

## Chapter 2

# Cancer Stem Cells in Breast Cancer

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**Abstract** Breast cancer is one of the leading causes of cancer-related deaths among women worldwide. While it is highly treatable during the primary stages, the disease is often lethal if it successfully metastasizes. Breast cancer stem cells (CSCs) show distinct similarities to normal breast stem cells, have been shown to be the driving force behind primary tumorigenesis, and are postulated to be the cells responsible for metastasis. Many groups have used the CD44<sup>+</sup>CD24<sup>-</sup> and/or ALDH<sup>+</sup> phenotype for breast CSC isolation; however, this definition does not apply to all breast cancers and needs further refining. As CSCs have been shown to be therapy resistant, identification of additional markers will aid in the isolation of a pure CSC population, which can then be used to elucidate effective treatments. This chapter will discuss normal breast stem cells, breast CSC identification, the relationship between normal mammary stem cells and breast CSCs, and the clinical implications of the CSC population in breast cancer.

### Abbreviations

ABCG2    ATP-binding cassette sub-family G member 2  
ALDH     Aldehyde dehydrogenase

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BCRP1	Breast cancer resistance protein 1
BMP	Bone morphogenic protein
BRCA1	Breast cancer susceptibility gene
CD	Cluster of differentiation
CSC	Cancer stem cell
CXCR4	Chemokine receptor 4
DCIS	Ductal carcinoma in situ
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
ER	Estrogen receptor
ESA	Epithelial specific antigen
HA	Hyaluronic acid
HER2	Human epidermal growth factor receptor 2
HSC	Hematopoietic stem cell
IHC	Immunohistochemistry
LCIS	Lobular carcinoma in situ
Lin	Lineage
MaSC	Mammary epithelial stem cell
MDR1	Multi drug resistance pump 1
MMTV	Mouse mammary tumor virus
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NOD/SCID	Non-obese diabetic/severe combined immune deficiency
PR	Progesterone receptor
RA	Retinoic acid
RAR	Retinoic acid receptor
RXR	Retinoid X receptor
SDF	Stromal derived factor
TGF- $\beta$	Transforming growth factor beta
T-IC	Tumor-initiating cells

## 2.1 Breast Cancer

### 2.1.1 Statistics

Excluding nonmelanoma skin cancers, breast cancer is the most frequently diagnosed cancer and the second highest cause of cancer-related deaths among both Canadian and American women [1, 2]. On a global scale, breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of total cancer cases and 14% of cancer deaths [3].

### ***2.1.2 Initiation and Disease Progression***

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 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 [4, 5]. 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 in the in situ stage, treatments are highly effective (DCIS) if even necessary (LCIS) [6–8]. Prognosis worsens when the tumor invades adjacent tissues and gains the potential to metastasize. Metastatic disease is the aspect of breast cancer that is responsible for the majority of breast cancer-related deaths.

Breast cancer tumors exhibit two levels of heterogeneity: different tumor subtypes [9, 10] and functional differences at the cellular level within the tumor [11, 12]. Among patients and even among different tumors within the same patient, breast tumor subtype can vary in many ways: through histopathology (i.e., where the tumor is located and the type of cellular morphology), molecular pathology (ER/PR/HER2 status and other cellular markers), and through variability of genetic composition and expression (loss or gain of chromosomal material, oncogene expression, or mutation carriers) [9]. Through the use of gene expression analysis, six breast tumor subtypes have been identified, each having different characteristics and prognosis. These include two unique luminal subtypes (A and B); basal-like; HER2-overexpressing; normal breast-like; and the most recently identified, claudin-low subtype [13–15].

Cell populations that make up individual tumors are not homogenous, but are in fact functionally heterogeneous. The two categories consist of the tumor-initiating cells (T-ICs), capable of tumor propagation and maintenance due to their ability to self-renew, and terminally differentiated cells that are not capable of producing large amounts of progeny and are not capable of tumor propagation [12, 16, 17]. These observed levels of heterogeneity are accounted for by the cancer stem cell (CSC) hypothesis, which postulates that cancers are hierarchically organized stemming from progenitor cells, or CSCs [18]. The hierarchal nature of the tumors mirrors that of the normal breast tissue for which a normal mammary epithelial stem cell (MaSC) has recently putatively been identified in human and murine tissues.

