymal transition (EMT)/ differentiation (N-cadherin, Vimentin, E-cadherin, CK8/18/19)[31]. We also examined expression of RARα, a key transcription factor in retinoid signalling[28], and expression of YB1, a protein that has been implicated as an important cell survival protein during development[32] (*Figures 3.8, 3.9, 3.10*, and *3.11*). Following treatment of MDA-MB-231 ALDH^{hi}CD44^{+} cells with DEAB or ATRA, no significant difference was observed in expression of any of these proteins relative to treatment with vehicle control. In the MDA-MB-468 ALDH^{hi}CD44^{+} cells, there was a significant increase in CK8/18/19 expression in ATRA-treated cells compared to vehicle control ($P<0.05$); however, there was no significant difference identified in expression of any other protein investigated.

3.3.5 ATRA therapy in combination with paclitaxel *in vivo* causes a decrease in primary tumor burden, but increases metastatic disease in the lungs

ATRA therapy in conjunction with chemotherapy has been used successfully to treat APL patients due to the synergistic effect of ATRA, which is able to differentiate the malignant myeloid cells, thus sensitizing them to chemotherapy[23,25,26]. Studies in breast cancer using ATRA as a single agent or in combination with the estrogen receptor antagonist Tamoxifen (Nolvadex®) have reported varied results, showing both positive and negative outcomes of ATRA therapy[45,46]; however, no studies to our knowledge have looked at the combined effects of ATRA and chemotherapy in breast cancer patients. In this experiment, the combination of ATRA and paclitaxel was tested *in vivo* to determine the effect of both primary and metastatic tumor burden following 6 weeks of combination therapy (*Figure 3.12*). Daily injections of dexamethasone were required in order to help the mice combat retinoid syndrome, which is a deleterious side effect of RA therapy in both mice and humans[33]. Although both ATRA treatment alone and paclitaxel treatment alone caused a decreased in primary tumor burden compared to placebo following 6 weeks of therapy, this did not reach statistically significant

Figure 3.8 Expression of drug resistance and differentiation proteins in response to DEAB or ATRA treatment. Stem-like $ALDH^{hi}CD44^{+}$ cells were isolated from MDA-MB-231 (*left panels*) and MDA-MB-468 (*right panels*) breast cancer cell lines and exposed to vehicle (EtOH), DEAB (100μM), or ATRA (5μM) in culture for one week. As described in the Materials and Methods and *Table 3.1*, cell lysates were collected and analyzed by western blot to examine the expression of proteins associated with drug resistance (GSTpi, Pgp, CHK1, CHK2, YB1) or the epithelial-to-mesenchymal transition (EMT)/differentiation (N-cadherin, Vimentin, E-cadherin, CK8/18/19), as well as RARα. Representative western blots (of n=3 per protein) are shown as cropped gel images. Quantitative densitometric analysis of expression levels is provided in *Figure 3.9, 3.10,* and *3.11.*

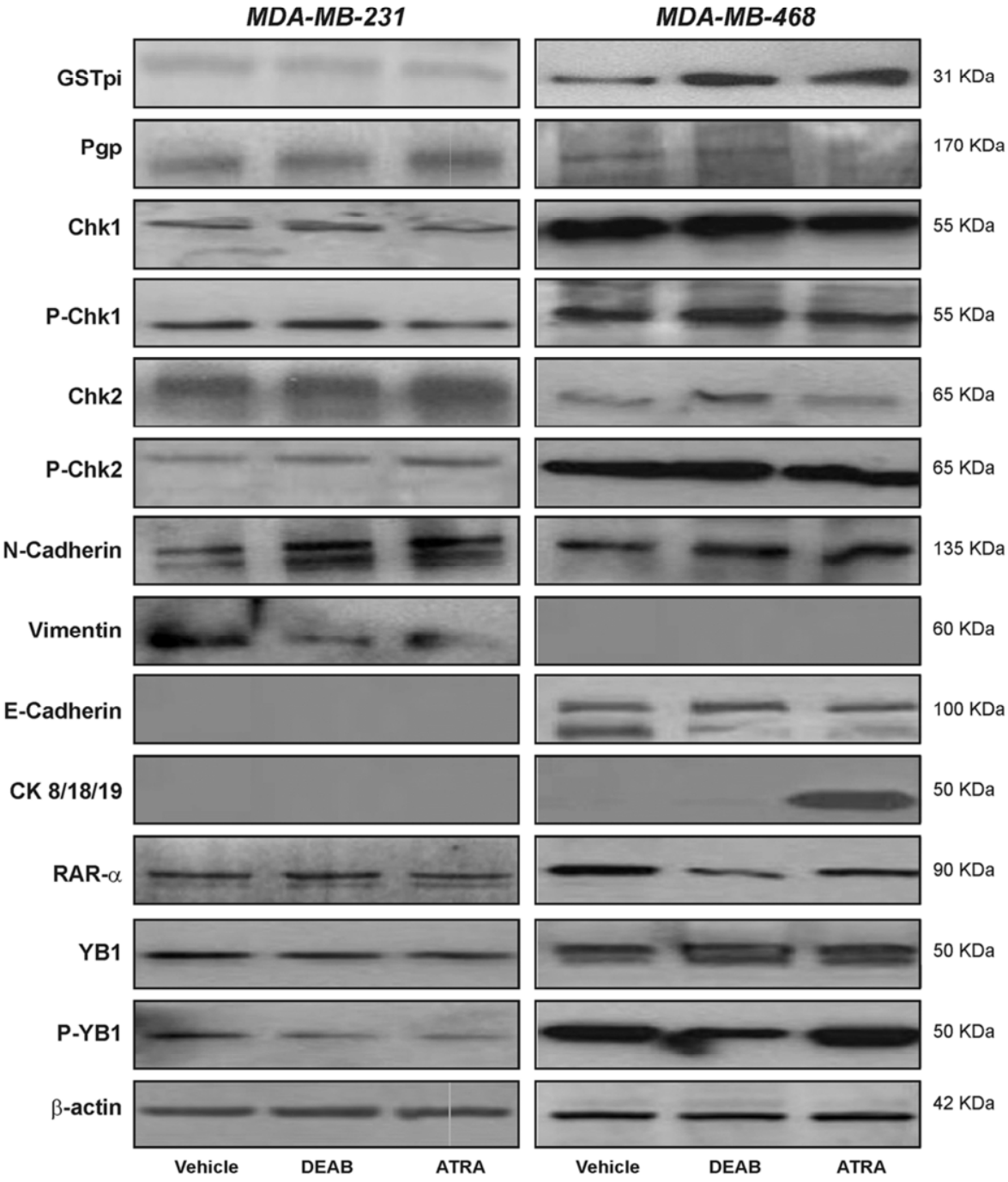

MDA-MB-231
MDA-MB-468
GSTpi
Pgp
Chk1
P-Chk1
Chk2
P-Chk2
N-Cadherin
Vimentin
E-Cadherin
CK 8/18/19
RAR-α
YB1
P-YB1
β-actin
31 KDa
170 KDa
55 KDa
55 KDa
65 KDa
65 KDa
135 KDa
60 KDa
100 KDa
50 KDa
90 KDa
50 KDa
50 KDa
42 KDa
Vehicle
DEAB
ATRA
Vehicle
DEAB
ATRA

Figure 3.9 Quantitative densitometric analysis of GSTPi, Pgp, CHK1, P-CHK1, CHK2, and P-CHK2 from *Figure 3.8* western blots. Stem-like $ALDH^{hi}CD44^{+}$ cells were isolated from MDA-MB-231 (*left panels*) and MDA-MB-468 (*right panels*) breast cancer cell lines and exposed to EtOH vehicle, DEAB (100μM), or ATRA (5μM) in culture for one week. Cell lysates were isolated and protein expression of the therapy-resistance proteins GSTPi, Pgp, CHK1, P-CHK1, CHK2, and P-CHK2 was analyzed by western blot analysis as described in the Materials and Methods and shown as representative images in *Figure 3.8*. Expression of each protein was normalized to the β-actin loading control and signal intensity was quantified using the computer-assisted densitometry program AlphaEase FCTM Software version 3.1.2 (Alpha Innotech Corporation, Miami FL). Data are compiled from n=3 blots per protein and are presented as the mean ± SEM.

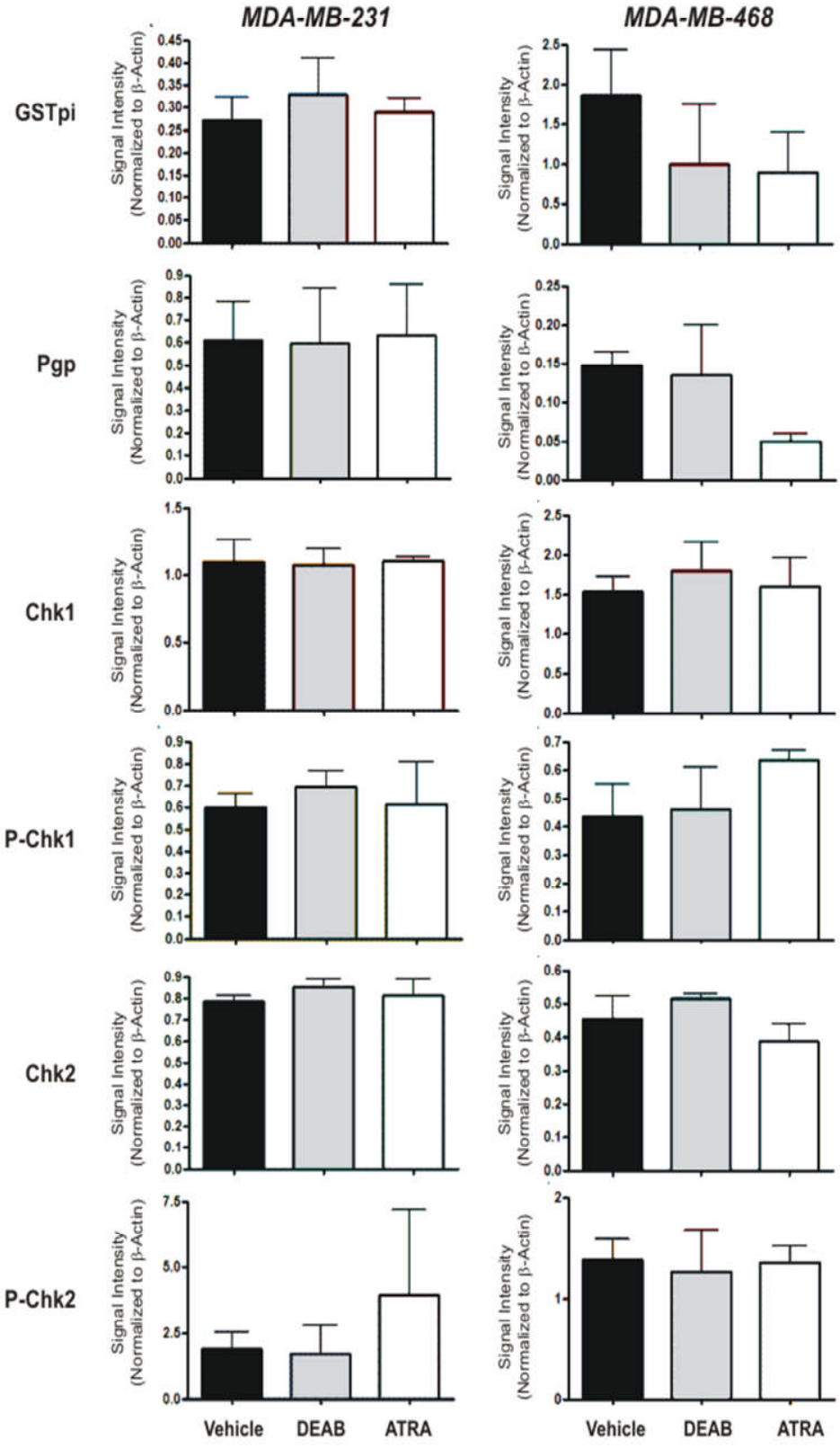

MDA-MB-231
MDA-MB-468
GSTpi
Pgp
Chk1
P-Chk1
Chk2
P-Chk2
Signal Intensity (Normalized to β-Actin)
Vehicle
DEAB
ATRA

Figure 3.10 Quantitative densitometric analysis of N-cadherin, Vimentin, E-cadherin and CK8/18/19 from *Figure 3.8* western blots. Stem-like $ALDH^{hi}CD44^{+}$ cells were isolated from MDA-MB-231 (*left panels*) and MDA-MB-468 (*right panels*) breast cancer cell lines and exposed to EtOH vehicle, DEAB (100μM), or ATRA (5μM) in culture for one week. Cell lysates were isolated and protein expression of the EMT/differentiation proteins N-cadherin, Vimentin, E-cadherin, and CK8/18/19 was analyzed by western blot analysis as described in the Materials and Methods and shown as representative images in *Figure 3.8*. Expression of each protein was normalized to the β-actin loading control and signal intensity was quantified using the computer-assisted densitometry program AlphaEase FCTM Software version 3.1.2 (Alpha Innotech Corporation, Miami FL). Data are compiled from n=3 blots per protein and are presented as the mean ± SEM. * = significantly different relative to vehicle control treatment ($P < 0.05$).

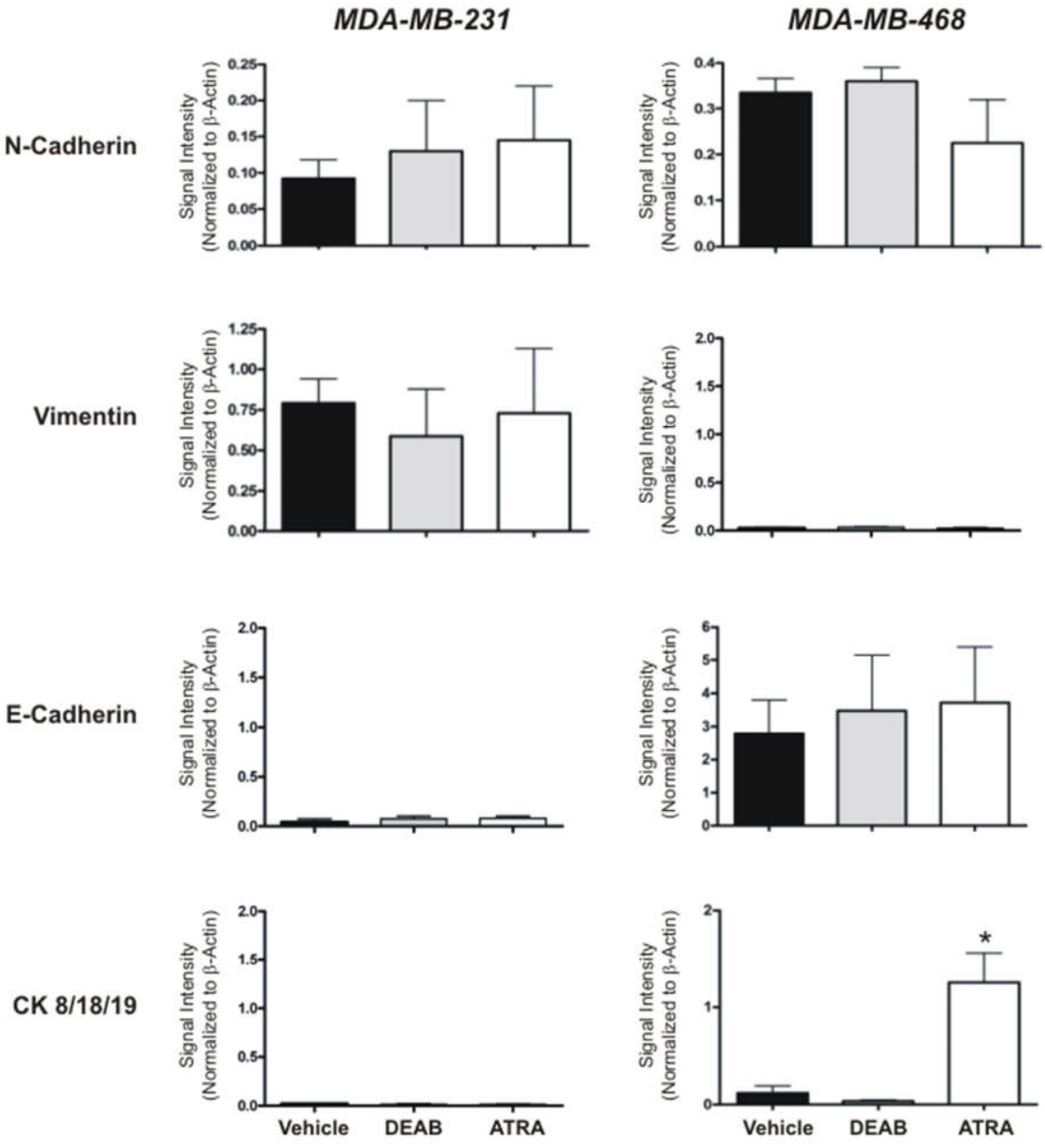

MDA-MB-231
MDA-MB-468
N-Cadherin
Vimentin
E-Cadherin
CK 8/18/19
Signal Intensity (Normalized to β-Actin)
Vehicle
DEAB
ATRA
*

Figure 3.11 Quantitative densitometric analysis of RARα, YB1, and P-YB1 from *Figure 3.8* western blots. Stem-like $ALDH^{hi}CD44^{+}$ cells were isolated from MDA-MB-231 (*left panels*) and MDA-MB-468 (*right panels*) breast cancer cell lines and exposed to EtOH vehicle, DEAB (100μM), or ATRA (5μM) in culture for one week. Cell lysates were isolated and protein expression of RARα, YB1, and P-YB1 was analyzed by western blot analysis as described in the Materials and Methods and shown as representative images in *Figure 3.8*. Expression of each protein was normalized to the β-actin loading control and signal intensity was quantified using the computer-assisted densitometry program AlphaEase FCTM Software version 3.1.2 (Alpha Innotech Corporation, Miami FL). Data are compiled from n=3 blots per protein and are presented as the mean ± SEM.