## **2.2 Normal Breast Organization and Mammary Stem Cells**

Recent studies point strongly to the existence of both murine and human MaSCs. Indeed, the dynamic nature of breast development throughout life dictates the need for some type of long-lived progenitor capable of multiple types of differentiation with a large capacity for cellular proliferation. The breast undergoes restructuring

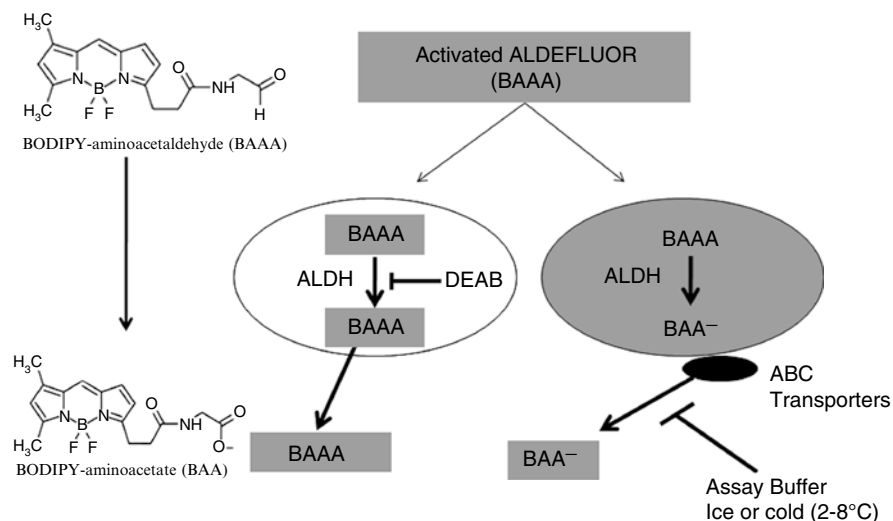
involving proliferation, remodeling, and differentiation in response to hormonal changes during embryogenesis, puberty and pregnancy [4]. A stem cell (defined as a cell capable of unlimited self-renewal and possessing the ability to produce at least one kind of differentiated progeny [19]) is likely the driving force behind this continual remodeling. Unlike pluripotent embryonic stem cells that are able to give rise to all cells of the body, these tissue-specific stem cells are multipotent – they are restricted to producing cells found within the breast tissue.

### ***2.2.1 Support for Normal Murine Mammary Stem Cells***

The first evidence of a potential mammary stem cell was observed by Deome et al. [20]. In their transplantation experiments, a sample of normal mammary tissue was implanted into a cleared mammary fat pad, resulting in outgrowths with normal mammary gland appearance. Further transplantation experiments demonstrated that single cells are capable of re-creating the entire heterogeneity of a mammary gland [21]. Single cell implantation experiments using sorted cells have verified that murine cells depleted of hematopoietic cells ( $\text{Lin}^-$ ) and expressing CD29 and/or CD49f in combination with CD24 are capable of self-renewal and differentiation into the breast cell lineages, forming a functional mammary gland [22, 23].

### ***2.2.2 Support for Normal Human Mammary Stem Cells***

An exact identification of a human mammary epithelial stem cell has yet to be solidified, but many groups have identified putative mammary epithelial progenitor cells. Technical challenges have arisen due to the complex nature of the hormonal requirements for MaSC differentiation and also for a suitable environment to support growth [24]. Work with human breast stem cells builds on the foundations of experiments investigating the murine population. Work by Kuperwasser et al. [24] has resulted in the development of a humanized murine fat pad that more accurately represents the human breast stroma. They demonstrated that fat pad injection with a mixture of irradiated and nonirradiated human mammary epithelial cells allows for the successful engraftment of the stromal cells and for the creation of a humanized environment [24, 25]. More recently, a new model has been described by Eirew et al. [26], whereby fibroblast and putative mammary stem cells are engrafted in a collagen plug under the murine kidney capsule. The outgrowths observed recapitulate the hierarchal nature of the normal human mammary gland. Through the use of these assays,  $\text{CD49f}^{\text{hi}}\text{EpCAM}^-$  has been established as the fraction containing the human breast stem cell population [26, 27]. To complement these cell surface markers, a functional marker, aldehyde dehydrogenase 1A1 ( $\text{ALDH}^+$ ) (Fig. 2.1) has been established as a functional marker for mammary stem cells [28] among others [29].



**Fig. 2.1** The Aldefluor<sup>®</sup> assay. The Aldefluor<sup>®</sup> assay is a fluorometric assay that detects the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1) (StemCell Technologies, Vancouver, BC, Canada). Cells are incubated with the intrinsically fluorescent ALDH substrate, BODIPY-aminoacetaldehyde (BAAA). BAAA is a neutral molecule and enters the cell through passive diffusion, where it is then converted into BAA<sup>-</sup> by ALDH and is unable to leave the cell due to its negative charge. The active removal of BAA<sup>-</sup> by ATP Binding Cassettes is quenched through the use of the assay buffer and through incubation of cells between 2 and 8°C. The resulting fluorescence of the cells is then assessed by flow cytometry, providing single cell analysis of ALDH activity. As a negative control, the activity of ALDH is quenched by the addition of diethylaminobenzaldehyde (DEAB), and the fluorescence of these cells is assessed by flow cytometry. The population observed in the DEAB sample is used to create the gate for the ALDH<sup>+</sup> cells, whereby cells are only included if they demonstrate higher levels of fluorescence compared to the DEAB sample. Adapted from StemCell Technologies ([www.stemcell.com](http://www.stemcell.com))

## 2.3 Identification of Cancer Stem Cells in Breast Cancer

The first identification of a CSC in solid tumors came from the work of Al-Hajj et al. [30] using cells isolated from pleural effusions and primary tumors of breast cancer patients. Cells with an ESA<sup>+</sup>CD44<sup>+</sup>CD24<sup>-/low</sup>Lin<sup>-</sup> phenotype were capable of forming tumors in numbers as low as 100 when injected into the mammary fat pad of nonobese diabetic/severe combined immune deficiency (NOD/SCID) mice, while tens of thousands of cells from other populations were nontumorigenic. Further work by Ginestier et al. [28] identified a small subset of CD44<sup>+</sup>CD24<sup>-</sup> cells which were ALDH<sup>+</sup> and were able to initiate tumor formation in NOD/SCID mice with as few as 20 cells injected. These cells recreated the heterogeneity of the initial tumor, exhibiting nontumorigenic populations in addition to the tumorigenic cells. This recapitulation could be repeated upon serial passaging in naïve NOD/SCID mice, demonstrating both differentiation and self-renewal potential [28]. The presence of ALDH expressing cells in tumors has been correlated with poor prognosis in breast

cancer patients [28, 31, 32]. Additionally, the CD44<sup>+</sup>CD24<sup>-</sup> population appears to be enriched in basal-like tumors (ER, PR, HER2 negative) and in BRCA1 tumors [33], both of which have been associated with poor patient prognosis [34, 35]. The presence of a CSC population has also been verified in breast cancer cell lines and primary tumor samples [36].

Due to the functional stem cell-like characteristics of these cells, the term “cancer stem cell” is a fitting descriptor. However, it does not mean that these cells are indeed stem cells re-wired, although they may be. A consensus on the definition of CSCs was created by the leaders in the field to be “a cell within a tumor that possess the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor” [37]. It is hypothesized that CSCs arise either from a normal tissue stem cell that has acquired mutations that make it tumorigenic or from a more differentiated progenitor or mature cell that has dedifferentiated and acquired the ability to self-renew in addition to the tumorigenic mutations. While the described phenotype is not an absolute definition of the breast CSC population, it provides a basis for further work.