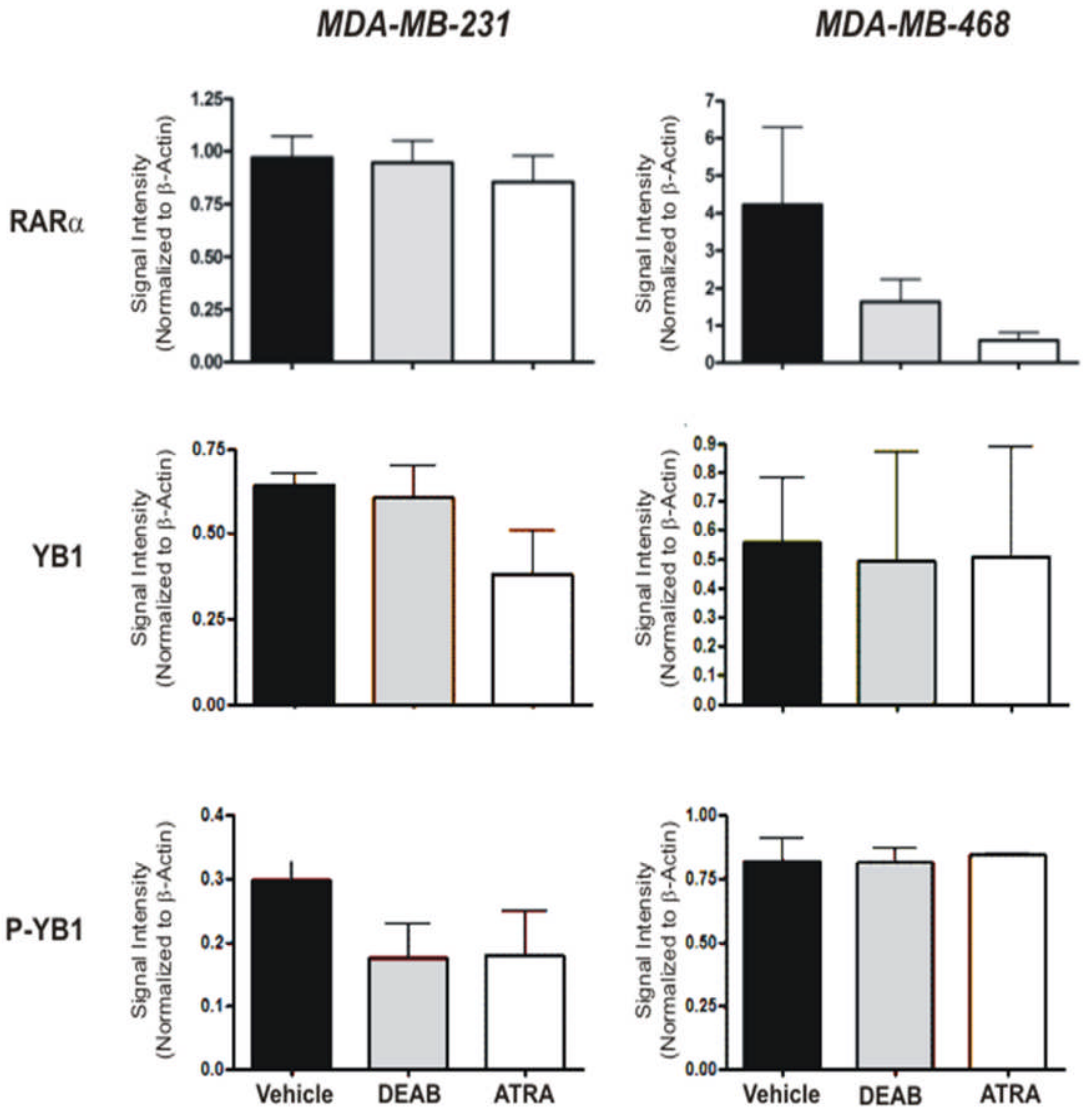
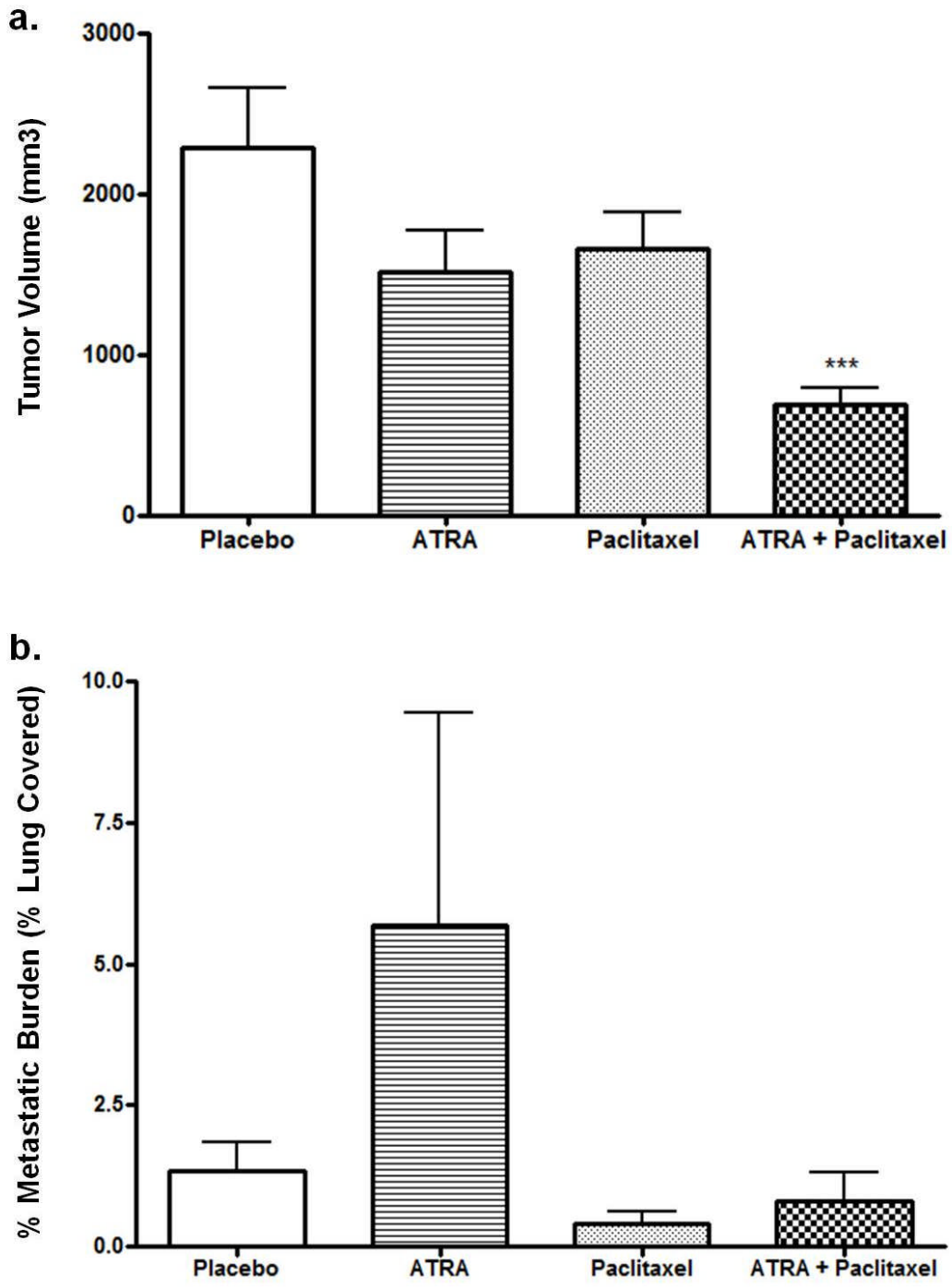

MDA-MB-231
MDA-MB-468
RARα
YB1
P-YB1
Signal Intensity (Normalized to β-Actin)
1.25
1.00
0.75
0.50
0.25
0.00
7
6
5
4
3
2
1
0
0.9
0.8
0.7
0.6
0.4
0.3
0.2
0.1
0.0
Vehicle
DEAB
ATRA

Figure 3.12 ATRA therapy in combination with paclitaxel *in vivo* causes a decrease in primary tumor burden, but increases metastatic disease in the lungs. One x 10^6 MDA-MB-231 cancer cells were injected into the mammary fat pad 6-7 week old female NOD/SCID mice (n=8 mice/group). Tumors were allowed to grow to a mean tumor volume of 500 mm^3, and then the mice were randomized into groups and treated with either placebo, ATRA (160mg/kg/day), placebo + paclitaxel (i.p., 5mg/kg), or ATRA (160mg/kg/day) + paclitaxel once weekly for 6 weeks. Dexamethasone (i.p, 2mg/kg daily) was used to help the mice survive the ATRA therapy. Primary tumor measurements were taken weekly for 6 weeks. After 6 weeks of therapy, mice were sacrificed and primary tumors were measured ***(a)*** and then flash-frozen in OCT for cryosectioning. Tissues from distant organs were collected and assessed for differences in metastatic involvement using H&E staining. Lung tissues were sectioned and stained with H&E in order to determine mean metastatic burden ***(b)***.


a.
Tumor Volume (mm3)
3000
2000
1000
0
***
Placebo
ATRA
Paclitaxel
ATRA + Paclitaxel
b.
% Metastatic Burden (% Lung Covered)
10.0
7.5
5.0
2.5
0.0
Placebo
ATRA
Paclitaxel
ATRA + Paclitaxel

levels. However, the combination of ATRA and paclitaxel lead to a significant decrease in primary tumor burden compared to placebo mice (*Figure 3.12A*). In terms of metastatic disease, neither paclitaxel nor the combination of paclitaxel + ATRA decreased the metastatic burden in the lungs compared to placebo animals (*Figure 3.12B*). Furthermore, ATRA therapy as a single agent tended to increase the metastatic burden in the lungs compared to placebo animals, however, this increase did not reach statistical significance.

3.4 Discussion

Treatment failure in the metastatic setting is the main cause of breast cancer-related death[3]. Therapy fails because a small subset of cells are either intrinsically resistant or acquire resistance to chemotherapy and/or radiation therapy during disease progression. It is therefore essential to identify and target these resistant metastatic cells in order to improve the success of breast cancer therapy. We recently identified stem-like $ALDH^{hi}CD44^{+}$ cells that demonstrate enhanced tumorigenic and metastatic capacity *in vitro* and *in vivo*[7]. In the current study we hypothesized that these $ALDH^{hi}CD44^{+}$ breast cancer cells are also more resistant to standard cancer therapy, and that inhibiting ALDH activity via treatment with ATRA or DEAB sensitizes these cells to treatment.

The novel results presented here indicate that $ALDH^{hi}CD44^{+}$ cells are more resistant to both chemotherapy (anthracyclines and taxanes) and radiation. $ALDH^{hi}CD44^{+}$ cells also express higher levels of many therapy resistance proteins (p-glycoprotein, GSTpi, and/or CHK1) relative to $ALDH^{low}CD44^{-}$ cells, which may help explain some of the mechanisms underlying the observed therapy resistance. Our findings are consistent with other studies demonstrating that tumor-initiating cells in various cancers are resistant to chemotherapy[15,21,34,35] or radiation therapy[11-13]. Taken together with our previous work[7], the results of this study suggest a very challenging paradigm whereby cells that initiate primary breast tumors may ultimately also

become the cells that both initiate distant metastases and contribute to treatment resistance. However, the best approach to target these resistant cells requires further elucidation.

We chose to test the approach of targeting ALDH because of its high activity in resistant $ALDH^{hi}CD44^{+}$ cells and its known role in self-protection and detoxification[9]. Although ALDH activity has been shown to render cancer cells exquisitely resistant to cyclophosphamide chemotherapy[27,36-39], it has been unclear until this point whether ALDH activity could also protect cells from other chemotherapeutics and/or from ionizing radiation. However, there is some supporting evidence suggesting that this could be the case. For example, it has been shown that ALDH1 expression increases in primary breast cancer tumors following chemotherapy[34,35], and that high ALDH1 expression correlates with poor patient outcome[8]. Additionally, ALDH activity plays a well characterized role in differentiation via its role in the retinoic acid pathway, and it is possible that through differentiation, resistant cells could become more sensitive to treatment[28].

We therefore took two approaches to targeting ALDH activity, either by treatment with DEAB to specifically and directly block ALDH activity, or by treatment with ATRA to indirectly target ALDH activity via the retinoic acid pathway. When $ALDH^{hi}CD44^{+}$ cells were pre-treated with DEAB or ATRA, a significant initial sensitization to doxorubicin, paclitaxel, and radiation therapy was observed, in many cases to a level equivalent to that of $ALDH^{low}CD44^{-}$ cells. However, only DEAB pre-treatment was able to also reduce the long term re-growth/colony-forming ability of chemotherapy- or radiation-treated $ALDH^{hi}CD44^{+}$ cells, whereas cells pre-treated with ATRA were able to re-grow just as well as non-ATRA treated cells. These results indicate that specifically blocking ALDH activity is key for sensitizing resistant $ALDH^{hi}CD44^{+}$ cells to therapy. As discussed earlier, ATRA can down-regulate ALDH activity through an indirect route, but in our breast cancer cells, we saw that this ALDH down-regulation was short-lived as cells began showing increasing ALDH activity again as early as 48 hours following treatment. Therefore, a more direct targeted approach may be necessary.

The fact that ATRA did not maintain therapy sensitization over the long term was surprising because of the clear role it plays in other cancers such as APL in not only sensitizing cells to therapy, but also in malignant cell differentiation[40-42]. Additionally, ATRA has been shown to induce growth inhibition of MCF-7 breast cancer cells, which accumulate in the G1 phase and undergo apoptosis[43]. Interestingly, ATRA-mediated growth inhibition has been correlated with the presence of functional estrogen receptors, and the effect of ATRA can be enhanced by the use of Tamoxifen (an anti-estrogen drug)[43-45]. In fact, it has been shown that ATRA can inhibit estrogen-stimulated protein synthesis[45]. This is further supported Phase I/II clinical trials investigating the use of ATRA in breast cancer. In a single institution Phase II study, 17 patients with hormone refractory, metastatic breast cancer were administered 150mg/m^2 oral ATRA. Of those 17, only one patient experienced a partial response, which lasted only 4 months. Three other patients experienced stable disease for anywhere between 2-4 months[46]. However, in a different Phase I/II study, patients with measurable disease or evaluable non-measurable disease were given differing doses of ATRA (70-230mg/m^2/day, resulting in corresponding plasma concentrations of 1-3μM ATRA) on alternating weeks during Tamoxifen treatment. Of the 7 patients with measurable disease, 2 experienced a partial response to the combination therapy of ATRA and Tamoxifen. Of the 18 patients with evaluable, non-measurable disease, 7 experienced a partial response for 6 months or more[47]. In the present study, we used breast cancer cells that were estrogen receptor (ER) negative, and thus it is possible that ATRA can only exert its full effect in the presence of ER. However, when ATRA was used in conjunction with paclitaxel to treat ER negative primary breast tumors in mice in the present study, the combination caused a significant reduction in primary tumor burden. It should be noted, however, that this combination did not reduced metastatic tumor burden. In fact, ATRA as a single agent actually increased metastatic burden in the lungs in our mouse model, though not to statistically significant levels. This is supported by clinical data, where the Phase III Beta-Carotene and Retinol Efficacy Trial (CARET) was stopped prematurely

because no beneficial effect of retinoid treatment was observed, and in fact the treatment group experienced a 28% higher incidence of lung cancers compared to the placebo group[48,49]. Additional research is clearly required in order to gain a greater understanding of the retinoic acid pathway and ALDH before ATRA can be used successfully to treat breast cancer in the clinic.

In order to better understand how DEAB and ATRA were influencing sensitization to therapy, we analyzed the expression of various therapy resistance and differentiation/EMT proteins. Surprisingly, cells that were pre-treated with DEAB or ATRA showed no change in expression of any of the drug resistance proteins investigated (Pgp, GSTpi, CHK1, CHK2), suggesting that these proteins play a minimal role in ALDH-mediated therapy resistance of $ALDH^{hi}CD44^{+}$ breast cancer cells or that we may be missing the appropriate window of time for observing transient expression changes in these proteins in response to DEAB or ATRA. Of the differentiation/EMT markers, only CK8/18/19 was significantly increased following ATRA treatment of the MDA-MB-468 cells, suggesting some influence of ATRA in driving a more epithelial/differentiated phenotype in these cells. Alternatively, recent evidence suggests that ALDH activity may protect against cancer cell death caused by reactive oxygen species (ROS)[50]. Since radiation and chemotherapy are known to induce oxidative stress[51], it is possible that inhibition of this protective function of ALDH activity using DEAB or ATRA may serve to sensitize $ALDH^{hi}CD44^{+}$ stem-like cells to therapy via enhanced exposure to ROS and downstream cell death pathways. Future studies in our laboratory are aimed at investigating this.

In the present study, we investigated $ALDH^{hi}CD44^{+}$ cells isolated from two human breast cancer cell lines with distinct genetic backgrounds; moderately metastatic MDA-MB-231 cells and weakly metastatic MDA-MB-468 cells. Interestingly, $ALDH^{hi}CD44^{+}$ cells from both cell lines behaved in a similar manner from the point of view of chemotherapy and radiation resistance, suggesting that the $ALDH^{hi}CD44^{+}$ phenotype is appropriate for identifying resistant breast

cancer cells even in the presence of different genetic backgrounds. However, there were some subtle differences between cell lines with regards to response to DEAB and ATRA, both from the perspective of ALDH inhibition and therapy sensitization. These differences may be due to cell-line specific mechanisms related to retinoid signalling and other associated pathways, and this will also be investigated in future studies.

In summary, the novel findings presented here indicate that stem-like $ALDH^{hi}CD44^{+}$ breast cancer cells preferentially survive both chemotherapy and radiation therapy, and express high levels of therapy resistance proteins. Furthermore, targeting of ALDH activity using either DEAB or ATRA results in significant sensitization to therapy, in many cases to the same level of sensitization as the non-resistant $ALDH^{low}CD44^{-}$ cells. However, only the DEAB-treated cells were able to sustain this sensitization over the long-term, indicating that selectively blocking ALDH activity is the key to targeting these resistant cells. This idea was further validated *in vivo* where only primary tumors and not metastatic tumors responded to combination therapy with ATRA and paclitaxel. To the best of our knowledge, this is the first report that provides clear functional evidence that ALDH activity influences breast cancer cell resistance to anthracyclines (i.e. doxorubicin) and taxanes (i.e. paclitaxel) as well as to radiation, suggesting a much broader role for ALDH in treatment response than previously reported. Further investigation of the mechanisms involved in ALDH detoxification of these agents is required in the future, and translating this knowledge to the clinic via development of a direct ALDH inhibitor that is safe for human use could potentially have important implications for the management and treatment of metastatic breast cancer.

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Chapter 4

Differential functional roles of ALDH1A1 and ALDH1A3 in mediating metastatic behavior and therapy resistance of human breast cancer cells

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Croker AK, Pardhan S, Leong HS, and Allan AL, in preparation.

Abstract

Metastasis is the main cause of lethality associated with breast cancer, and this is due mainly to an incomplete understanding of the mechanisms driving metastasis and ineffective treatment of metastatic disease. Stem-like $ALDH^{hi}CD44^{+}$ breast cancer cells have been show to play major role in both metastasis and therapy resistance; however, the mechanisms underlying these behaviors remain poorly understood. ALDH1 expression has been correlated with poor patient outcome and metastatic disease, indicating a potential functional role in cancer progression. Therefore, the objective of the current study was to determine the role of ALDH1 in mediating breast cancer metastasis and therapy resistance. We used siRNA to knockdown expression of either the ALDH1A1 or ALDH1A3 isozyme in MDA-MB-468 and SUM159 human breast cancer cell lines in order to characterize the impact on ALDH activity as measured by the Aldefluor® assay; malignant/metastatic behavior *in vitro* (proliferation, adhesion, migration, invasion); therapy resistance *in vitro*; and tumorigenic and metastatic behavior *in vivo*. We observed that knockdown of ALDH1A3 but not ALDH1A1 in breast cancer cells significantly decreased ALDH activity as measured by the Aldefluor® assay ($p<0.05$). Knockdown of ALDH1A1 resulted in a significant reduction in breast cancer cell proliferation, adhesion, migration, and invasion *in vitro*, and reduced extravasation and metastasis in the chick embryo chorioallantoic membrane (CAM) *in vivo* model compared to control ($p<0.05$). In contrast, knockdown of ALDH1A3 did not alter breast cancer cell proliferation or invasion/extravasation *in vitro*, but did result in a significant increase in both *in vitro* adhesion and migration ($p<0.05$) and a significant decrease in metastasis in the CAM assay ($p<0.05$) compared to control. Furthermore, knockdown of ALDH1A1 but not ALDH1A3 in breast cancer cells led to significantly increased sensitivity to both chemotherapy and radiation ($p<0.05$). These novel results suggest that ALDH1 may play a functional role in mediating breast cancer metastasis and therapy resistance, and that different enzyme isoforms within the ALDH1 family differentially impact these cell behaviors.

4.1 Introduction

Breast cancer is a leading cause of death in women, due primarily to ineffective treatment of metastatic disease. In order to reduce mortality from breast cancer, it is therefore essential to learn more about the metastatic process, and in particular, mechanisms that may contribute to therapy resistance in the metastatic setting[1-3].

Metastasis is a multi-step process that involves tumor cell escape from the primary tumour, migration through the body, adhesion and extravasation at the secondary site, initiation of micrometastatic growth and maintenance of growth into clinically detectable macrometastases[1,4,5]. Given the complex nature of this process, it is not surprising that metastasis is highly inefficient, with the main rate-limiting steps being initiation and maintenance of growth at secondary sites[1,4,5]. Taken together with the heterogeneous nature of solid tumours, this metastatic inefficiency suggests that only a small subset of cells can successfully navigate the metastatic cascade and eventually re-initiate tumour growth to form metastases. We have previously identified a subset of breast cancer cells with high aldehyde dehydrogenase (ALDH) activity and expression of CD44, and demonstrated that these $ALDH^{hi}CD44^{+}$ cells have enhanced tumor-initiating and metastatic abilities both *in vitro* and *in vivo*[6]. Subsequent studies by Charafe-Jauffret et al. (2009, 2010) further demonstrated that $ALDH^{hi}$ cells isolated from breast cancer cell lines also had increased metastatic potential *in vivo*, supporting the premise that $ALDH^{hi}CD44^{+}$ cells may have a role as metastasis-initiating cells[7,8]. More recently, we have demonstrated that these $ALDH^{hi}CD44^{+}$ cells are also significantly more resistant to chemotherapy and radiation therapy, and that the observed therapy resistance may occur, at least in part, via ALDH-dependent mechanisms[9].