## 2.4 Markers Used to Identify CSCs

In order to elucidate the functions and the populations of CSCs within solid tumors, the phenotypic definition of a CSC must first be established. Selectable markers are either found on the cell surface or confer functional properties that are characteristics of normal stem cells that have extended to malignant stem cell populations. As previously mentioned, the current definition of a breast CSC is CD44<sup>+</sup>CD24<sup>-</sup> and/or ALDH<sup>+</sup>. In the following section, these markers and other putative CSC markers will be discussed.

### 2.4.1 *CD44*

CD44 is a multifunctional cell membrane protein that plays a role in both cell–cell and cell–extracellular matrix (ECM) interactions primarily through the binding of hyaluronan (HA). Other ligands of CD44 include collagen, fibronectin, fibrinogen, laminin, chondroitin sulfate, mucosal vascular addressin, seryglycin, osteopontin, class II major histocompatibility complex invariant chain, L-selectin, and E-selectin [38, 39]. As CD44 is widely expressed throughout the body, and its ligands are common, the successful binding of CD44 to its ligands often depends on an external stimulus. Alternative splicing and protein glycosylation gives rise to multiple CD44 isoforms that differ in size (85–230 kDa), functionality, and tissue localization [39, 40].

### 2.4.1.1 Function in Normal Tissue

Work by Lesley et al. [41] has identified three states of CD44: active, inducible, and inactive. The activity is dictated by the glycosylation status of the protein: the active form is least glycosylated and constitutively binds HA; inducible CD44 is moderately glycosylated and requires activation by monoclonal antibodies, cytokines, growth factors, or phorbol ester; and inactive CD44, the most glycosylated, is unable to bind HA (reviewed by Naor et al. [38]). Adding additional variability, the types of glycosylation may vary from isoform to isoform, using side chains such as heparin sulfate and chondroitin sulfate, resulting not only in variations of molecular weight but also in differentially charged environments that affect CD44 function [42].

The human CD44 gene consists of 19 exons, the first 5 of which are constant [39]. The middle 9 exons (v2–v10) are variable regions which may be removed depending on the variant expressed. The next three exons (16–18) are constant, and the last two exons (19 and 20) are variable. Exons 1–17 encode the extracellular domain of the protein, while 18 encodes the transmembrane domain, and 19 and 20 encode the cytoplasmic tail [43]. Individual cells are capable of altering the splicing of CD44, allowing for much diversity. The standard form, CD44s, is the smallest of the isoforms (37 kDa unglycosylated; 80–100 kDa when glycosylated [42]), and was first identified on hematopoietic cells [44] and is therefore additionally termed hematopoietic CD44, or CD44H [38]. Further research has highlighted CD44s expression in a variety of tissues including the epidermis, liver, pancreas, lung, and central nervous system. The distribution of variant CD44 (CD44v) isoforms is much more restricted and apparently tissue specific (reviewed by Sneath [42]). Nomenclature for CD44v isoforms depends on the variant expressed. A CD44v expressing only variant exon 6 would be called CD44v6.

CD44 is involved in cell–ECM and cell–cell interactions. In cell–ECM interactions, CD44 functions through the binding of its previously mentioned ligands, which may facilitate cellular functions such as adhesion and migration. Additionally, CD44 binding of HA causes the internalization of the CD44–HA complex and the lysosomally facilitated degradation of HA [45]. In cell–cell interactions, CD44 allows for the aggregation of cells through the binding of exogenous or endogenous HA [42]. CD44s has also been implicated in the lymph node homing and activation of lymphocytes through its binding of mucosal addressin. The standard and variant forms of CD44 are also involved in myelopoiesis and lymphopoiesis, angiogenesis, chemokine and growth factor presentation, and growth and apoptosis signaling [39, 42, 46].

In normal breast tissue, expression of CD44s and CD44v has been observed by immunohistochemistry (IHC) to be in the myoepithelial layer, while the remaining epithelial cells are CD44<sup>−</sup> [47–50]. Normal breast stromal elements have been observed to express only CD44s [47]. These IHC observations also apply to clinical tumor specimens, as high levels of mainly CD44v have been observed. The correlation between CD44 expression and patient prognosis varies from study to study, likely due to differences in technique, isoform, and the breast cancer population studied (reviewed by Herrera-Gayol and Jothy [51]).