The ALDH superfamily of enzymes is involved in detoxification and/or bioactivation of various intracellular aldehydes in a $NAD(P)^{+}$-dependent manner. Of particular biological importance, the ALDH1 family of enzymes (namely, ALDH1A1 and ALDH1A3) plays an important role in oxidizing vitamin A (retinal) to retinoic acid (RA) through an alcohol

intermediary. RA functions as a ligand for nuclear retinoid receptors and leads to transactivation and transrepression of target genes, and is finally degraded by CYP26 enzymes[10]. In cancer, high ALDH1 expression has been shown to correlate with poor prognosis in breast cancer patients[11], and has been associated with metastasis development and poor clinical outcome[7,8,12]. In terms of ALDH1, ALDH1A1 was shown to be expressed more in breast cancer patients who presented with positive lymph nodes and in patients who succumbed to their disease[13]. In a meta-analysis that looked at almost 900 breast cancer cases compared to over 1800 control samples, Zhou et al (2010) found that ALDH1A1 expression was significantly associated with a high histological grade, ER/PR negativity, HER2 positivity, and worse overall survival[14]. Furthermore, when ALDH1A1^{bright} cells in various tumors, including breast, were targeted with ALDH1A1-specific CD8+ T cells, which resulted in elimination of the ALDH1A1^{bright} cells, an inhibition of tumorigenic and metastatic growth was observed[15]. Alternatively, an interesting study by Marcato et al. (2011) demonstrated that ALDH1A3 (and not ALDH1A1) expression in patient breast tumors correlated significantly with tumor grade, metastasis, and cancer stage, indicating that even within the ALDH1 family, alternate isoforms may function differently[16]. In terms of therapy resistance, Tanei et al (2009) conducted a study looking at 108 breast cancer patients who received neoadjuvant paclitaxel and epirubicin-based chemotherapy[17]. When ALDH1A1^{+} and CD24^{-}CD44^{+} expression was compared between core needle biopsies (pre-treatment) and subsequent excision (post-treatment), there was a significant increase in ALDH1A1 positive cells, but no change in CD24^{-}CD44^{+} cells, indicating that ALDH1A1^{+} cells may play a significant role in resistance to paclitaxel/epirubicin treatment[17].

Interestingly, ALDH activity has been shown to be involved in self-protection of normal stem cells and in resistance to the chemotherapeutic drug cyclophosphamide[18]. In the treatment of acute promyelocytic leukemia (APL), the differentiation agent all-trans retinoic acid (ATRA) is used clinically in combination with chemotherapy[19,20]. Increased levels of RA signaling from ATRA treatment can not only suppress levels of ALDH1, but also drives the differentiation of

promyelocytes into neutrophils, causing enhanced sensitivity to cyclophosphamide[21,22]. Additionally, ATRA has been shown to modulate cell growth, apoptosis, and differentiation in breast cancer cells[11,23].

Retinoic acid has recently been found to signal through two different pathways, resulting in distinctly opposite outcomes[24]. Traditionally, it was thought that RA signaled through cellular retinoic acid-binding protein-2 (CRABP2), transporting RA to RA receptors (RARs) in the nucleus and causing transcription of genes resulting in cell differentiation and reduced cell proliferation and survival[24]. More recently, an alternative pathway of RA action was identified involving binding and transport of RA into the cell nucleus by FABP5, resulting in increased cell survival and proliferation via activation of the peroxisome proliferator-activated receptor (PPARδ/γ)[24]. Interestingly, which of these divergent pathways is activated depends on the ratio of the respective transport proteins (FABP5:CRABP2). A low ratio leads to predominant RAR activation, whereas high ratio results in preferential activation of PPARδ/γ[24]. This may explain why some cancers respond to ATRA therapy and others do not[25]. In theory, retinoids may only inhibit tumor growth if the CRABP2/RAR pathway is predominant in the tumor cells, and this idea is supported by data showing that diverting RA from PPARβ/γ to RAR is sufficient to overcome RA resistance in mammary carcinomas[25]. Clinical studies have shown that CRABP2 protein levels were inversely correlated with tumor grade and were actually lowest in grade IV tumors[26]. Furthermore, there was a significant association between CRABP2 staining and the absence of lymphatic permeation in head and neck squamous cell carcinomas (HNSCC)[27]. Additionally, the absence of CRABP2 expression has been associated with worse disease outcome because patients with CRABP2-negative tumors had a higher risk of locoregional recurrence or distant metastases compared to patients with CRABP2-positive tumors. Alternatively, expression of FABP5 in breast cancer has been shown to inhibit RA-induced differentiation and instead induce survival and proliferation of tumor cells after diverting RA to the PPARδ/γ pathway[28]. Clinically, the expression of FABP5 has been associated with higher

grade tumors, and has shown consistent over-expression in short-term cancer survivors compared to long-term survivors[29]. Furthermore, FABP5 has been shown to cluster exclusively in high proliferative tumor regions and is associated with nestin (a stem cell related intermediate filament)[26]. In glioblastoma (GBM), high ratios of FABP5:CRABP2 exists and it has been hypothesized that even endogenous RA may be sufficient to promote malignant progression through activation of multiple anti-apoptotic signaling cascades mediated by FABP5/PPARδ/γ[29].

The goal of the current study was to test the hypothesis that ALDH1 is not simply a marker of highly aggressive breast cancer cells and poor patient prognosis, but that it also contributes functionally to metastatic behavior and therapy resistance. Furthermore, we wanted to begin to elucidate the molecular mechanisms underlying the functional role of ALDH1 in breast cancer metastasis, including the involvement of different ALDH1 isoforms (ALDH1A1 and ALDH1A3) and chaperone proteins (CRABP2 and FABP5). The novel findings presented here indicate that ALDH1 is functionally involved in breast cancer metastasis and therapy resistance, although the effects of ALDH1 appear to be isoenzyme-specific. Furthermore, the role that ALDH1 plays in metastasis and therapy resistance may not be RA-specific, suggesting that ALDH1 may have other important physiological functions beyond oxidizing aldehydes.

4.2 Materials and Methods

4.2.1 Cell culture, reagents, and therapy conditions

MDA-MB-468 cells were a kind gift from Dr. Janet Price, M.D. Anderson Cancer Center, Houston, TX[30], and were maintained in αMEM + 10% fetal bovine serum (FBS). The 468GFP subline was previously generated by stable transfection with the pEGFP-C2 expression vector (BD Biosciences Clontech, Palo Alto, CA) using LipofectAMINE™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines[31]. SUM159 cells were obtained from Asterand USA (Detriot, MI) and were maintained in Hams:F12 + 5% fetal bovine serum (FBS). CellTracker™ 5-chloromethylfluorescein diacetate (CMFDA; Invitrogen, Carlsbad, CA) was used

to label SUM159 cells for use in fluorescence microscopy analysis of the CAM assay. Both MDA-MB-468 and SUM159 cell lines were authenticated via third party testing of 9 short tandem repeat (STR) loci on June 14, 2010 (CellCheck, RADIL, Columbia, MO). Media was obtained from Invitrogen (Carlsbad, CA). FBS was obtained from Sigma (St. Louis, MO). All tissue culture plastic was obtained from NUNC (Roskilde, Denmark).

All-*trans* retinoic acid (ATRA) and diethylamino-benzaldehyde DEAB (Sigma) were constituted in 100% ethanol and diluted in either Hams:F12 (SUM159 cells) or α-MEM (MDA-MB-468) media at 5μM (ATRA) or 100μM (DEAB). Doxorubicin (Novopharm Limited, Toronto, ON) and paclitaxel (Biolyse Pharma Corporation, St. Catherines, ON) were obtained from the London Regional Cancer Program pharmacy and diluted in either Hams:F12 or α-MEM to a concentration of 0.4μM (doxorubicin) or 0.2μM (paclitaxel). Radiation was administered to cells using a Cobalt-60 irradiator (Theratron 60, Atomic Energy of Canada Limited). SUM159 cells received 2x15Gy and MDA-MB-468 cells received 2x5Gy of radiation. All chemotherapy and radiation dose levels were selected based on LC_{50} values determined in previous experiments[9].

4.2.2 siRNA knockdown of ALDH1A1 and ALDH1A3

ON-TARGET plus SMART pool small interfering RNAs (siRNA) (Dharmacon Thermo Scientific, Lafayette, CO) were used to knockdown human ALDH1A1 and ALDH1A3 in both the SUM159 and MDA-MB-468 breast cancer cell lines. All siRNAs were suspended in sterile RNAse-free water at a concentration of 25μM. Scrambled control (20-50μl/ml), ALDH1A1 (20μl/ml), ALDH1A3 (50μl/ml) siRNAs were diluted into serum-free Opti-MEM (Invitrogen, Carlsbad, CA). Lipofectamine RNAiMax reagent (Invitrogen, Carlsbad, CA) was also diluted into Opti-MEM at a concentration of 20μl/ml in all cases. Lipofectamine and siRNA concentrations were determined based on preliminary experiments which indicated the greatest knockdown of the proteins of interest (*data not shown*). The Lipofectamine media and siRNA media were then combined and incubated for 20 minutes before adding 2ml of media per 100mm plate and 1ml of media per 60mm plate. The transfections yielded the following cell populations used in further

experiments: 468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, and 159ALDH1A3^{low}.

4.2.3 RNA isolation and quantitative RT-PCR

Total RNA was extracted from MDA-MB-468 and SUM159 breast cancer cells with TRIzol reagent (Invitrogen) according to the manufacturer's protocol. The RNA concentration was measured using NanoDrop™ spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE) at the wavelengths of 260/280nm. Total RNA was reverse transcribed using Superscript III (Invitrogen) and the Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany). The forward and reverse primers for selected genes are listed in *Table 4.1*. Relative quantification of ALDH1A1 and ALDH1A3 gene expression in MDA-MB-468 and SUM159 breast cancer cells was determined by quantitative PCR. The Brilliant® II SYBR® Green qPCR Low ROX Master Mix (Agilent Technologies, Eugene, OR) was used for the analysis. The qPCR cycles for ALDH1A1, ALDH1A3, FABP5, CRABP2, GAPDH are described in *Table 4.2*. The delta Ct method was used to calculate relative gene expression levels, and expression of the housekeeping gene GAPDH was used for normalization of ALDH1A1 and ALDH1A3 expression.

4.2.4 Western blotting

Cell lysates were extracted from cell populations (468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low}) using lysis buffer containing PMSF (100mM), benzamidine (100mM), leupeptin (1mg/ml), pepstatin A (1mg/ml), and aprotinin (1mg/ml). Ten micrograms of protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%) and transferred onto polyvinylidene difluoride membranes (PVDF; Immobilon™, Millipore; Bedford, MA, USA). Blocking and antibody dilution was done using 5% skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST). Anti-human primary antibodies against N-cadherin (CDH2), vimentin, E-cadherin (CDH1), cytokeratins 8, 18,

***Table 4.1*.** **Forward and reverse primer sequences for GAPDH, ALDH1A1, ALDH1A3, CRABP2, and FABP5**

Gene	Primer Sequence	Product (bp)	Company
GAPDH – forward	5'- CAT GTT CGT CAT GGG TGT GAA CCA -3'	24bp	Sigma
GAPDH – reverse	5'- ATG GCA TGG ACT GTG GTC ATG AGT -3'	24bp	Sigma
ALDH1A1 – forward	5' - CGT TGG TTA TGC TCA TTT GGA A - 3'	22bp	IDT
ALDH1A1 – reverse	5' - TGA TCA ACT TGC CAA CCT CTG T - 3'	22bp	IDT
ALDH1A3 – forward	5' – ATG TGG GAA AAC CCC CTG TG – 3'	20bp	IDT
ALDH1A3 – reverse	5' – GAA TGG TCC CAC CTT CAC CTC – 3'	21bp	IDT
CRABP2 – forward	5' – TGC TGA GGA AGA TTG CTG TG – 3'	20bp	IDT
CRABP2 – reverse	5' – CCC ATT TCA CCA GGC TCT TA – 3'	20bp	IDT
FABP5 – forward	5' – GAG TGG GAT GGG AAG GAA AG – 3'	20bp	IDT
FABP5 – reverse	5' – GAT CCG AGT ACA GGT GAC ATT G – 3'	22bp	IDT

***Table 4.2*.** **qPCR conditions for ALDH1A1, ALDH1A3, CRABP2, FABP5, and GAPDH genes**

Gene	qPCR Conditions	Number of Cycles
GAPDH	45 Seconds 60^{o}C 45 Seconds 72^{o}C 60 Seconds 95^{o}C	40
ALDH1A1	60 Seconds 55^{o}C 60 Seconds 72^{o}C 60 Seconds 95^{o}C	45
ALDH1A3	60 Seconds 57^{o}C 60 Seconds 72^{o}C 60 Seconds 95^{o}C	45
CRABP2	60 Seconds 55^{o}C 60 Seconds 72^{o}C 60 Seconds 95^{o}C	45
FABP5	60 Seconds 55^{o}C 60 Seconds 72^{o}C 60 Seconds 95^{o}C	45

19 (CK8/18/19), aldehdyde dehydrogenase-1A1 (ALDH1A1), aldehyde dehydrogenase-1A3 (ALDH1A3), fatty acid binding protein-5 (FABP5), cellular retinoic acid binding protein-2 (CRABP2), and β-actin loading control are described in *Table 4.3*. Secondary antibodies used include goat anti-mouse (Calbiochem, Gibbstown, NJ), mouse anti-rabbit (Calbiochem, Gibbstown, NJ), antibodies conjugated to horseradish peroxidase (1:2000 dilution for all proteins except E-cadherin; 1:10,000). Protein expression was visualized using the Amersham ECL Plus Western Blot Detection System (GE Healthcare, Baie d'Urfe, QC) and BIOMAX MS scientific imaging film (Kodak, Rochester, NY), and developed in the Kodak M35A X-OMAT Processor (Kodak, Rochester, NY). Expression of each protein was normalized to the β-actin loading control and signal intensity was quantified (n=3 blots per protein) using the computer-assisted densitometry program AlphaEase FC™ Software version 3.1.2 (Alpha Innotech Corporation, Miami FL).

4.2.5 Analysis of ALDH activity

To assess ALDH activity of the different engineered cell lines, the ALDEFLUOR® assay (StemCell Technologies, Vancouver, BC) was used as described previously[32-34]. Briefly, MDA-MB-468 and SUM159 cells were harvested, placed in ALDEFLUOR® assay buffer (2 x 10^6/ml), and incubated with the ALDEFLUOR® substrate for 45 minutes @ 37^{o}C to allow substrate conversion. As a negative control for all experiments, an aliquot of ALDEFLUOR®-stained cells was immediately quenched with 1.5-mM diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor. Cells were analyzed using the green fluorescence channel (FL1) on a Beckman Coulter EPICS XL-MCL flow cytometer.

Table 4.3 Details of anti-human antibodies used for western blot analysis of proteins related to ALDH, RA Signaling, and EMT markers

Protein/Antibody	Commercial Source	Lot	Dilution
Aldehyde Dehydrogenase-1A1	Abcam	GR3098-4	1:1000
Aldehyde Dehydrogenase- 1A3	Abcam	GR2728-7	1:500
Fatty Acid Binding Protein-5 (FABP5)	R&D Systems	WGU0111061	1:2000
Cellular Retinoic Acid Binding Protein-2 (CRABP2)	Abcam	GR98701-1	1:500
N-Cadherin	Abcam	812783	1:1000
Vimentin	Millipore	LV1700725	1:1000
E-Cadherin	BD Biosciences	09705	1:20000
Cytokeratins 8/18/19	Abcam	794363	1:1000
β-actin	Sigma	083K4834	1:5000

4.2.6 Cell proliferation assays

Cell populations (468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low}) were plated at a density of 5.0 x 10^4 cells/60 mm plate (n=3 for each time point) and maintained in regular growth media. Every 48 hours for 14 days, triplicate cultures were trypsinized and counted by hemacytometer. Doubling time of each cell population was estimated during the exponential growth phase according to $T_d = 0.693t/\ln (N_t/N_0)$, where t is time (in hr), N_t is the cell number at time t, and N_0 is the cell number at initial time.

4.2.7 Cell adhesion assays

Cells were plated onto sterile 96-well non-tissue culture plates (Titertek, Flow Laboratories Inc.; McLean, VA) treated with either 5µg/ml of human vitronectin (Sigma; MDA-MB-468 cells), 20µg/ml of human laminin (Sigma; SUM159 cells), or PBS (negative control), using 1x10^4 cells/well (n=3) for each cell population (468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low}). Vitronectin and laminin were chosen based on previous experiments in our laboratory that have demonstrated that MDA-MB-468 and SUM159 cells differentially express integrin receptors for laminin and vitronectin respectively ([35] and our unpublished data). Cells were allowed to adhere for 5 hours, after which the media was removed and non-adhered cells were rinsed away. Adhered cells were fixed with 2% gluteraldehyde and stained using Harris' hematoxylin. Five high powered fields (HPF) (200x) were counted for each well, and mean numbers of adhered cells/field were calculated.

4.2.8 Cell migration and invasion assays

Transwell plates (6.5mm, 8µm pore size; Becton Dickinson; Franklin Lakes, NJ) were coated with 6 µg/well of gelatin (Sigma) (for migration assay) or 10 µg/well of Matrigel (Becton Dickinson) (for invasion assay) as described previously[36,37]. Control (0.01% BSA) or chemoattractant (5% FBS) media was placed in the bottom portion of each well. 5 x 10^4 cells of each cell population (468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low}) were plated on top of the transwells. Cells were allowed to migrate for 24

hours or invade for 28 hours, after which the upper transwell was removed, inverted, fixed with 1% gluteraldehyde, and stained with Harris' hematoxylin. Non-migrated or non-invaded cells on the inner surface of the transwell were carefully removed using a cotton swab. Five HPF were counted for each well, and mean numbers of migrated or invaded cells/field were calculated.

4.2.9 Cell survival assays

Cell populations (468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low}) were plated at a density of 5x10^5 cells in 6-well plates (n=3/treatment group) and maintained in normal growth medium for 24 hours. Cells were then treated with either normal media alone (control), chemotherapy (paclitaxel [0.2μM]; doxorubicin [0.4μM]), or radiation (2x5Gy, MDA-MB-468; or 2x15Gy, SUM159), and cells were cultured for a further 72 hours. Cells were then harvested and viable cells were quantified using trypan blue exclusion and manual counting on a hemocytometer using light microscopy.

4.2.10 Chick embryo chorioallantoic membrane (CAM) assay

For assessment of *in vivo* invasive/metastatic behavior, chick embryo chorioallantoic membrane (CAM) assays were used as described previously[38,39]. Briefly, fertilized White Leghorn eggs were manually opened over sterile plastic weigh boats, and intact embryos were carefully spilled into the weigh boats and maintained *ex ovo*. In this manner, the CAM, which is normally located immediately underneath the eggshell *in ovo,* becomes exposed as a free-floating organ atop the developing embryo. The weigh boats were covered by bottoms of square Petri dishes (100 x 100mm; Nunc, Rochester NY), and embryos were maintained in a humidified cell culture incubator at 37^{o}C with 90% humidity. Embryos were used at day 9 (micrometastasis assay) and day 12 (extravasation assay).

Green-fluorescent protein (GFP) labeled MDA-MB-468 cell populations (468CON, 468ALDH1A1^{low}, and 468ALDH1A3^{low}) or CellTracker^{TM} CMFDA-labeled SUM159 cell populations (159CON, 159ALDH1A1^{low}, and 159ALDH1A3^{low}) were trypsinized, washed in PBS, and resuspended in PBS at 1 x 10^6 cells/ml for the extravasation assay (468CON n=18;

468ALDH1A1^{low} n=16; 468ALDH1A3^{low} n=17; 159CON n=18; 159ALDH1A1^{low} n=17; 159ALDH1A3^{low} n=11) or 3 x 10^6 cells/ml for the micrometastasis assay (468CON n=9; 468ALDH1A1^{low} n=11; 468ALDH1A3^{low} n=8; 159CON n=13; 159ALDH1A1^{low} n=14; 159ALDH1A3^{low} n=14). Cells were then injected into the CAM as described previously[38,39]. Briefly, using a micro-needle and a dissection microscope, 100µl of cell suspension was injected into the tributaries or secondary tributaries of major veins of the chicken embryo. After the needle was pulled out of the embryo, kimwipes were used to dab the injection site to remove blood and cancer cells that may have spilled onto the surface of the CAM. A fluorescence microscope was used in order to verify successful injection of cancer cells throughout the capillary plexus of the CAM.

4.2.10.1 Extravasation assay

After cell injection, a portion of the CAM was sectioned off using aluminum foil and the number of cells arrested in the sectioned-off area was manually counted using a fluorescence microscope at 20X magnification. The embryos were then returned to the incubator for 24 hours, after which time the number of extravasated cells in the sectioned off area were manually counted using a fluorescence microscope. Percent extravasation was calculated by dividing the number of initial cells by the number of successfully extravasated cells in the CAM.

4.2.10.2 Micrometastasis assay

After injection, the chicken embryos were returned to the incubator for 7 days to allow the formation of micrometastatic tumors. After 7 days, the number of micrometastatic tumors that developed following the i.v. injection were manually counted using a fluorescence microscope at 4X magnification.

4.2.11 Statistical analysis

All experiments were performed following at least three separate transfections with at least 3 biological replicates included within each experiment. In all cases, quantitative data was compiled from all experiments. Statistical analysis was performed using GraphPad Prism 4.0

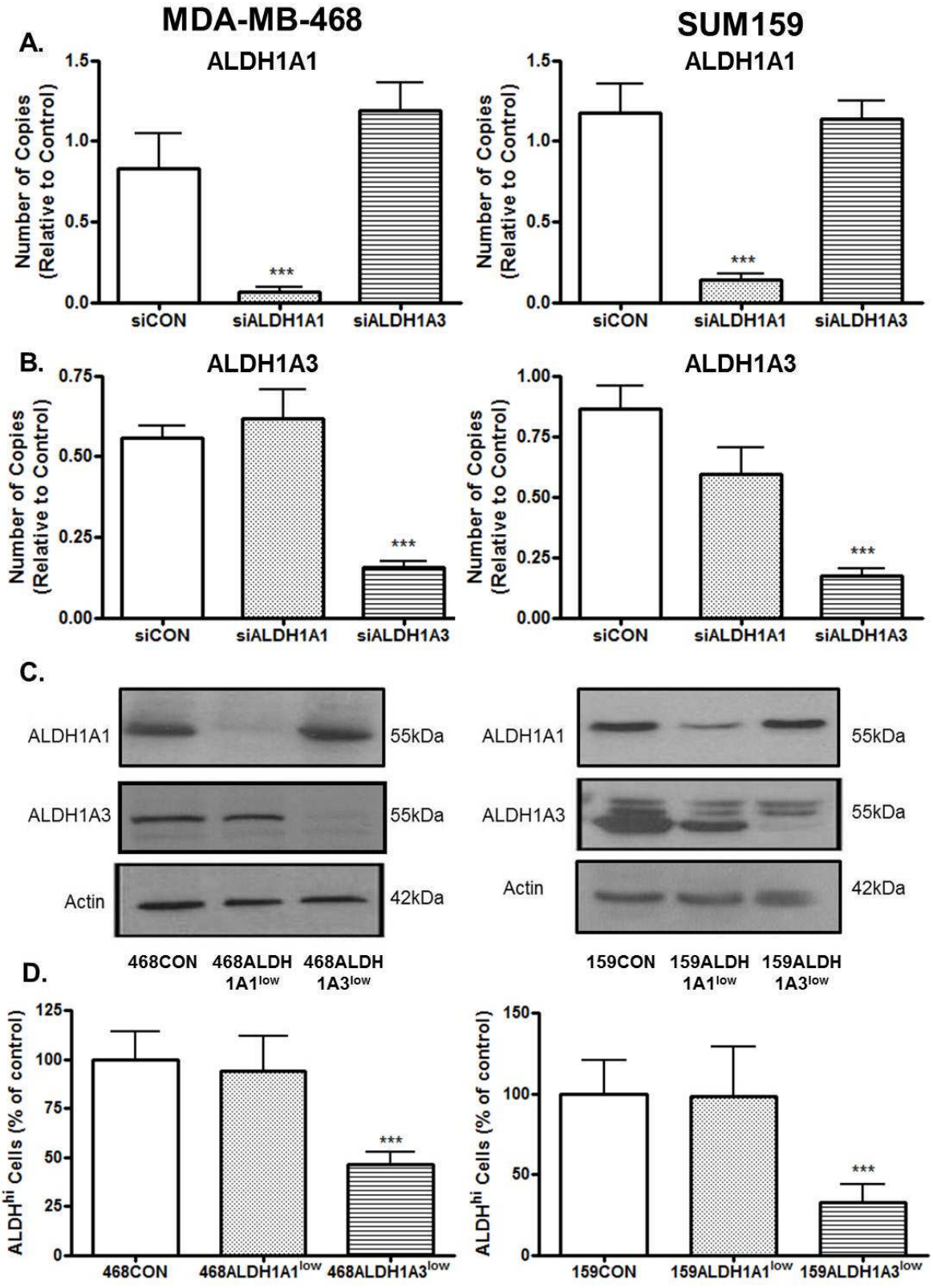
software© (San Diego, CA) using either t-test (for comparison between 2 groups) or ANOVA with Tukey post-test (for comparison between more than 2 groups). Differences between means were determined using the Student's t-test when groups passed both a normality test and an equal variance test. When this was not the case, the Mann-Whitney Rank-Sum test was used. Unless otherwise noted, data is presented as the mean ± SEM. In all cases, *P* values of ≤0.05 were regarded as being statistically significant.

4.3 Results

4.3.1 Decreased expression of ALDH1A3 but not ALDH1A1 reduces ALDH activity as measured by the ALDEFLUOR® Assay

siRNA was used to knockdown expression of two ALDH1 isoforms (ALDH1A1 and ALDH1A3) in MDA-MB-468 and SUM159 breast cancer cells. The knockdown was confirmed by quantitative RT-PCR and Western blotting (*Figure 4.1A-C*). There has been some debate over which ALDH1 isoform is responsible for the ALDH activity measured in the ALDEFLUOR® assay (StemCell Technologies), with some groups suggesting that ALDH1A1 is responsible for the ALDH activity while others believe that it is ALDH1A3[16,40]. In order to determine whether ALDH activity was reduced following ALDH1 knockdown, 468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, and 159ALDH1A3^{low} cell populations were labeled using the ALDEFLUOR® assay (*Figure 4.1D*). Compared to their respective controls, the 468ALDH1A3^{low} and 159ALDH1A3^{low} cell populations did demonstrate a significant decrease in ALDH activity ($p<0.001$), while 468ALDH1A1^{low} and 159ALDH1A1^{low} cell populations did not exhibit a change in ALDH activity ($p>0.05$). This data supports previous observations by Marcato et al. (2011), and indicates that the ALDH1A3 isozyme is the major contributor to ALDH activity as measured by the ALDEFLUOR® assay[16].

Figure 4.1 Decreased expression of ALDH1A3 but not ALDH1A1 reduces ALDH activity as measured by the Aldefluor® assay. MDA-MB-468 (*left panels*) or SUM159 *(right panels*) human breast cancer cells were transfected with 100pmol siRNA targeted towards ALDH1A1, ALDH1A3, or a scrambled control using Lipofectamine to generate the following cell lines: 468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, and 159ALDH1A3^{low}. After 4 days, RNA, cell lysates, or cells were collected and qRT-PCR ***(A,B),*** Western blots ***(C),*** or Aldefluor® assays ***(D)*** were performed to assess ALDH1 gene expression, protein expression, and enzyme activity (respectively). Data represents the mean +/- SEM. *** = significantly different than respective siCON, 468CON, or 159CON control ($p<0.001$).


MDA-MB-468
SUM159
A.
ALDH1A1
Number of Copies (Relative to Control)
1.5
1.0
0.5
0.0
***
siCON
siALDH1A1
siALDH1A3
B.
ALDH1A3
0.75
0.50
0.25
0.00
1.00
0.75
0.50
0.25
0.00
C.
ALDH1A1
ALDH1A3
Actin
55kDa
42kDa
468CON
468ALDH 1A1low
468ALDH 1A3low
159CON
159ALDH 1A1low
159ALDH 1A3low
D.
ALDHhi Cells (% of control)
125
100
75
50
25
0
150
468CON
468ALDH1A1low
468ALDH1A3low
159CON
159ALDH1A1low
159ALDH1A3low

4.3.2 Decreased expression of ALDH1A1 or treatment with ATRA/DEAB reduces breast cancer cell proliferation

We next wanted to assess whether downregulating ALDH1 had a functional effect on malignant breast cancer cell behavior *in vitro*. First, differences in cellular growth characteristics between cell populations for both cell lines were assessed in response to direct knockdown of ALDH1 (ALDH1A1 or ALDH1A3) by siRNA, or by indirect chemical inhibition of ALDH1 using ATRA or DEAB (*Figure 4.2*). In contrast to the ALDH activity results, 468ALDH1A1^{low} and 159ALDH1A1^{low} cells demonstrated significantly decreased growth in normal culture relative to respective control cells, whereas 468ALDH1A3^{low} and 159ALDH1A3^{low} cells showed no difference in proliferation compared to control cells. Lag times (time to reach exponential growth phase) were also observed to be longer for 468ALDH1A1^{low} and 159ALDH1A1^{low} cells versus respective control cells (9 vs. 5 days for MDA-MB-468 cells; 5 days vs. 3 days for SUM159 cells) (*Figure 4.2A*). Cells treated with ATRA and DEAB both demonstrated decreased growth in normal culture relative to respective control cells (*Figure 4.2B*). Interestingly, while DEAB-treated cells always proliferated more slowly than control, ATRA treated cells grew no differently from control until Day 9 in MDA-MB-468 cells and Day 5 in SUM159 cells.

4.3.3 Adhesion and migration of human breast cancer cells *in vitro* is differentially influenced by decreased expression of ALDH1A1 or DEAB treatment versus decreased expression of ALDH1A3 or ATRA treatment

In addition to cell proliferation, we also assessed the influence of ALDH1 downregulation on breast cancer cell adhesion and migration *in vitro* (*Figure 4.3*). 468ALDH1A1^{low} and 159ALDH1A1^{low} cells were observed to be significantly less adherent to vitronectin (MDA-MB-468) or laminin (SUM159) (*Figure 4.3A*), and significantly less migratory towards serum (*Figure 4.3C*) than respective control cells ($p<0.01$). MDA-MB-468 cells treated with DEAB displayed identical patterns, with DEAB treated cells being significantly less adhesive (*Figure 4.3B*) and less migratory compared to control cells ($p<0.01$) (*Figure 4.3C*). DEAB-treated SUM159 cells demonstrated a significant decrease in migration ($p<0.001$) (*Figure 4.3D*), and a trend towards

Figure 4.2 Decreased expression of ALDH1A1 or treatment with ATRA/DEAB reduces breast cancer cell proliferation *in vitro*. MDA-MB-468 (*left panels*) and SUM159 (*right panels*) breast cancer cells were treated with control siRNA (siCON) or ALDH-specific siRNA (siALDH1A1 or siALDH1A3) for 96 hours to generate the following cell lines: 468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low} ***(A)*** or cells were treated with 5µM ATRA or 100µM DEAB ***(B)***. 1 x 10^5 viable cells/plate were seeded into 60mm dishes (n=3/time point) and allowed to grow for up to 13 days. At each time point, cells were trypsinized and counted using a hemacytometer. Data represents the mean +/- SEM. * = significantly different than respective 468CON or 156CON cells ($p<0.05$).

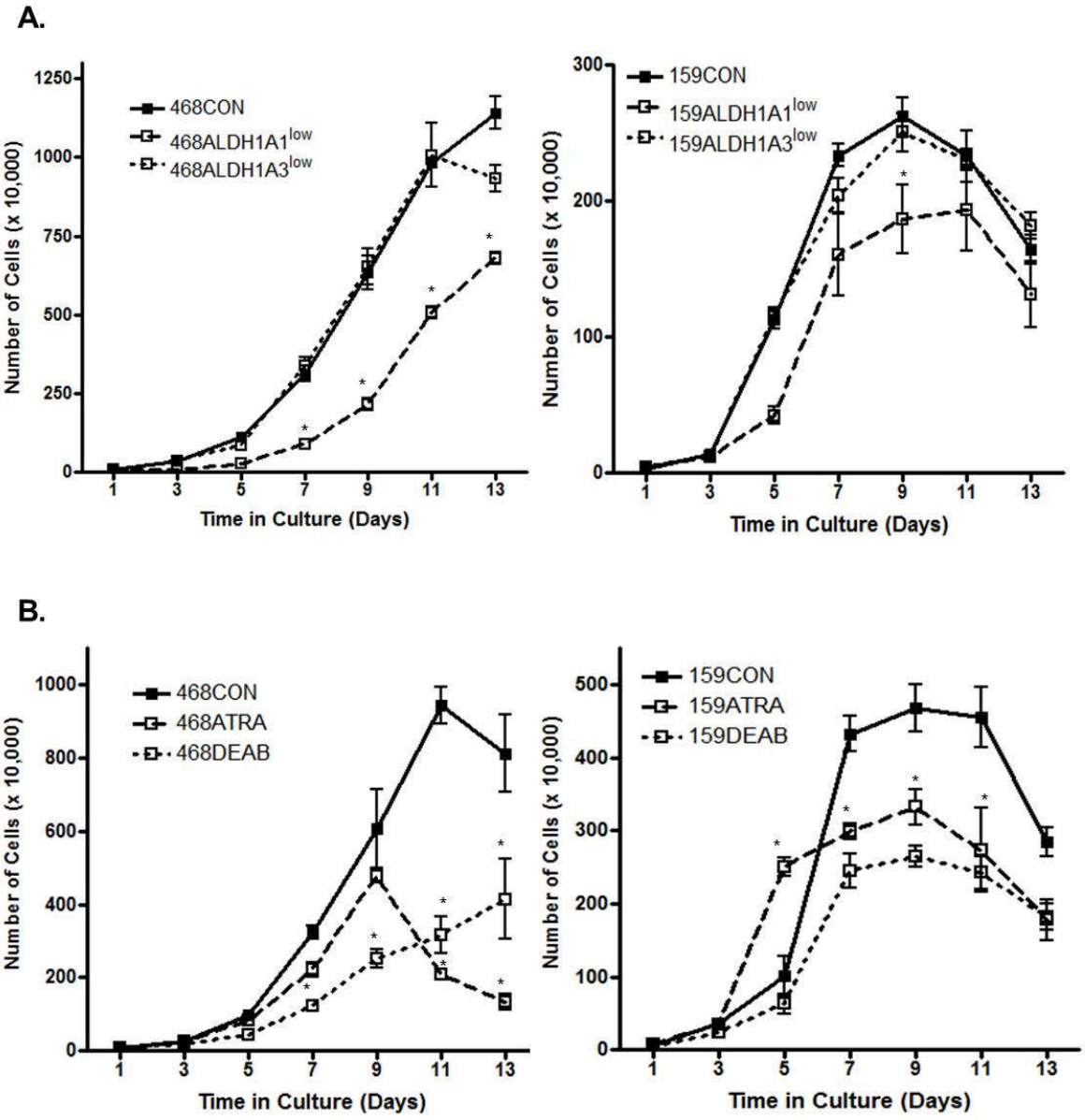
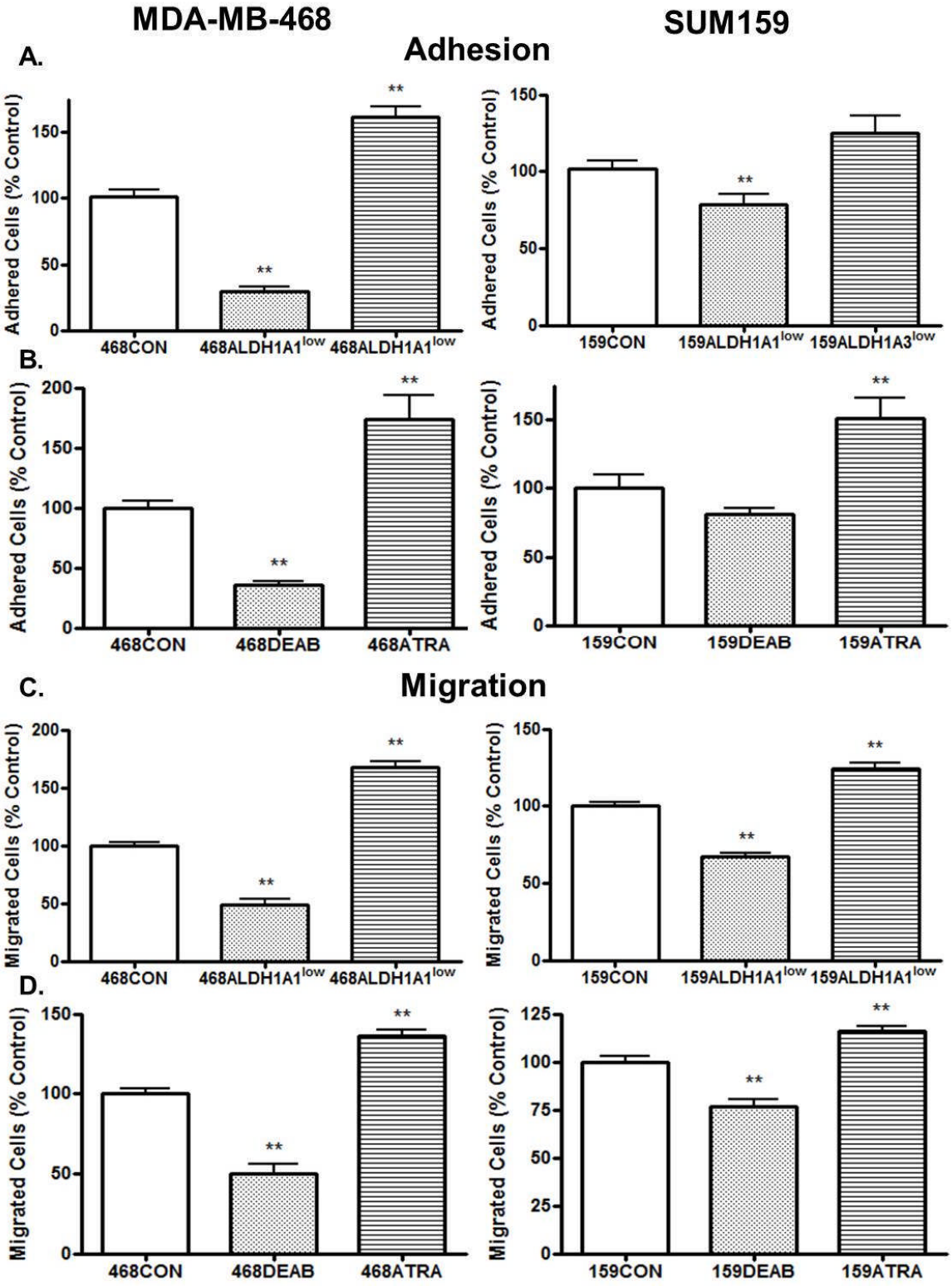

A.
468CON
468ALDH1A1low
468ALDH1A3low
Number of Cells (x 10,000)
Time in Culture (Days)
159CON
159ALDH1A1low
159ALDH1A3low
Number of Cells (x 10,000)
Time in Culture (Days)
B.
468CON
468ATRA
468DEAB
Number of Cells (x 10,000)
Time in Culture (Days)
159CON
159ATRA
159DEAB
Number of Cells (x 10,000)
Time in Culture (Days)

Figure 4.3 Adhesion and migration of human breast cancer cells is differentially influenced by decreased expression of ALDH1A1 or DEAB treatment versus decreased expression of ALDH1A3 or ATRA treatment. MDA-MB-468 (*right panels*) and SUM159 *(left panels*) breast cancer cells were treated with control siRNA (siCON) or ALDH-specific siRNA (siALDH1A1 or ALDH1A3) for 96 hours to generate the following cell lines: 468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low} ***(A,C)***; or treated with 5µM ATRA or 100µM DEAB ***(B,D)***. For adhesion assays ***(A,B)***, 96-well non-tissue culture plates were coated with PBS (neg. control) or vitronectin (VN; 5 mg/ml). 1 x 10^4 viable cells were added to each well and allowed to adhere for 5h. For migration assays ***(C,D)***, 5.0 x 10^4 viable cells were allowed to migrate towards PBS (neg. control) or 5% FBS (chemoattractant) for 24hrs. Adhered and migrated cells were fixed, stained, and 5 fields of view/well were counted. Data represents the mean +/- SEM. ** = significantly different than respective 469CON or 159CON cells ($p<0.05$).