#### 2.4.1.2 First Implications in Cancer and Potential Role in CSCs

CD44 was first implicated in cancer when a nonmetastatic cell line acquired metastatic potential upon transfection with CD44v4-v7, a variant previously found to be expressed by a metastatic rat pancreatic adenocarcinoma. Studies have demonstrated that CD44s is involved in breast cancer cell adhesion, motility, and invasion; whereas CD44v6 is involved solely in cell motility [52]. CD44 most likely acts in tumorigenesis by allowing for more efficient colony formation through increased adhesion to its multitude of ligands in the surrounding environment, its ability to aggregate cells, its induction of cellular growth signals via intracellular signaling partners, and by facilitating the degradation of the surrounding ECM and basal lamina, allowing a path for cellular migration and tumor expansion (reviewed in [42, 51]). Notably, CD44 has been shown to interact with matrix metalloproteinases, activating them and attaching them to the cell surface of tumor cells, thus enabling efficient tumor cell invasion through collagen IV [53, 54]. It is also thought that CD44 plays a distinct role in tumor metastasis; however, the absolute mechanism remains elusive due to the many isoforms and variable functions in different environments [53]. A possible component is revealed through the observation that CD44v4 has been shown to mediate breast cancer transendothelial metastasis through its binding to E-selectin [54]. Contradicting studies show that the presence of CD44s reduced metastasis, potentially explained through the masking of HA from other receptors [55].

The function of CD44 in breast CSCs has yet to be fully elucidated; however, it is likely that the molecule plays a role in enabling CSCs to be the metastasis-initiating cells observed by Croker et al. [56] and Charafe-Jauffret et al. [31, 57]. Recent evidence has shown that CD44 plays a role in protection against apoptosis [58], an important characteristic for a tumor-initiating and metastasis-initiating cell. Additionally, CD44's dual ability for cell-cell and cell-ECM adherence could confer an advantage for CSCs as they travel through the bloodstream and arrive at and enter their secondary site [53]. Within the last few years, much work has been done on the HA-CD44 interaction, revealing that it promotes growth through an EGFR-MAP/ERK (MEK)-dependent mechanism in head and neck cancer [59], and through a HER2- $\beta$ -catenin-dependent manner in ovarian cancer [60]. In breast and ovarian cancers, the HA-CD44 interaction has been shown to activate transcription of Nanog (an embryonic stem cell transcription factor) transcription, which proceeds to activate Rex1, SOX2, and Multi-drug resistance pump 1 (MDR1) [61], all stem cell-related products. These responses to HA-CD44 binding may provide insight into the observed properties of breast CSCs, especially with regard to their therapy resistance.

There is no distinct rule regarding CD44 isoforms and functions within cancer. In some cases, CD44 variants are involved in promoting malignancy, while in others it is the standard form [62]. A further exception to the rule is the observation that CD44 can in fact act as a metastasis suppressor, holding the tumor within the primary site [55, 63]. Diaz and colleagues suggest that the expression of CD44s in node-negative invasive cancer may be associated with increased disease-free survival [64].

Further studies must be done to investigate the functional aspects of CD44 expression in CSC populations through transfection experiments introducing CD44 into non-CSC populations, and more relevantly, through knockdown experiments looking at loss of function due to downregulated CD44 expression.

### **2.4.2 CD24**

Like CD44, CD24 is a glycosylated cellular adhesion molecule, with a weight ranging from 30 to 70 kDa depending on the glycosylation present [65]. It was first described as a B-cell surface protein, but has since been found to be expressed by other hematopoietic cells, the developing brain and pancreas, as well as by a large number of epithelial cells such as keratinocytes and renal tubular cells [65, 66]. Of particular interest, CD24 is emerging as a marker of malignant cells either due to its expression or lack thereof.