MDA-MB-468
SUM159
A.
Adhesion
Adhered Cells (% Control)
468CON
468ALDH1A1low
468ALDH1A1low
159CON
159ALDH1A1low
159ALDH1A3low
B.
468CON
468DEAB
468ATRA
159CON
159DEAB
159ATRA
C.
Migration
Migrated Cells (% Control)
468CON
468ALDH1A1low
468ALDH1A1low
159CON
159ALDH1A1low
159ALDH1A1low
D.
468CON
468DEAB
468ATRA
159CON
159DEAB
159ATRA

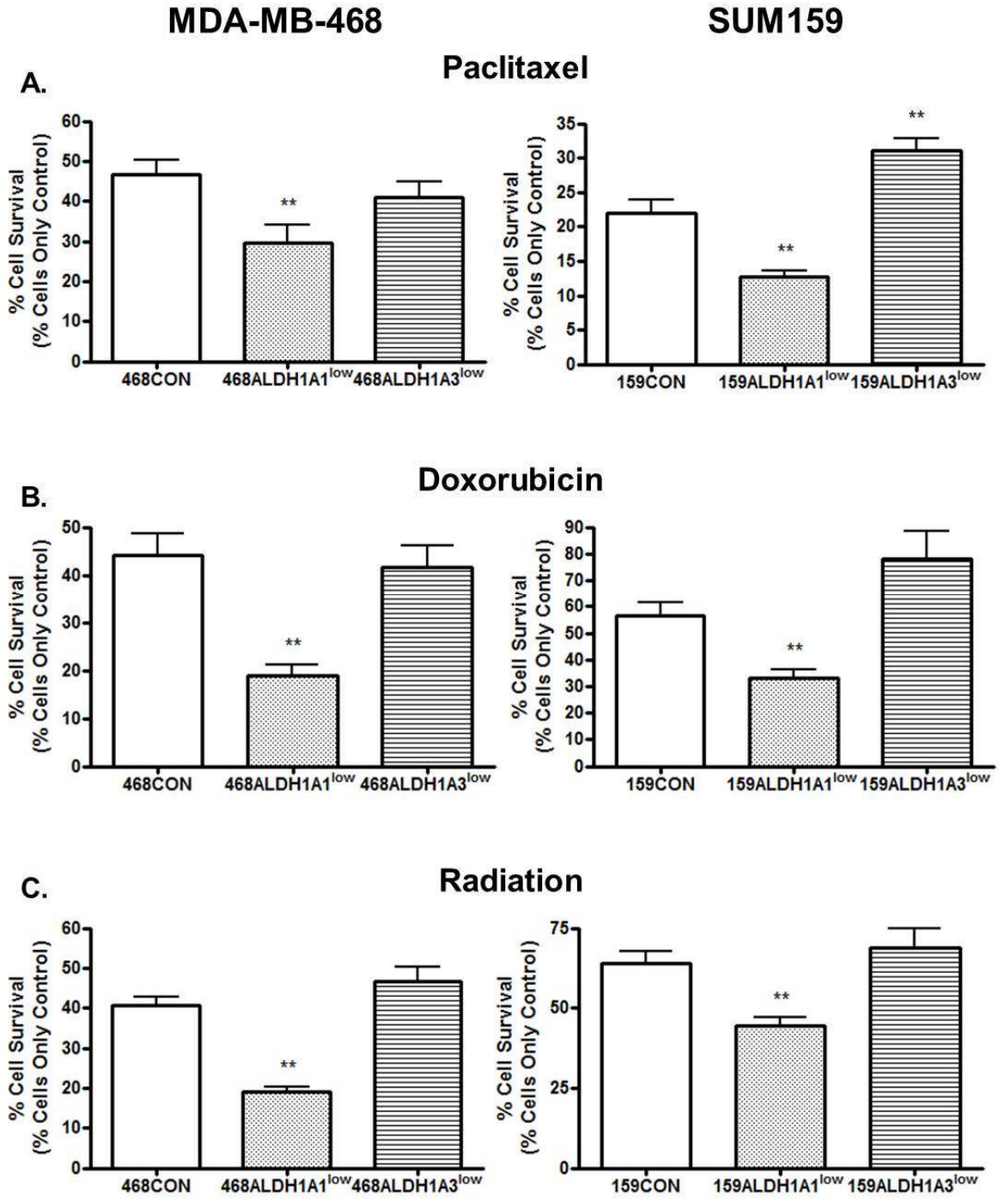
decreased adhesion, although this decrease did not reach statistically significant levels (*Figure 4.3B*). In contrast, 468ALDH1A3^{low}, 159ALDH1A3^{low} cells, and MDA-MB-468 and SUM159 cells treated with ATRA were observed to be significantly more adherent to vitronectin (MDA-MB-468) or laminin (SUM159) ($p<0.01$) (*Figure 4.3A,B*), and significantly more migratory towards serum (*Figure 4.3C,D*) than respective control cells ($p<0.01$).

4.3.4 Decreased expression of ALDH1A1 but not ALDH1A3 sensitizes breast cancer cells to chemotherapy and radiation *in vitro*

We have previously observed that ALDH^{hi}CD44^{+} breast cancer cells are significantly more resistant to chemotherapy and radiation therapy, and that this therapy resistance may occur, at least in part, via ALDH1-dependent mechanisms[9]. Taken together with the known role of ALDH activity in stem cell self-protection and detoxification[41], we hypothesized that a reduction in ALDH1 expression might sensitize MDA-MB-468 and SUM159 cells to chemotherapy and radiation. To test this, ALDH1A1 or ALDH1A3 were targeted using siRNA in MDA-MB-468 and SUM159 cells, and then treated with paclitaxel, doxorubicin, or radiation (*Figure 4.4*). ALDH1A1 knockdown caused a significant sensitization of both MDA-MB-468 and SUM159 cells to paclitaxel (*Figure 4.4A*), doxorubicin (*Figure 4.4B*), and radiation therapy (*Figure 4.4C*) ($p<0.01$). In contrast, ALDH1A3 knockdown did not reduced therapy resistance compared to control cells, and in fact, even resulted in an increase in cell survival in SUM159 cells exposed to paclitaxel (*Figure 4.4A*) ($p<0.01$).

We previously demonstrated that ALDH^{hi}CD44^{+} breast cancer cells become significantly more sensitive to both chemotherapy and radiation if treated in combination with ATRA or DEAB, and we hypothesized that this was potentially due to an ALDH1-dependent mechanism[9]. The data presented here suggests that ALDH1A1 may have been the isozyme responsible for the decrease in therapy resistance associated with ALDH^{hi}CD44^{+} breast cancer cells treated with ATRA or DEAB.

Figure 4.4 Decreased expression of ALDH1A1 but not ALDH1A3 sensitizes breast cancer cells to chemotherapy and radiation. MDA-MB-468 cells (*left panels*) and SUM159 cells (*right panels*) were treated with control siRNA (siCON) or ALDH-specific siRNA (ALDH1A1 or ALDH1A3) for 96 hours to generate the following cell lines: 468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low}. Cell populations were plated on 6-well plates at 5x10^5 cells, allowed to adhere, and were then treated with paclitaxel (0.2µg/ml) ***(A)***, doxorubicin (0.2µg/ml) ***(B)***, or radiation (2x5Gy; MDA-MB-468 or 2x15Gy; SUM159) ***(C)***. Data represents the mean +/- SEM. ** = significantly different than respective 468CON or 159CON cells + paclitaxel, doxorubicin, or radiation ($p<0.01$).


MDA-MB-468
SUM159
A.
Paclitaxel
% Cell Survival
(% Cells Only Control)
468CON
468ALDH1A1low
468ALDH1A3low
159CON
159ALDH1A1low
159ALDH1A3low
**
B.
Doxorubicin
C.
Radiation

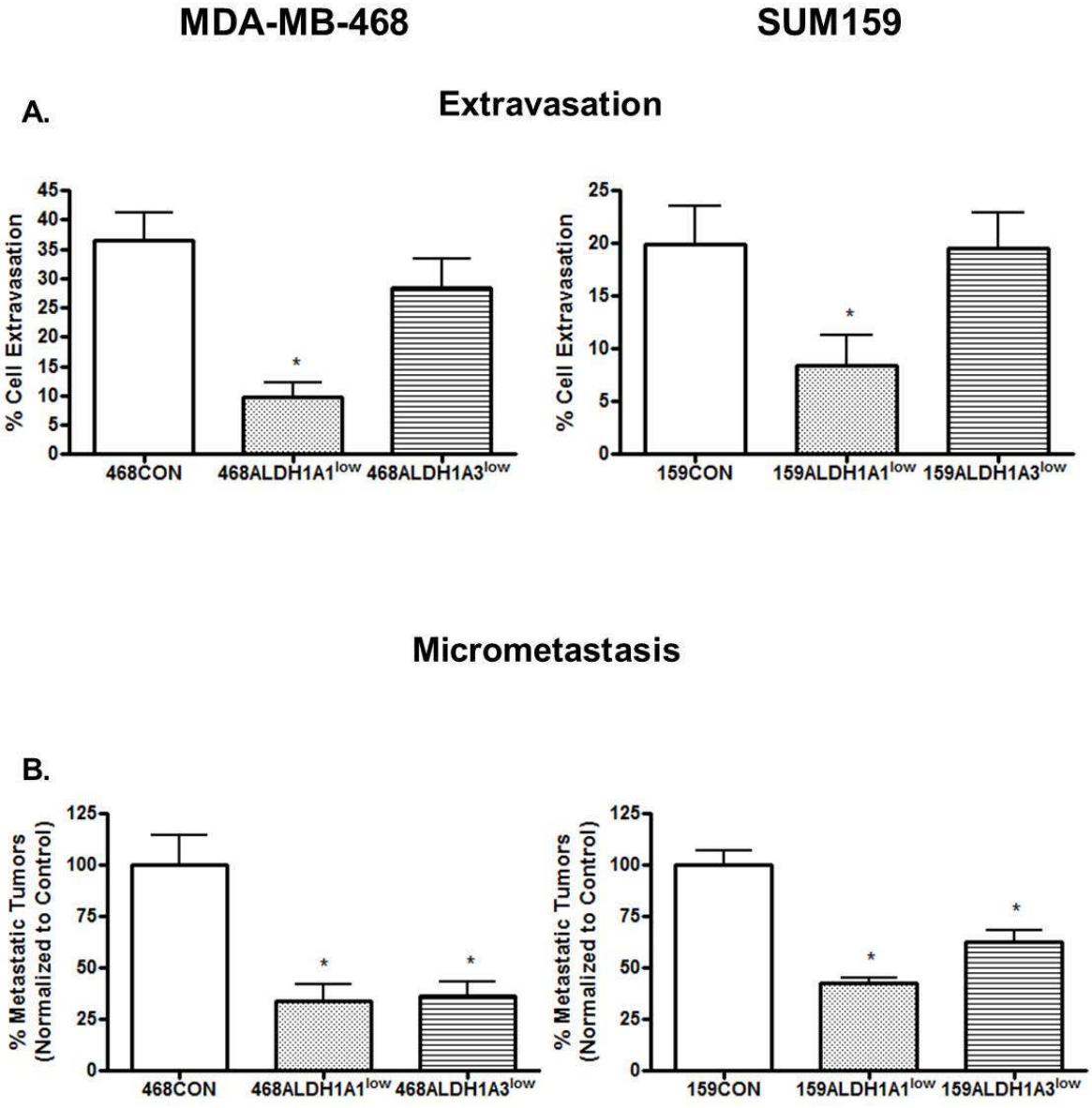
4.3.5 Decreased expression of ALDH1A1 and ALDH1A3 reduces *in vivo* metastatic ability of breast cancer cells in the chick CAM assay

In order to assess the metastatic ability of ALDH-deficient cell populations *in vivo*, GFP-labeled MDA-MB-468 cell populations (468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low} cells) or CMFDA-labeled SUM159 cell populations (159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low} cells) were inoculated on the CAM of 9- or 12-day-old chicken embryos, and the percentage of breast cancer cell extravasation into the CAM and formation of micrometastases in the chicken embryo were analyzed (*Figure 4.5*). 468ALDH1A1^{low} cells demonstrated a significant decrease in extravasation compared to 468CON cells ($p<0.001$), whereas there was no significant difference observed in the extravasation of 468ALDH1A3^{low} cells compared to control cells ($p>0.05$) (*Figure 4.5A*). Similarly, 159ALDH1A1^{low} cells demonstrated a significant decrease in extravasation compared to 159CON cells ($p<0.05$), whereas there was no significant difference observed in the extravasation of 159ALDH1A3^{low} cells compared to control cells ($p>0.05$) (*Figure 4.5A*). The number of micrometastases that formed in the chicken embryo was counted one week following inoculation in order to determine the metastatic potential of cell populations (468CON, 468ALDH1A1^{low}, and 468ALDH1A3^{low} populations or 159CON, 159ALDH1A1^{low}, and 159ALDH1A3^{low} populations). Both 468ALDH1A1^{low} and 468ALDH1A3^{low} cell populations demonstrated a significant decrease in the number of micrometastatic populations that were able to form compared to 468CON cells ($p<0.001$) (*Figure 4.5B*). Similarly, both 159ALDH1A1^{low} and 159ALDH1A3^{low} cell populations demonstrated a significant decrease in the number of micrometastatic populations that were able to form compared to 159CON cells ($p<0.001$) (*Figure 4.5B*).

4.3.6 The functional effects of ALDH1A1 and ALDH1A3 in metastasis and therapy resistance may not be related to differentiation or RA signaling

In an attempt to determine how ALDH1 is contributing functionally to the metastatic behaviour and therapy resistance of breast cancer cells, we investigated whether the expression of classical “de-differentiation” or EMT markers (N-cadherin, E-cadherin, vimentin,

Figure 4.5 Decreased expression of ALDH1A1 and ALDH1A3 reduces *in vivo* metastatic ability of breast cancer cells in the chick CAM assay. GFP-labeled MDA-MB-468 or CMFDA-labeled SUM159 cell populations were transfected with 100pmol (MDA-MB-468) or 400pmol (SUM159) siRNA targeted towards ALDH1A1, ALDH1A3, or scrambled control using Lipofectamine to generate the following cell lines: 468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low}. After 4 days, 1x10^5 (extravasation assay) or 2x10^5 (micrometastasis assay) cells were injected into chicken embryos and the % cell extravasation or micrometastatic formation was observed over 24hrs-7days. Extravasation: 468CON n=18; 468ALDH1A1^{low} n=16; 468ALDH1A3^{low} n=17; 159CONn=18; 159ALDH1A1^{low}n=17; 159ALDH1A3^{low}n=11. Micrometastasis: 468CON n=9; 468ALDH1A1^{low} n=11; 468ALDH1A3^{low} n=8; 159CONn=13; 159ALDH1A1^{low}n=14; 159ALDH1A3^{low}n=14. ALDH1A1^{low} cells demonstrated a decreased ability to extravasate into the chicken embryo compared to control, whereas no change was observed in the ALDH1A3^{low} cells. One week following injections, the number of micrometastases that formed were counted. Both ALDH1A1^{low} and ALDH1A3^{low} cell populations had a decreased ability to form micrometastases in the chicken embryo. Data represents the mean +/- SEM. * = significantly different than respective 468CON and 159CON cells ($p<0.05$).


MDA-MB-468
SUM159
A.
Extravasation
% Cell Extravasation
45
40
35
30
25
20
15
10
5
0
*
468CON
468ALDH1A1low
468ALDH1A3low
% Cell Extravasation
25
20
15
10
5
0
*
159CON
159ALDH1A1low
159ALDH1A3low
Micrometastasis
B.
% Metastatic Tumors (Normalized to Control)
125
100
75
50
25
0
*
*
468CON
468ALDH1A1low
468ALDH1A3low
% Metastatic Tumors (Normalized to Control)
125
100
75
50
25
0
*
*
159CON
159ALDH1A1low
159ALDH1A3low

cytokeratin8/18/19) were altered following knockdown of ALDH1A1 or ALDH1A3 (*Figure 4.6*). No changes were observed in the expression of any EMT protein in either the MDA-MB-468 or SUM159 cells with altered ALDH1 expression ($p>0.05$) with the exception of vimentin in the SUM159 cells, which demonstrated the expression of a different vimentin banding pattern compared to the 159CON and 159ALDH1A1^{low} cells. We also hypothesized that the effects of retinoic acid signaling may be a mechanism of action for ALDH1 involvement in breast cancer metastasis. Retinoic acid can signal through FABP5 to increase the aggressiveness, metastatic potential, and survival of cells, but only when over-expressed compared to CRABP2 levels[24,28]. We looked at the relative levels of CRABP2 and FABP5 in the MDA-MB-468 and SUM159 cells and found that in the MDA-MB-468 cells, CRABP2 was expressed more than FABP5 (*Figure 4.7A*). In the SUM159 cells, however, FABP5 was expressed more than CRABP2 (*Figure 4.7B*). However, the differential expression of the CRABP2 and FABP5 chaperone proteins between the two cell lines does not explain the observed functional differences between ALDH1A1 versus ALDH1A3, which were not cell line specific.

4.4 Discussion

Breast cancer is a leading cause of death in women, and this is due primarily to ineffective treatment of metastatic disease[1,35]. Our group has previously demonstrated that stem-like ALDH^{hi}CD44^{+} cells play a key role in breast cancer metastasis[12]. Furthermore, these ALDH^{hi}CD44^{+} cells were also shown to be highly resistant to chemotherapy and radiation compared to their ALDH^{low}CD44^{-}counterparts[9]. Additionally, it has been shown that ALDH1 expression is correlated with worse patient prognosis and also with a high incidence of metastasis[7,11,16]. It seems clear that ALDH^{hi} cells are important players in breast cancer metastasis; however, the actual functional contribution of ALDH1 in breast cancer metastasis requires further investigation, and this was the goal of the current study.

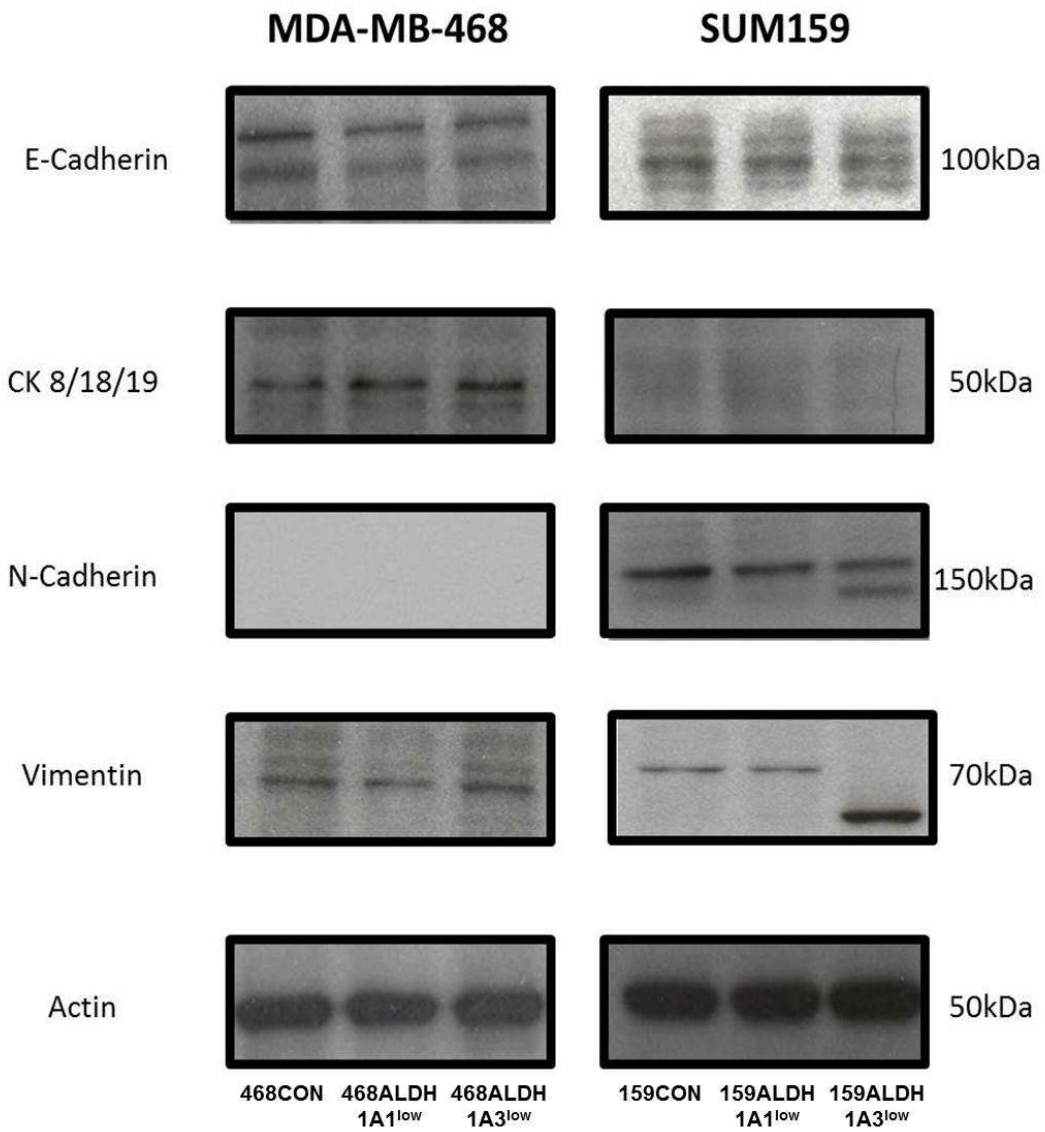


Figure 4.6 Decreased expression of ALDH1A1 and ALDH1A3 does not alter the expression of epithelial-to-mesencymal transition (EMT) markers. MDA-MB-468 and SUM159 breast cancer cells were transfected with 100pmol (MDA-MB-468) or 400pmol (SUM159) siRNA targeted towards ALDH1A1, ALDH1A3, or scrambled control using Lipofectamine to generate the following cell lines: 468CON, 468ALDH1A1^{low}, 468ALDH1A3^{low}, 159CON, 159ALDH1A1^{low}, 159ALDH1A3^{low}. After 4 days, cell lysates were collected and western blots were performed using antibodies and conditions outlined in *Table 4.3*.

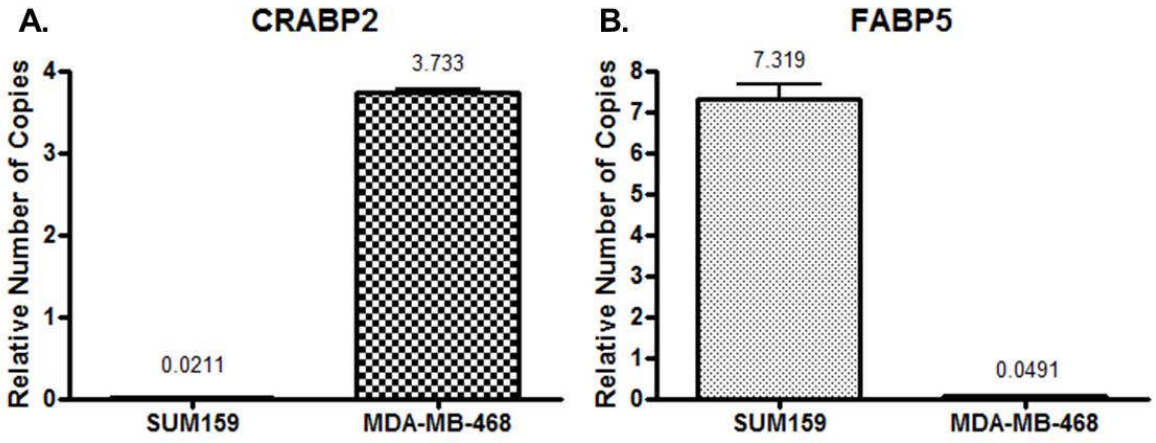


Figure 4.7 Expression of CRABP2 and FABP5 in MDA-MB-468 and SUM159 breast cancer cells. Total RNA was isolated from MDA-MB-468 and SUM159 breast cancer cells as described in the Materials and Methods. Quantitative RT-PCR was performed to identify relative expression levels of CRABP2 and FABP5 in the two cell lines. There was significantly more CRABP2 expression (over 150-fold more) in the MDA-MB-468 cells compared to the SUM159 cells. Alternatively, there was significantly more FABP5 expression (over 150-fold more) in the SUM159 cells compared to the MDA-MB-468 cells.

The Aldefluor® assay is often used in order to isolate ALDH^{hi} cancer cells[7-9,11,12,42,43]. In this assay, cells are incubated in a buffer containing a fluorescent aldehyde substrate (bodipy-aminoacetylaldehyde). The aminoacetylaldehyde is taken up into the cells via passive diffusion. Once inside the cell, intracellular ALDH oxidizes the aminoacetylaldehyde into aminoacetate, which is negatively charged, and therefore retained inside the cell, causing the cells to fluoresce[43]. Interestingly, when ALDH1A1 was knocked down in both MDA-MB-468 and SUM159 cell lines, there was no observable change in ALDH activity as measured by the Aldefluor® assay; however, when ALDH1A3 was knocked down, there was an approximate 50% reduction in ALDH activity measured by the Aldefluor® assay. This is consistent with breast cancer studies done by Marcato et al (2011), which found that ALDH1A3 knockdown was better correlated with a decrease in Aldefluor® activity compared to ALDH1A1 and ALDH2[16]. Interestingly, ALDH1A1 and ALDH7A1 have been reported to drive the Aldefluor® activity in other tumor types (i.e. ALDH1A1 in lung and ALDH7A1 in prostate)[43,44], indicating that the ALDH1 isoform(s) responsible for Aldefluor® activity may be tumor-specific. Furthermore, in the present study, even after ALDH1A3 knockdown, there was still approximately 50% normal ALDH activity, indicating that other ALDH isozymes might be involved. Taken together, these results suggest that many ALDH isoforms may contribute to the ALDH activity measured by the Aldefluor® assay, and potentially that different isoforms may contribute to ALDH activity in different tumor types.

We previously reported that ALDH^{hi}CD44^{+} cells demonstrated enhanced proliferation[12], and previous work in lung and liver cancers has suggested that a decrease in ALDH expression can cause a decrease in proliferation[45-47]. In the current study, we used DEAB to inhibit overall ALDH activity and found that breast cancer cells with low ALDH activity demonstrated decreased proliferation. In order to determine whether different ALDH isoforms contributed to proliferation, we used siRNA to specifically knock down ALDH1A1 or ALDH1A3. Interestingly, ALDH1A1^{low} cells behaved similarly to DEAB-treated cells, demonstrating decreased

proliferation, whereas ALDH1A3^{low} cells showed no change in proliferation. Taken together, these results demonstrate that ALDH activity, and in particular, ALDH1A1 activity, functionally contributes to cellular proliferation in breast cancer cells. We also treated cells with ATRA to elucidate the role of RA signaling in proliferation of the breast cancer cells. There was no observable change in proliferation until the second week of the assay when there was a significant decrease in proliferation.

ALDH1 expression (in particular ALDH1A3 expression) has been correlated with an increased incidence of metastasis[7,11,16]. Interestingly, cells with decreased ALDH1A1 expression demonstrated decreased abilities to adhere, migrate, and invade/extravasate; whereas cells with decreased ALDH1A3 expression demonstrated increased abilities to adhere and migrate, but no change in invasive capabilities compared to control cells. In the actual formation of metastases *in vivo*; however, both ALDH1A1^{low} and ALDH1A3^{low} cells demonstrated a decrease in metastatic potential, with an approximate 50% reduction in the number of micrometastases that were able to form in the chicken embryo compared to control cells. Taken together, this data suggests that both ALDH1A1 and ALDH1A3 contribute functionally to breast cancer metastasis; however, they may do so in different ways. It is possible that ALDH1A1^{low} cells may have difficulty traveling through the metastatic cascade to a secondary site since these cells are less able to adhere and migrate, whereas ALDH1A3^{low} cells may experience difficulty initiating and sustaining metastatic growth in the secondary site. This data both supports and contradicts previous work by Marcato et al (2011) that showed that ALDH1A3 and not ALDH1A1 correlated with metastatic disease in breast cancer patients[16]. Furthermore, the ALDH1A3 results are supported by a study done in prostate cancer that showed that ALDH7A1 was functionally involved in prostate cancer metastasis to bone[44]. ALDH7A1^{low} cells demonstrated no change in proliferation or even primary tumor growth compared to control cells; however, a significant decrease in the number of metastases to the bone was observed. Moreover, the authors noted that the inability of ALDH7A1^{low} cells to grow in the bone may have been due to the bone

microenvironment. For example, the authors found that treating cells with TGFβ significantly increased ALDH7A1 expression, whereas cells treated with bone morphogenetic proteins (BMP2, BMP4, or BMP7) resulted in a decrease in ALDH7A1 expression[44]. It is possible that in breast cancer, ALDH1A3 is required in order for interactions within the microenvironment that allow cell survival and metastatic tumor formation in the secondary site.

We previously observed that $ALDH^{hi}CD44^{+}$ cells demonstrate high levels of therapy resistance, and that pre-treatment targeting of ALDH either directly (using DEAB) or indirectly (using ATRA) could sensitize these resistant cells to both anthracycline and taxane chemotherapy, as well as radiation[9]. In the current study, we directly targeted specific ALDH1 isoforms using siRNA and tested the effect on therapy response. Interestingly, when ALDH1A1 expression was decreased, there was a significant sensitization of the cancer cells to both chemotherapy and radiation. Cells with decreased ALDH1A3 expression, however, showed no change in therapy resistance to either chemotherapy or radiation. These results suggest that ALDH1A1 can contribute functionally to therapy resistance, though the mechanisms by which this occurs remain unclear. There is, however, some evidence that ALDH1A1 is correlated with the expression of the pregnane-X-receptor (PXR) and the nuclear factor erythroid 2-related factor 2 (Nrf2)[48]. PXR is a nuclear receptor whose primary function is to sense the presence of foreign toxic substances, and, in response, up-regulate the expression of proteins involved in detoxification and clearance of the substance (i.e. GSTpi and CYP3A4)[49]. Nrf2, on the other hand, is a transcriptional activator that binds to the antioxidant response element (ARE) in the promoter region of target genes, and is important for the coordinated up-regulation of genes in response to oxidative stress[50]. A study by Alnouti and Klaassen (2008) demonstrated that PXR ligands and Nrf2 activators did not change ALDH1A3 expression levels; however, their expression did significantly increase ALDH1A1 mRNA expression in both rat and human cell lines[48]. This suggests a possible mechanism of action for ALDH1A1 in the protection of cancer cells from chemotherapy and radiation, although more research is required to validate this idea.

Undergoing epithelial to mesenchymal transition (EMT) has been hypothesized to be important for successful metastatic cells to acquire the more motile phenotype required to migrate and invade, as well as for resistance to chemotherapy[44]. In the present study, ALDH1A1^{low} cells demonstrated no change in classical EMT markers (E-cadherin, N-cadherin, CK8/18/19, and vimentin), and thus it is unlikely that differentiation/EMT mechanisms can explain the decreased adherent or migratory phenotypes observed in these cells. ALDH1A3^{low} cells showed no change in epithelial markers (E-cadherin or CK8/18/19), but did show an alteration in the vimentin banding pattern such that no band was detected at approximately 60kDa, but instead, a smaller band was detected at approximately 40kDa. The significance of this is unknown, although it is currently under investigation.

We also hypothesized that the effects of retinoic acid signaling may be a mechanism of action for ALDH1 involvement in breast cancer metastasis. When we treated MDA-MB-468 or SUM159 cells with the ALDH inhibitor DEAB, we found that the cells behaved similarly to ALDH1A1^{low} cells in that both cell populations demonstrated decreased adhesion and migration compared to controls. Conversely, treating cells with ATRA caused the cells to behave similarly to ALDH1A3^{low} cells in that both cell populations demonstrated increased adhesion and migration. DEAB treated cells would have lower levels of RA due to decreased ALDH activity, and obviously, treating cells with ATRA increases the amount of available RA. This suggests that relative cellular levels of RA may contribute to the metastatic phenotypes observed in ALDH1A1^{low} and ALDH1A3^{low} cells.

Retinoic acid can signal through FABP5 to increase the aggressiveness, metastatic potential, and survival of cells, but only when over-expressed compared to CRABP2 levels[24,28]. Not surprisingly, the highly metastatic SUM159 cells expressed over 150-fold more FABP5 compared to the weakly metastatic MDA-MB-468 cells, suggesting that more aggressive breast cancer cell lines might have a higher expression of FABP5, and less metastatic cell lines might have a higher CRABP2 expression. This differential expression of CRABP2 and FABP5 in the

MDA-MB-468 and SUM159 cells suggest that these two cell lines should behave differently in response to RA; however, our results showed that both cell lines responded similarly to ATRA treatment. Furthermore, the differential expression of CRABP2 and FABP5 between the two cell lines does not explain the observed functional differences between knockdown of ALDH1A1 versus ALDH1A3, since the results were isozyme-specific versus cell line-specific. Taken together, this data suggest that RA signaling is probably not the major mechanism responsible for the changes in metastatic behavior and therapy response that we observed in this study.

Overall, the results of this study suggest that ALDH1 plays a functional role in both breast cancer metastasis and therapy resistance; although ALDH1A1 and ALDH1A3 seemed to contribute to these behaviors in different ways. Furthermore, our results suggest that the functional effects of ALDH1 knockdown might not be due to RA signaling or aldehyde detoxification, which are the two main known functions of ALDH1. This suggests that the ALDH1 family of enzymes may possess other, as yet unknown, mechanisms of contributing to the metastatic capacity of breast cancer cells. Finally, the observation that ALDH1A3 knockdown only caused a 50% reduction in ALDH activity suggests that other ALDH enzymes may be involved in Aldefluor® activity. It would therefore be interesting to determine the role of other ALDH enzymes in breast cancer metastasis (i.e. ALDH7A1)[44].

Further elucidation of the mechanisms by which ALDH1A1, ALDH1A3 and other ALDH isozymes contribute to breast cancer metastasis and the translation of this knowledge to the clinic could have potentially important implications for the management and treatment of breast cancer. Furthermore, additional investigation of the ALDH1A1-specific therapy resistant mechanisms is required, and translating this knowledge to the clinic through development of either a direct ALDH1A1 inhibitor or an ALDH1A1-related inhibitor that is safe for human use could have important implications for the management of both primary and metastatic breast cancer. Finally, it is well known that treating breast cancer before metastasis is observed (i.e. in the adjuvant setting) is significantly correlated with better patient survival[9,12,51]. Given that

ALDH1 has been both correlated with metastatic disease and shown to functionally contribute to metastasis, it may be beneficial to use assessment of ALDH1 expression in the primary tumor as a clinical tool for identifying breast cancer patients with a high risk of metastasis and stratifying them for aggressive therapy to prevent disease recurrence or progression.

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Chapter 5

Overall Discussion

Breast cancer metastasis is a leading cause of death in women[1,2]. Metastasis is involved in most of the lethality associated with breast cancer because our current cancer therapies fail in the metastatic setting[3]. When I undertook the studies presented in this thesis, stem-like cancer cells had been shown to be highly tumorigenic in most tumor types; however, the role that these cells played in metastasis was unknown[4-11]. Furthermore, while it was known that normal stem cells have many self-protection mechanisms[12-17], it was unclear whether stem-like cancer cells also retained self-protection abilities and whether or not these cells were responsible for therapy failure and disease relapse in patients. Finally, while ALDH1 had been used to identify aggressive, stem-like cancer cells in many different tumor types, and had been correlated with worse patient prognosis, higher tumor grade, and a higher incidence of metastasis, it was unclear whether ALDH1 functionally contributed to either breast cancer metastasis and/or therapy resistance[18-20]. This thesis sought to answer these questions.

5.1 Summary of key experimental findings

1. Commonly studied human breast cancer cell lines contain subpopulations of stem-like cells based on both putative CSC marker expression (CD44/CD24 and CD133), and functional stem cell properties (enhanced ALDH activity). Furthermore, the “stem-like” cell content of the cell lines seemed to be associated with their aggressiveness (i.e. the more aggressive/metastatic the cell line, the greater the $CD44^{+}$ and/or $ALDH^{hi}$ cell content). This suggests that breast cancer cell lines may provide a suitable model system for investigating stem-like cancer cells.

2. $ALDH^{hi}CD44^{+}$ cancer cells isolated from MDA-MB-231 and MDA-MB-468 human breast cancer cell lines demonstrate enhanced malignant/metastatic behavior *in vitro* and *in vivo* compared to $ALDH^{low}CD44^{-}$ cells.
3. $ALDH^{hi}CD44^{+}$ breast cancer cells isolated from MDA-MB-231 and MDA-MB-468 human breast cancer cell lines preferentially survive both chemotherapy and radiation, and express higher levels of therapy-resistance proteins compared to $ALDH^{low}CD44^{-}$ cells.
4. Reduction of ALDH activity in $ALDH^{hi}CD44^{+}$ breast cancer cells using ATRA, DEAB, or siRNA targeted towards ALDH1A1 results in significant sensitization to therapy, indicating that blocking ALDH activity is key to targeting these resistant cells.
5. ALDH1 plays a functional role in both breast cancer metastasis and therapy resistance; although the ALDH1A1 and ALDH1A3 isozymes seem to contribute to these behaviors in different ways.
6. The observed functional effects of ALDH1 knockdown may not be due to RA signaling or aldehyde detoxification, which are the two main known functions of ALDH1; suggesting a new function of the ALDH1 enzyme.
7. Finally, ALDH1A3 but not ALDH1A1 contributes to ALDH activity as measured by the Aldefluor® assay in MDA-MB-468 and SUM159 human breast cancer cell lines.

5.2 Implications of experimental findings

5.2.1 Cell lines provide a valuable proof-of-principle model system for investigating the role of stem-like cells in metastasis

When we first began this work, it was assumed that cancer cell lines were clonogenic populations; all identical cells that had been passaged for many years. What we actually observed was that breast cancer cell lines, like patient tumors, contain subpopulations of highly aggressive cells with a stem-like phenotype. We observed that subsets of cells within cancer cells lines express cancer stem cell markers ($CD44^{+}CD24^{-}$, CD133), while others do not.

Furthermore, there is differential ALDH activity in breast cancer cell lines, with only a small percentage of cells being identified as having high ALDH activity. When we looked at the relative proportion of cells in each cell line with an $ALDH^{hi}CD44^{+}$ phenotype, we found that the most aggressive and metastatic cells lines contained the highest proportion of cancer cells with CSC markers, and the least aggressive cell lines contained the lowest proportion of cells expressing CSC markers. Although Chapter 2 reports this observation in 4 different human breast cancer cell lines, during the course of this thesis we made the same observation in a total of seven cell lines (*Figure 5.1*). Combined with the functional data presented in Chapter 2 (discussed further below), this demonstrates that commonly used breast cell lines contain subpopulations of cells with phenotypic and functional stem-like properties. Thus, the purification of these cells from cell lines can provide a valuable proof-of-principle model system for investigating their role in metastasis and therapy resistance and a useful alternative to using human breast cancer patient tumor samples, which are difficult to acquire and propagate. Subsequent studies by other groups have also provided support for this type of model system[11,18,20-28].