#### **2.4.2.1 Functions in Normal Tissue**

CD24 has been putatively implicated in B-cell maturation and the determination of T and B lymphoid progenitors to survive and proliferate. It has additionally been defined as an important T-cell co-stimulatory molecule, although the exact mechanism remains to be elucidated [66]. The CD24-bound oligosaccharides act as a ligand for P-selectin, a cell adhesion molecule expressed by activated blood vessel endothelial cells and activated platelets. This interaction may facilitate tumor passage through the blood stream, and has been shown to mediate breast cancer cell rolling on P-selectin through the blood stream [67].

#### **2.4.2.2 Implications in Cancer and Potential Role in CSCs**

A study investigating tumor invasiveness found that downregulation of CD24 correlated with increased invasion in mammary cancer cell lines; however, a study in a glioma mouse model demonstrated opposite results [66]. These studies have been mirrored by many contradicting studies demonstrating that the presence of CD24 both enhances [65] and inhibits breast cancer cell invasion and metastasis (reviewed by Giatromanolaki et al. [36]). Additionally, work by Schabath et al. [68] demonstrated that low CD24 expression might enhance the growth ability and metastatic potential of breast tumor cells, as CD24 closely regulates the CXCR4 response. This would suggest that the low level of CD24 expression in the CSC population increases the metastatic potential of these cells. Interestingly, Rappa and Lorico [69] noted that within the breast cancer MA-11 cell line, tumorigenicity did not differ between sorted CD44<sup>+</sup>CD24<sup>-</sup> and CD44<sup>+</sup>CD24<sup>high</sup> populations, and that both populations were capable of producing cells with heterogeneous CD24 expression.

Whether or not CD24 is simply a marker of CSCs or actually plays a functional role in CSC cell behavior has yet to be established. However, the molecule plays a role in many functions that may influence tumorigenicity, and the functionality of this molecule in CSCs requires further study.

### 2.4.3 Lineage Markers

In the original identification of the breast CSC, cells positive for lineage markers CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b were discarded during flow cytometry in order to exclude normal human leukocytes, endothelial cells, mesothelial cells, and fibroblasts from the population being analyzed [30]. Work by Sheridan et al. has highlighted that CD10 is expressed on several breast cancer cell lines, and that perhaps CD10 should be excluded from the lineage criteria, as it has been defined as a marker of basal cells and might provide a further subdivision for the breast CSC population [70, 71].

### 2.4.4 Additional Cell Surface Markers

While the CD44<sup>+</sup>CD24<sup>-</sup> selection criterion appears to enrich the tumor-initiating capability of breast cancer cells, it is not a definitive identification of these cells, nor does it apply to all breast cancers. Thus, other groups have been investigating other potential markers to further narrow down the CSC phenotype.

As discussed previously, the mouse mammary stem cell markers have been established as Lin<sup>-</sup>CD29<sup>hi</sup>CD49<sup>fhi</sup> ( $\alpha$ 6-integrin) and human mammary stem cells putatively identified as CD49<sup>fhi</sup>EpCAM<sup>-</sup>. It is notable that a subpopulation in the human breast cancer line MCF-7 was recently identified as overexpressing  $\alpha$ 6-integrin. These cells were capable of propagation as mammospheres, resisted pro-apoptotic agents and exhibited increased tumorigenicity when compared to the whole population, and as few as 1,000 cells were capable of tumor formation. Furthermore, knockdown of  $\alpha$ 6-integrin caused the loss of mammosphere capability and tumorigenicity [72].

In mouse models, CD29 and CD61 have been highlighted as potential proteins active in driving luminal cell fate. Within the CD24<sup>+</sup> population, CD29 differentiates between luminal committed (CD29<sup>low</sup>) and mammary stem cells (CD29<sup>high</sup>) [23]. The addition of CD61 allows for further division of the luminal committed cells into progenitors (CD61<sup>+</sup>) and mature differentiated cells (CD61<sup>-</sup>) [73]. Recent work in a mouse model of luminal breast cancer (MMTV-WNT1) demonstrated that the selection of the CD61<sup>+</sup> population resulted in a much more tumorigenic population when compared to the CD61<sup>-</sup> population [74].