5.2.2. $ALDH^{hi}CD44^{+}$ cells play a functional role in breast cancer metastasis

One of the major goals of this thesis was to determine whether stem-like breast cancer cells were responsible for metastasis. As previously mentioned, metastasis is an incredibly complex process, and as such, is a very inefficient process, with the rate-limiting steps being the initiation of growth and sustaining that growth in the secondary site[3,29,30]. There have been many hypotheses attempting to explain the rate-limiting step, and most have done so by suggesting that only certain cells can survive in certain microenvironments, and so even if a cancer cell could reach a secondary site, whether or not it will be able to grow there will depend on the interactions between the cell and the microenvironment[3,30,31]. Our hypothesis was slightly

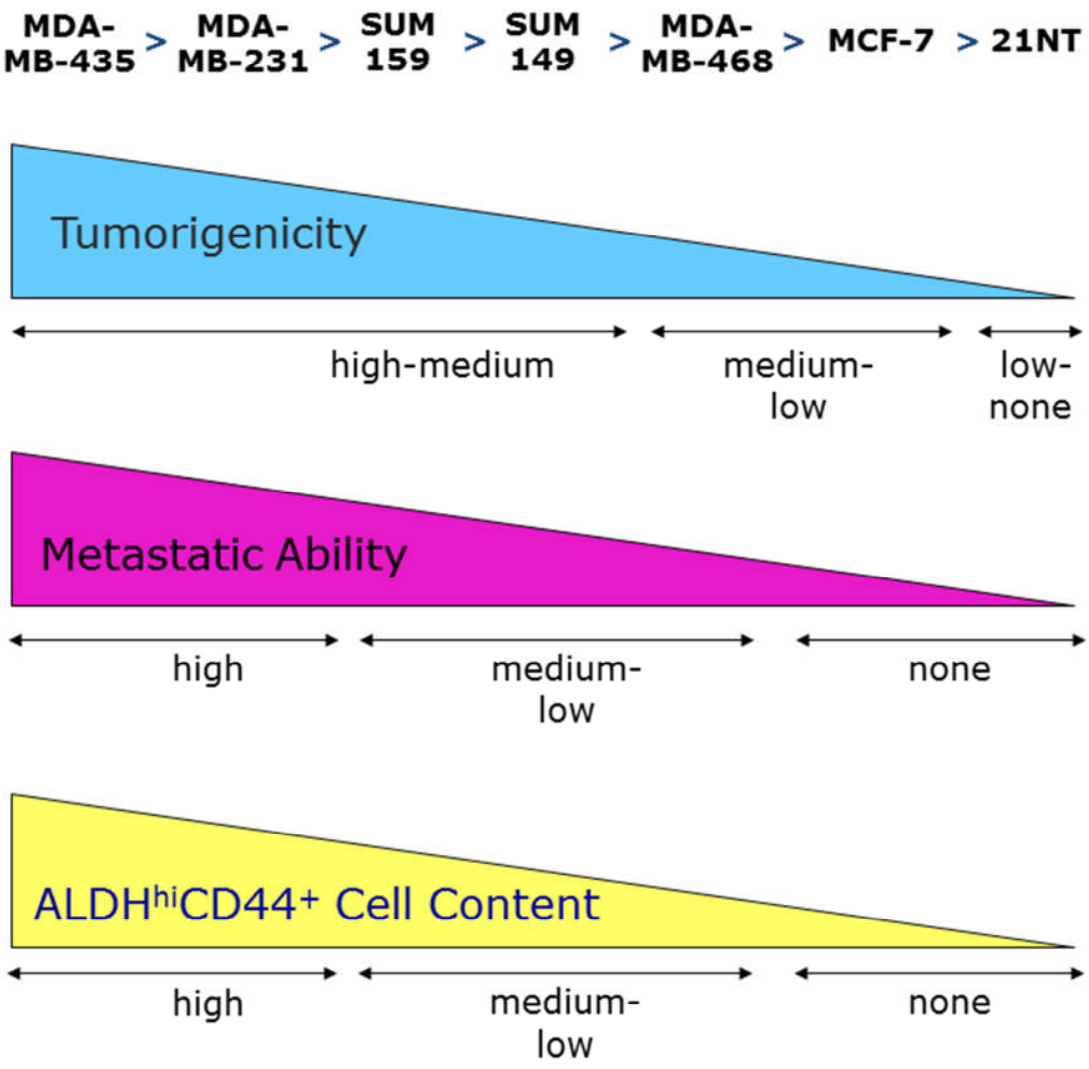

Figure 5.1 ALDH^{hi}CD44^{+} cell content relates to breast cancer cell line aggressiveness. Seven different breast cancer cell lines with differing tumorigenic and metastatic abilities were analyzed for ALDH^{hi}CD44^{+} cell content. Interestingly, cell lines with the highest ALDH activity and CD44 expression (highest ALDH^{hi}CD44^{+} cell content) were the most aggressive and metastatic cell lines. Conversely, the cell lines with the lowest (or no) ALDH^{hi}CD44^{+} cell content were the least tumorigenic and metastatic cell lines. These results suggest that the ALDH^{hi}CD44^{+} cell content relates to breast cancer cell line aggressiveness.

simpler. We believed that whether a cell could initiate growth in the secondary site would depend on the cell itself and whether or not the cell contained stem-like properties. Since there is usually only a small population of stem-like cells in a breast tumor[32,33], of the number of cells that escape the primary tumor, only a small percentage of these cells might be capable of initiating metastases.

Collectively, our data suggests that $ALDH^{hi}CD44^{+}$ cells may represent these successful metastasis-initiating cells in breast cancer. $ALDH^{hi}CD44^{+}$ breast cancer cells exhibited increased proliferation, adhesion, migration, and invasion *in vitro*. Furthermore, in an experimental metastasis model of breast cancer, we injected either $ALDH^{hi}CD44^{+}$ cells or $ALDH^{low}CD44^{-}$ cells into the tail vein of immunocompromised mice and observed that $ALDH^{hi}CD44^{+}$ cells successfully metastasized not only to the lung, but also to the pancreas, liver, spleen, and kidney. In contrast, $ALDH^{low}CD44^{-}$ cells formed either tiny microscopic metastases or no metastases in the lung, and they were not able to metastasize to any other organ. We then injected $ALDH^{hi}CD44^{+}$ cells or $ALDH^{low}CD44^{-}$ cells into the mammary fat pad of immunocompromised mice and investigated primary tumor initiation and metastatic capacity. $ALDH^{hi}CD44^{+}$ cells formed larger primary tumors and metastatic tumors in the lungs compared to $ALDH^{low}CD44^{-}$ cells, and only $ALDH^{hi}CD44^{+}$ cells were also able to metastasize to other distant organs such as the spleen and liver.

These results indicate that $ALDH^{hi}CD44^{+}$ breast cancer cells not only have tumor-initiating capacity, but also metastasis-initiating (and maintaining) abilities. This supports the idea that metastasis is inefficient not necessarily solely because of harsh microenvironmental conditions, but also because only certain cells possess the ability to initiate and sustain metastatic growth in a secondary site. This has interesting clinical implications for identifying risk factors for metastasis in patients. It has previously been observed that high ALDH expression in primary breast tumors is correlated with worse patient prognosis[18,19], and this supports the idea that $ALDH^{hi}$ cells are successful metastasis-initiating cells. This would suggest that patients who

have high ALDH expression/activity in their primary tumors may require a more aggressive treatment approach, and that targeting and eliminating $ALDH^{hi}CD44^{+}$ cells may be imperative for blocking metastasis in breast cancer patients.

5.2.3 $ALDH^{hi}CD44^{+}$ cells play a functional role in breast cancer therapy resistance

Therapy failure in the metastatic setting is the main cause of breast cancer related deaths [3]. Subsequent to our findings that $ALDH^{hi}CD44^{+}$ cells played an important role in breast cancer metastasis, we next wanted to investigate whether these cells were also resistant cells that contributed to therapy failure and relapse. We determined that $ALDH^{hi}CD44^{+}$ cells were more resistant to both chemotherapy (anthracyclines and taxanes) and radiation. In addition, $ALDH^{hi}CD44^{+}$ cells expressed higher levels of many therapy-resistance proteins (p-glycoprotein, GSTpi, and/or CHK1) relative to $ALDH^{low}CD44^{-}$ cells, providing additional resistance to therapy-induced cytotoxicity. Our findings are consistent with other studies demonstrating that tumor-initiating cells in various cancers are resistant to chemotherapy[34-37] or radiation[38-40]. Taken together with our previous work[33], the results of this study suggest a challenging paradigm whereby cells that initiate primary breast tumors may ultimately also become the cells that both initiate distant metastases and contribute to treatment resistance. It is therefore imperative to target $ALDH^{hi}CD44^{+}$ cells directly or somehow sensitize these cells to therapy.

Targeting ALDH seemed like a reasonable place to start since it has been demonstrated that ALDH1 expression increases in primary breast tumors following chemotherapy[35,37], and that high ALDH1 expression correlates with poor patient outcome[19]. Furthermore, ALDH activity has been shown to render cancer cells exquisitely resistant to cyclophosphamide chemotherapy[41-45], although it has been unclear until this point whether ALDH activity could also protect cells from other chemotherapeutics and/or from radiation. Additionally, ALDH activity plays a well-characterized role in cell differentiation through its function in the RA pathway, and it is possible that through differentiation, resistant cells could become more sensitive to treatment[19]. When

$ALDH^{hi}CD44^{+}$ cells were pre-treated with either DEAB (a direct inhibitor of ALDH activity) or ATRA (a differentiation agent that indirectly reduces ALDH expression), they became significantly more sensitive to chemotherapy or radiation therapy, in most cases to the same level as the $ALDH^{low}CD44^{-}$ cells. Interestingly, however, only DEAB-treated cells were able to maintain this sensitization over the long-term, suggesting that specifically blocking ALDH activity may be key to sensitizing resistant cells to therapy.

In an attempt to determine whether blocking ALDH caused any other changes in the cellular drug resistance protein arsenal, we looked at expression of other DRPs in $ALDH^{hi}CD44^{+}$ cells following DEAB or ATRA treatment. It has been previously reported that $ALDH^{hi}$ cells in Ewing's sarcoma co-expressed ABCB1, and that it was this drug transporter that contributed to the therapy resistance seen in $ALDH^{hi}$ cells[23]. In our study, however, it was determined that ATRA or DEAB treatment caused no change in classical DRP markers, suggesting that the therapy resistance seen in $ALDH^{hi}CD44^{+}$ cells may be due to ALDH-specific mechanisms. This further supports the premise that targeting ALDH in high ALDH-expressing tumors could be of therapeutic benefit.

5.2.4 ALDH1A1 and ALDH1A3 have differential functional effects in mediating breast cancer metastasis and therapy resistance

ALDH enzymes are involved in detoxification and/or bioactivation of various intracellular aldehydes to the corresponding acids in a $NAD(P)^{+}$-dependent manner. Aldehydes are highly reactive electrophilic compounds that have a long lifespan, and while they can play a vital role in physiological processes, they also have the capacity for mutagenic, carcinogenic, and cytotoxic detrimental effects[46-48]. In particular, the ALDH1 family of enzymes plays an important role in oxidizing vitamin A (retinal) to retinoic acid (RA) through an alcohol intermediary. RA functions as a ligand for nuclear retinoid receptors and leads to transactivation and transrepression of target genes, and is finally degraded by cytochrome P450 enzymes (CYP26)[49]. In cancer, ALDH1 expression has also been correlated with worse patient prognosis, and high ALDH

activity is now often used to identify aggressive stem-like cells in cancer[18,19]. For this reason, we hypothesized that ALDH1 was not simply a marker of aggressive cancer cells, but that it was, in fact, functionally contributing to the aggressive and metastatic phenotype of these cells.

Two ALDH1 isozymes (ALDH1A1 and ALDH1A3) were investigated for contribution to metastatic behavior and/or therapy resistance. We had previously demonstrated that blocking ALDH activity with DEAB caused an increase in sensitivity of $ALDH^{hi}CD44^{+}$ cells to both chemotherapy and radiation. When ALDH1A1 was knocked down in breast cancer cells, there was a similar sensitivity to chemotherapy and radiation as that seen with DEAB. In contrast, when ALDH1A3 was knocked down, there was no change observed in therapy resistance. This indicates that only specific ALDH1 isozymes are responsible for the therapy resistance observed in $ALDH^{hi}CD44^{+}$ breast cancer cells. This could have important consequences in designing novel therapeutic strategies for cancer treatment.

Interestingly, the different ALDH1 isozymes also seemed to play differing roles in mediating different metastatic behaviours. ALDH1A1 knockdown caused a decrease in cellular proliferation, adhesion, migration, and invasion compared to control cells; whereas ALDH1A3 knockdown caused no changes in proliferation or invasion, and actually increased cellular adhesion and migration. In both cases, however, there was a significant decrease in the formation of micrometastases that were able to form *in vivo*. These results suggest that both ALDH1A1 and ALDH1A3 may play a role in breast cancer metastasis; however, these roles are likely distinct from each other. In the case of ALDH1A1, it seems that $ALDH1A1^{low}$ cells may experience difficulties in getting to a secondary site since adhesion, migration, and invasion abilities are important properties of metastatic cells. Additionally, since $ALDH1A1^{low}$ cells demonstrated a decreased ability to proliferate; therefore, initiating and maintaining aggressive cell growth by $ALDH1A1^{low}$ cells at a secondary site may be limited. In the case of ALDH1A3, it would seem more likely that the decreased metastatic ability of $ALDH1A3^{low}$ cells would stem from difficulties in establishing a tumor at the secondary site. Since $ALDH1A3^{low}$ cells

demonstrate an increase in both adhesion and migration, and no change in invasion compared to control cells, it seems unlikely that these cells would encounter difficulties in arriving at a secondary site. Because there was no change observed in proliferation compared to controls, it is surprising that ALDH1A3^{low} cells would have difficulty proliferating at a secondary site, but perhaps ALDH1A3 aids in the initiation of metastatic tumors. It is also possible that ALDH1A3 is essential for survival in a new microenvironment. Future investigation of the isozyme-specific role of ALDH1 in metastasis and therapy resistance is required in order to answer these questions.

5.3 Possible limitations to the study

The results presented in this thesis represent an important contribution to the cancer stem cell literature in breast cancer; however, as with every study, there are possible limitations to the described work. These limitations are discussed below.

In Chapter 2, we wanted to discern whether breast cancer cell lines could be a suitable model to study stem-like breast cancer cells. This was an important goal for us because cell lines are much more readily available than primary patient samples. We found that there were populations of cells that expressed CSC markers in breast cancer cell lines, and that these cells were more tumorigenic and more metastatic than cancer cells lacking these markers, thus validating the use of cell line models in the study of CSCs. We therefore used breast cancer cell lines throughout this thesis; however, cell lines as a model system are not without their limitations. It would have been interesting and important to compare our results from cells lines to results from using primary breast tumor samples; however, obtaining the amount of tumor tissue necessary to repeat all the described experiments in primary samples would have been difficult.

When we isolated stem-like breast cancer cells, we used the traditional $CD44^+CD24^-$ phenotype, but we also wanted to use a normal stem cell marker that had a functional component as opposed to simply being a cell surface marker. We therefore included the use of ALDH activity such that we isolated $ALDH^{hi}CD44^+CD24^-$ cells from different breast cancer cell lines. These $ALDH^{hi}CD44^+CD24^-$ cells showed increased tumorigenic and metastatic abilities in our hands. Interestingly, Ginestier et al (2007)[19] demonstrated that $CD44^+CD24^-$ cell populations (6.08% of total tumor) and $ALDH^+$ cell populations (4.34% of total tumor) overlap only slightly, with a very small proportion (~1%) of total tumor being comprised of $ALDH^{hi}CD44^+CD24^-$ cells. Importantly, $ALDH^{hi}CD44^+CD24^-$ cells were shown to be the most tumorigenic population, supporting our use of this phenotype to isolate metastasis-initiating cells. However, we did not examine the cellular hierarchy in our breast cancer cell lines to determine which population ($CD44^+CD24^-$ cells vs. $ALDH^{hi}$ cells vs. $ALDH^{hi}CD44^+CD24^-$ cells) was the most tumorigenic/metastatic in our model system.

In all of our experiments in Chapters 2 and 3, we studied $ALDH^{hi}CD44^+$ (stem-like) and $ALDH^{low}CD44^-$ (non-stem-like) breast cancer cells in isolation in order to compare their metastatic and therapeutic resistance capabilities. However, there have been some recent studies suggesting that these populations may work in tandem. For example, Emminck et al. (2011) found that the non-stem-like cells in colorectal cancer actually formed a type of stem cell niche for the stem-like cancer cells which protected them from therapy[50]. It would have been interesting to determine whether there was some kind of functional relationship between the $ALDH^{hi}CD44^+$ and $ALDH^{low}CD44^-$ cell populations in our studies. Also, it would have been interesting to determine the role (if any) of the intermediate cells in the breast cancer cell lines (i.e. the 80% of cells “left over” following FACS).

For our *in vivo* experiments, we used NOD/SCID and NOD/SCID/IL2γR null mice, which are severely immunocompromised mice. Immunocompromised mice are commonly used in cancer studies so that human cancer cells can be injected into mice and not be targeted and

eliminated by the mouse immune system. Additionally, xenotransplantation into immunocompromised mice has been used to demonstrate the “stemness” of normal stem cells and CSCs[51]. There has been some controversy in the CSC literature that the CSC hypothesis has only been supported because of the use of immunocompromised mouse models; that the low frequency of tumor-initiating cells may be an artifact of the model instead of the reality of tumorigenesis in humans[52,53]. The growth of human tumor cells in the mouse environment is a complex process. The growth of tumor cells requires a group of support cells such as fibroblasts, endothelial cells, macrophages, and mesenchymal cells. The cytokines and receptors that coordinate these interactions are mostly incompatible between human and mouse[51,54]. Therefore, it is possible that the phenotypic association with tumor formation in a xenograft model may represent the ability to obtain stromal support in a foreign environment, instead of the ability for self-renewal[51]. Additionally, immunosuppressing animals has been shown to result in more efficient tumor growth, regardless of stem cell abilities[53]. A study by Quintana et al. (2008) demonstrated that xenotransplantation assay conditions can increase the detection of tumorigenic melanoma cells by several orders of magnitude[53]. In limiting dilution assays, approximately 25% of unselected melanoma cells from 12 different patients, including cells from primary and metastatic melanomas, were able to form tumours in immunocompromised mouse models[53]. Also, while we did not look at CSC markers in patient samples, other groups have shown that expression of CSC markers in patient tumors is correlated with worse patient prognosis and a higher incidence of metastasis, supporting the idea that ALDH1 activity and CD44 expression identify aggressive and metastatic tumor cells[18,19,55] even in human patients with intact (albeit not always robust) immune systems.

“Humanized mouse models” have been developed over the past 2 decades in order to study the human immune system for various reasons (i.e. transplantation, vaccine development, autoimmunity)[56-59]. NOD/SCID mice are injected with human $CD34^+$ HSCs, resulting in systemic reconstitution of virtually all mouse tissues with human-derived hematopoietic cells[57,58]. Mice

with humanized immune systems are beginning to be used to study tumor initiation and metastasis with varying degrees of success[58,60]. While humanized mouse models represent a giant leap forward in solving the NOD/SCID tumor model, they are not without limitations. Most humanized mouse models exhibit an abundance of B cells, but lack the HLA class I and II expression in mouse thymus, which is required to support the selection of T cells, resulting in a lack of T cells in these mice[56,57]. Despite this, mice with humanized immune systems represent an interesting model in which to study human cancer cells *in vivo*, and it would be worthwhile to determine whether the results we obtained using immunocompromised mice would be similar to results obtained using mice with a competent human immune system; however, at present, this model is not ready for practical use.

In our therapy studies, we used 5μM of ATRA in order to attempt to induce differentiation or reduce ALDH expression in breast cancer cells. In clinical settings, 5μM translates to a very high dose (about 5 times higher than is normally used) and would probably lead to high toxic side effects resulting in retinoid syndrome[61]. In our studies, we were simply looking to establish proof-of-principle, but if a combination therapy regimen of ATRA + chemotherapy were ever considered, it would have first be determined that a much lower dose of ATRA were capable of sensitizing $ALDH^{hi}CD44^{+}$ cells to therapy in pre-clinical model systems.

Finally, in Chapter 4 we used a transient transfection approach to reduce levels of ALDH1A1 and ALDH1A3 in our breast cancer cells. Ideally, we would have liked to have generated stable cell lines with decreased ALDH1A1 or ALDH1A3 expression which might have given us more reliable and reproducible results. Also, stably transfected cell lines with sustained knockdown of proteins of interest can be injected into a mouse model for long term tumorigenicity and metastasis studies, as opposed to the chick CAM model used in our study, which was chosen because it has a much more rapid *in vivo* read-out. We did attempt to generate stable cell lines, and were able to stably transfect both MDA-MB-231 and MDA-MB-468 cells with ALDH1A1 siRNA; however, the protein levels of ALDH1A1 did not decrease (in

the case of MDA-MB-231 cells) or did not decrease to low enough levels to cause changes in behavior (MDA-MB-468 cells). In the future, we would like to try and target ALDH1A1 and ALDH1A3 using a lentivirus-mediated knockdown approach.

5.4 Future directions

The work presented in this thesis is far from completed, and the limitations described above, along with the questions that the studies presented here have left us with illustrates the exciting challenges that lie ahead.

The work presented in this study used breast cancer cell lines, and while it was important to demonstrate that cell lines were a useful model to study stem-like breast cancer cells, it will also be important to validate our results in breast cancer patient samples. While this has already been done by other groups using the $ALDH^{hi}$ phenotype[18,19], it would be interesting to incorporate the use of ALDH/CD44 expression in breast cancer patient tumor samples as a biomarker in future clinical trials in order to determine whether the presence of $ALDH^{hi}CD44^{+}$ cells correlates with increased metastatic risk and/or therapy resistance/failure.

Furthermore, in this thesis, we focused on the role of ALDH in breast cancer metastasis and therapy resistance; however, there is growing evidence that suggests that CD44 can also play a role in metastasis, and possibly even therapy resistance[62-67]. There are currently ongoing projects in our laboratory that are investigating the role of CD44 in breast cancer metastasis and therapy resistance.

Another attractive question that stems from ongoing studies in our laboratory has to do with the seed and soil hypothesis of metastasis. It would be interesting to determine whether $ALDH^{hi}CD44^{+}$ cells are highly metastatic regardless of which organ they metastasize to, or whether they display organ tropism based on interactions with the microenvironment of the secondary site. Ongoing projects in our lab are attempting to address this important question.

In terms of the ALDH1A1/ALDH1A3 experiments discussed in this thesis, it will be crucial that stable cell lines with reduced ALDH1A1 or ALDH1A3 levels be developed in the future in order to determine the metastatic potential of these cells in a mouse model. Furthermore, with stable cell lines it will be easier to investigate the actual mechanisms by which ALDH1A1/ALDH1A3 are mediating breast cancer metastasis and therapy resistance, which will be important in identifying any therapeutic strategies to target ALDH1 in breast cancer patients.

It would also be interesting to investigate the possible role(s) other ALDH isoforms play in breast cancer metastasis and/or therapy resistance. So far in breast cancer, only ALDH1 has been associated with poor patient prognosis; however, ALDH7A1 was shown to play a major role in prostate cancer metastasis, indicating that isoforms other than simply ALDH1 may be involved in metastasis.

Finally, the retinoic acid pathway has become a very prominent pathway in carcinogenesis, especially since the discovery of the dual effects of RA signaling resulting from the activation of two different nuclear receptors[68]. One of the main physiological roles of ALDH1 is to catalyze the development of RA from vitamin A. RA can then signal through either CRABP2 to induce differentiation and growth arrest, or FABP5 to induce cell survival and proliferation. FABP5 expression in triple negative breast cancer has been correlated with poor patient survival[69]. Furthermore, a higher expression of FABP5 in glioblastomas was shown to correlate with shorter survival times and a higher proliferation index, indicating that FABP5 may play an important role in tumorigenesis[70]. It was our hypothesis that ALDH1A1/ALDH1A3 would play a role in metastasis and/or therapy resistance through a RA-mediated mechanism. While we found that this was probably not the case, the role that RA signaling may play in metastasis and/or therapy resistance would be interesting to study and could result in potentially important therapeutic strategies to target metastatic disease.

5.5 Final conclusions

Throughout this project, we were able to uncover novel and important knowledge regarding breast cancer metastasis and the therapy resistance associated with metastatic disease. Firstly, we established that breast cancer cell lines are a useful tool to study stem-like cells in cancer. This was surprising as most groups assumed that cell lines were simply clonogenic cell populations. We were the first group in literature to show that selection and isolation of stem-like breast cancer cells on the basis of ALDH activity can enhance for functional cell properties that contribute to metastasis. Based on the high rate of therapy failure in the metastatic setting, we also asked whether our current cancer therapies are, in fact, targeting the right cells, and discovered that they are not. Overall, this thesis suggests that there is a population of aggressive, stem-like $ALDH^{hi}CD44^{+}$ cells that are responsible for tumor initiation, metastatic disease, and therapy resistance in breast cancer. It is therefore imperative to target these cells in order to successfully treat breast cancer. At the present time, our current cancer therapies are ineffective in eradicating $ALDH^{hi}CD44^{+}$ cells, potentially due to the high expression of DRP in the $ALDH^{hi}CD44^{+}$ cellular compartment, but also because of high ALDH activity. In addition, it was discovered that two ALDH1 isozymes play an important role in metastatic progression and therapy resistance. This will have important consequences in the struggle to target and eliminate the resistant $ALDH^{hi}CD44^{+}$ cells. Our results suggest that ALDH1 may make an excellent therapeutic target; however, this will have to be done carefully since ALDH1 is an essential enzyme found in most stem and progenitor cells. ALDH1 is also essential in detoxifying aldehydes and in the formation of biologically important molecules such as RA. Carefully designed future clinical studies aimed at determining the feasibility, safety, and efficacy of targeting ALDH1 in breast cancer patients will be imperative to determine if the knowledge gained in this thesis can be successfully translated to the clinic.

5.6 References

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Appendices

Appendix 1. Animal Protocol Approval for Chapter 2

Western

12.19.07
*This is the 1st Renewal of this protocol
*A Full Protocol submission will be required in 2010

Dear Dr. **Hess**

Your Animal Use Protocol form entitled:

Characterization of the angiogenic potential of aldehyde dehydrogenase expressing stem cells from human bone marrow.

has been approved by the Animal Use Subcommittee.
This approval is valid from **01.01.08 to 12.31.08**
The protocol number for this project remains as **2006-126-12**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 1 YEAR **Highest Pain Level:** D

Species	Other Detail	Housing/Use Locations	Animal # Total for 1 Year
Mouse	NOD/SCID/B2 M null	RACF / REB#3	300
Mouse	NOD/SCID/ MPS VII	RACF / REB#3	210
Mouse	NOD/SCID	RACF / REB#3	100
Mouse	NOD/SCID/ IL2 R gamma null	RACF / REB#3	100

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol - D Hess, K Levac, M Pickering
Approval Letter - K Levac, M Pickering

The University *of* Western Ontario
Animal Use Subcommittee/University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA - N6A 5C1
PH: 519-661-2111 ext. 86770 • F: 519-661-2028 • www.uwo.ca/animal

Appendix 2. Animal Protocol Approval for Chapter 3

Western

09.14.09

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2013

Dear Dr. Allan:

Your Animal Use Protocol form entitled:

Role of ALDH+/CD44+ stem-like cells in breast cancer progression and treatment

Funding Agency Ontario Institute for Cancer Research - OICR Project#08Nov-230

has been approved by the University Council on Animal Care. This approval is valid from **09.14.09 to 09.30.10.**

The protocol number for this project is **2009-064**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 4 Years

Species	Strain	Other Detail	Pain Level	Animal # Total for 4 Years
Mouse	Various As outlined in protocol	Female	D	700

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. D Goodale, K Bothwell, P Coakwell

The University *of* Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86770 ● FL 519-661-2028 ● www.uwo.ca / animal

Curriculum Vitae

Education Background

- PhD Candidate, University of Western Ontario, London, ON, 2007-Current
  Department: Anatomy and Cell Biology
  Supervisor: Dr. Alison Allan
  Thesis Topic: $ALDH^{hi}CD44^{+}$ cells in breast cancer metastasis and therapy resistance

- Bachelor of Science, University of Western Ontario, London, ON 2002-2007
  Program: Honors Specialization in Biology

Scholarships/Awards

National

- Banting and Best CIHR Doctoral Fellowship - $105,000, London, ON, 2009-2012
- Canadian Breast Cancer Foundation Fellowship - $27,000, London, ON 2008-2009

Provincial

- Ontario Graduate Scholarship - $15,000 (declined), London, ON, 2008-2009

Local

- CIHR-Cancer Research and Technology Transfer Fellowship, $11,800, London, ON, 2007-2008
- Translational Breast Cancer Research Unit Fellowship, $11,800, London, ON, 2007-2008
- Graduate Thesis Research Award, $1500, London, ON, 2008
- Best Platform Presentation 2012, London Health Research Day ($600)
- Best Platform Presentation 2010, Lawson Research Day ($400)
- Best Poster Presentation 2008 Oncology/CIHR-CaRTT Research and Education Day
- Best Poster Presentation 2009 Oncology/CIHR-CaRTT Research and Education Day
- Best Poster Presentation 2010 Oncology/CIHR-CaRTT Research and Education Day
- Altmann Research Award 2008 Department of Anatomy and Cell Biology ($100)
- Altmann Research Award 2010, Department of Anatomy and Cell Biology ($100)
- Best Poster Presentation 2012 – Honorable Mention, Canadian Student Health Research Forum, Winnipeg, MB June 12^{th}-14^{th}
- Best 4^{th} Year Thesis Award 2007, “Biology Day” UWO, London, ON ($100)
- Best Poster Presentation 2005, Ontario Veterinary College Summer Leadership Program, Guelph, ON ($100)
- Community Involvement Scholarship, GTA Union, London, ON, 2012 ($400)
- Western Humanitarian Award 2011 Nominee, London, ON
- CIHR Synapse Mentor Award 2012 Nominee
- Morris Kroll Memorial Scholarship in Cancer Research 2011, London, ON ($500)

- Lawson Leadership Award 2009, London, ON ($400)
- Lawson Leadership Award 2011, London, ON ($400)
- Schulich Travel Scholarship 2007 ($1500)
- Schulich Graduate Scholarship 2007-2012 ($7,000/year)

Publications

Peer-Reviewed Publications

- **Croker AK** & Allan AL. Inhibition of aldehyde dehydrogenase (ALDH) activity reduces chemotherapy and radiation resistance of stem-like $ALDH^{hi}CD44^{+}$ human breast cancer cells. *Breast Cancer Res Treat.* 2012, 133(1):75-87

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley BD, Hess DA, and Allan AL. High aldehyde dehydrogenase activity and expression of cancer stem cell markers selects for stem-like breast cancer cells with enhanced malignant and metastatic properties. *J Cell Mol Med.* 2009, 13(8b), 2236-2252.

- **Croker AK** & Allan AL. Cancer Stem Cells: Implications for the progression and treatment of metastatic disease. *J Cell Mol Med* 2008; 12:374-90.

Invited Book Chapters

- **Croker AK** & Allan AL. (2010). Chapter 23: Future Directions → Cancer Stem Cells as Therapeutic Targets. In: Allan, A.L., ed. *Cancer Stem Cells in Solid Tumors.* Springer Press, New York, NY 2011:403-429.

- **Croker AK***, Townsend J*, Allan AL, Chambers AF. (2009). Chapter 21: Tumor dormancy, metastasis, and cancer stem cells. Book title: Cancer and Stem Cells. Editors: Rebecca G. Bagley, Beverly A. Teicher.

National/International Meetings

- **Croker AK** & Allan AL. (2012). Functional role of aldehyde dehydrogenase (ALDH-1) in breast cancer metastasis and therapy resistance. Canadian Student Health Research Forum. Winnipeg, MB. June 12-14 2012 [Abstract/Poster Presentation]
  Selected to represent the Schulich School of Medicine and Dentistry at this national conference

- **Croker AK** & Allan AL. (2011). Functional role of aldehyde dehydrogenase (ALDH-1) in breast cancer metastasis and therapy resistance. Canadian Cancer Research Alliance – Canadian Cancer Research Conference. Toronto, Ontario. November 27-30 2011 [Abstract/Poster Presentation]

- **Croker AK** & Allan AL. (2010). Aldehyde dehydrogenase (ALDH) mediates chemotherapy and radiation resistance of stem-like $ALDH^{hi}CD44^{+}$ human breast cancer cells. 22nd Annual EORTC-NCI-AACR Symposium on Molecular Targets and Cancer Therapeutics, Berlin, Germany. Nov 16-19 2010 [Abstract/Poster Presentation]

- **Croker AK** & Allan AL. (2009). All-trans retinoic acid (ATRA) reduces chemotherapy and radiation resistance of stem-like $ALDH^{hi}CD44^{+}$ breast cancer cells. AACR Advances in Breast Cancer Conference, San Diego, CA. Oct 13-16 2009. [Abstract/Poster Presentation]

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley B, Hess D, & Allan AL. (2008). High aldehyde dehydrogenase activity and CD44 expression selects for stem-like breast cancer cells with enhanced malignant and metastatic activity. AACR Cancer Stem Cell Conference, Los Angeles, USA. February 12-15, 2008. [Abstract/Poster Presentation]

Local Meetings

- **Croker AK** & Allan AL. (2010). All *trans* retinoic acid (ATRA) reduces chemotherapy and radiation resistance of metastasis initiating $ALDH^{hi}CD44^{+}$ breast cancer cells. Lawson Research Day 2010. [Abstract/Oral Presentation].

- **Croker AK** & Allan AL. (2010). All-trans retinoic acid (ATRA) reduces chemotherapy and radiation resistance of stem-like $ALDH^{hi}CD44^{+}$ breast cancer cells. 7th Annual Oncology/CIHR-CaRTT Research and Education Day, the University of Western Ontario [Abstract/Poster Presentation]

- **Croker AK** & Allan AL. (2010). All trans retinoic acid (ATRA) reduces chemotherapy and radiation resistance of metastasis initiating $ALDH^{hi}CD44^{+}$ breast cancer cells. Murray Barr/Anatomy and Cell Biology Research Day 2010. [Poster Presentation]

- **Croker AK**, Hess D, & Allan AL. (2009). The effect of stem-like breast cancer cells on response to therapy. 6th Annual Oncology/CIHR-CaRTT Research and Education Day, the University of Western Ontario [Abstract/Poster Presentation]

- **Croker AK**, Goodale D, Hess D, & Allan AL. (2009). The effect of stem-like $ALDH^{hi}CD44^{+}$ breast cancer cells on response to therapy. Margaret Moffat Research Day 2009, UWO. [Poster Presentation]

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley B, Hess D, & Allan AL. (2008). High aldehyde dehydrogenase activity and CD44 expression selects for stem-like breast cancer cells with enhanced malignant and metastatic activity. Margaret Moffat Research Day, University of Western Ontario. [Abstract/Poster Presentation]

- **Croker AK**, Hedley B & Allan AL (2007). The effects of cancer stem cells on breast cancer malignancy and metastasis. Murray Barr Lecture/Anatomy and Cell Biology Research Day, the University of Western Ontario. [Poster presentation].

- **Croker AK**, Rosenthal T, & Friendship RM. (2005). A cross-sectional study of the prevalence of *Yersinia enterocolitica* and *Salmonella* in different age groups of swine. Ontario Veterinary College Summer Leadership Program Poster Competition. [Poster presentation].

Platform Presentations

- **Croker AK** & Allan AL. (2012). Functional role of aldehyde dehydrogenase-1 (ALDH1) in breast cancer metastasis and therapy resistance. 10th Annual Oncology Research and Education Day, Western University, London, ON.

- **Croker AK** & Allan AL. (2012). Functional role of aldehyde dehydrogenase-1 (ALDH1) in breast cancer metastasis and therapy resistance. Lawson Research Day 2012, Western University, London, ON.

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley B, Hess D, & Allan AL. (2011). The effect of stem-like ALDH^{hi}CD44^{+} breast cancer cells on breast cancer metastasis and response to therapy. Anatomy and Cell Biology Seminar Series, Western University, London, ON.

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley B, Hess D, & Allan AL. (2010). The effect of stem-like ALDH^{hi}CD44^{+} breast cancer cells on breast cancer metastasis and response to therapy. Introductory Lecture ANATCELL 9520: Scientific Interchange, Western University, London, ON.

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley B, Hess D, & Allan AL. (2010). The effect of stem-like ALDH^{hi}CD44^{+} breast cancer cells on breast cancer metastasis and response to therapy. CRLP Student Seminar Series, London Regional Cancer Program, London, ON.

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley B, Hess D, & Allan AL. (2009). The effect of stem-like ALDH^{hi}CD44^{+} breast cancer cells on breast cancer metastasis and response to therapy. Introductory Lecture Biology 9313: Cancer Stem Cells, Western University, London, ON.

- **Croker AK** & Allan AL. (2009). The effect of stem-like breast cancer cells on response to therapy. CBCF-Run for the Cure Kickoff Campaign, Collingwood ON. June 21st, 2009.

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley B, Hess D, & Allan AL. (2009). The effect of stem-like $ALDH^{hi}CD44^{+}$ breast cancer cells on breast cancer metastasis and response to therapy. MDT Clinical Breast Rounds Meeting, LRCP.

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley B, Hess D, & Allan AL. (2009). The effect of cancer stem cells on breast cancer metastasis and response to therapy. CRLP Student Seminar Series, LRCP.

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley B, Hess D, & Allan AL. (2008). The effect of cancer stem cells on breast cancer metastasis and response to therapy. Murray Barr Research Day, UWO.

- **Croker AK**, Goodale D, Chu J, Postenka C, Hedley B, Hess D, & Allan AL. (2008). High aldehyde dehydrogenase activity and CD44 expression selects for stem-like breast cancer cells with enhanced malignant and metastatic activity. 5^{th} Annual Oncology Research and Education Day, University of Western Ontario.

- **Croker, AK** & Allan AL. (2007). The effect of cancer stem cells on breast cancer malignancy and metastasis. The University of Western Ontario Biology Day.

- **Croker, AK** & Allan AL. (2007). The effect of cancer stem cells on breast cancer malignancy and metastasis. Invited speaker for the Owen Sound Public Health Unit Cancer Awareness Meeting.

Teaching Experience

- Teaching Assistant for Medical Science 4900F/G Honors Thesis Course, University of Western Ontario, London, ON, 2009-Current
- Mentor for Partners in Experiential Learning Program
    - Emily Spearing, Grade 11, February-June 2010
    - Meryl Hodge, Grade 12, February-June 2011
    - Nicole DiNardo, Grade 12, February-March 2012

Other Contributions

Leadership Roles

- Sr. Coordinator for Let's Talk Science at Western, London, ON, 2008-Current
- Board Member, Women's Community House, London, ON, 2009-Current (Elected to the executive committee, position of secretary, 2012)
- Chair of Fund Development Committee, Women's Community House, London, ON, 2012-Current
- Research Representative, Rose in My Book Committee, London, ON, 2010-2012
- Outreach Coordinator, Translational Breast Cancer Research Unit, London, ON, 2010-Current

- Elected to position of Off-Campus Representative, Anatomy and Cell Biology Student Council, London, ON, 2010-2012
- Graduate Representative, Schulich School of Medicine and Dentistry Student Council, London, ON, 2010-Current
- Student Representative, Program Advisory Committee-CIHR-CaRTT Program, London, ON, 2009-2010

Community Outreach/Volunteer

- Student Reviewer, Journal of Laboratory Investigation (Impact Factor: 4.405), Editorial Internship Program (2009-Current)
- Bioscience Steward for the GTA Union (2009-2012)
- Lawson Research Day Organizing Committee (2010-2011)
- Facilitator for the Leadership Education Program (2008-2010)
- Captain of VRL's Labatt 24 hour relay team (2008 & 2009)
- "Expert", Consult the Expert Series (Scholarship Help) 2010-Current
- Co-Founder of the Trick or Eat Program at London Regional Cancer Program, London, ON 2008