Most recently, Meyer et al. [75] isolated a tumorigenic subset of CD44<sup>+</sup> cells from ER-negative breast cancers and found that CD49<sup>fhi</sup>CD133/2<sup>hi</sup> cells exhibited

xenograft-initiating capability, whereas the  $CD49^{neg/low}CD133/2^{neg/low}$  population did not. They noted that while this new population enriched for xenograft initiation in mouse mammary fat pads, capability varied between their samples. Additionally, other markers established as CSC markers for other cancers, such as CD133 (a marker for colon and brain cancer initiating cells [76, 77]), may be good candidates for further refining the breast CSC phenotype.

Although knowledge translation from murine models and from other cancers to breast cancer is anything but direct, results from these highlighted surface markers merit more investigation into their application on the human breast cancer front. Furthermore, the lack of identified markers for the human mammary gland stem cell highlights the need for more research and standardized assays in this area.

### 2.4.5 ALDH

A hallmark of cancer cells is the genomic instability that allows for the accrual of the multiple mutations necessary for a cell to become tumorigenic [78]. The additional selection criterion afforded by the Aldefluor® assay (Fig. 2.1) provides quantitative analysis of ALDH functionality within CSCs, and this is emerging as an important tool in the study of normal stem cells and CSCs. ALDH activity has been shown to be a functional marker of stem cells. As a result, it might be a common property of CSC populations across all subtypes of the cancer in question (unlike the  $CD44^+CD24^-$  phenotype). Interestingly, work by Ginestier et al. demonstrated that  $CD44^+CD24^-Lin^-Aldefluor^-$  cells were nontumorigenic [28], suggesting that the  $CD44^+CD24^-Lin^-$  phenotype is itself heterogeneous and does not contain strictly CSCs.

The aldehyde dehydrogenases are a large family of enzymes responsible for the oxidation of aldehydes into their corresponding carboxylic acids in a NAD(P)<sup>+</sup>-dependent manner [79]. Different subfamilies are responsible for many functions in the body such as facilitation of retinoic acid biosynthesis, metabolizing cyclophosphamides and its derivatives, and clearing toxic byproducts of reactive oxygen species [29, 80].

High ALDH activity has been used to isolate a variety of normal stem cells, most notably human hematopoietic (HSCs) [81, 82] and murine neural stem cells [83]. Additionally, ALDH activity has been reported to identify leukemic stem cells [84, 85], head and neck CSCs [86], colon CSCs [87], and normal and malignant breast epithelial stem cells [28]. Consequently, ALDH is emerging as an important marker of both normal and malignant stem cell populations. Gene expression studies in HSCs and IHC staining of normal and malignant breast tissue reveal that ALDH 1A1 is likely the isoform responsible for the observed ALDH activity within these stem cell populations [80].

In addition to the conferred resistance to cyclophosphamide and its derivatives, ALDH is responsible for the metabolism of retinal to retinoic acid (RA) [88, 89], and therefore plays an important role in cellular differentiation during development

[90, 91] and in stem cell self-protection from intracellular aldehydes for the duration of an organism's life [29]. The formed RA can proceed to interact with nuclear retinoic acid receptors (RAR) and retinoid X receptors (RXR). RA–RAR interactions cause downstream effects on histone deacetylases, which control the epigenetic regulation of gene expression [92]. It is thought that this ALDH-dependent gene regulation and drug resistance play a role in creating the CSC phenotype.

## 2.5 Comparison of Breast CSCs and Normal Mammary Stem Cells

Although CSCs may arise from a normal tissue stem cell that has undergone cancerous mutations, CSCs may also arise from a more differentiated progenitor that has acquired self-renewal capabilities. Putative pathways involved in mammary stem cell self-renewal include LIF, Hedgehog, Wnt, Notch, TGF $\beta$ , EGF, Prl/GH, and ER/PR (reviewed by Kalirai and Clarke [5]). Similarly, Notch, HOXB4, Wnt, and bone morphogenetic protein (BMP) signaling pathways are identified pathways regulating HSC self-renewal [90]. Notably, Notch has been identified as being upregulated in CD44<sup>+</sup> populations of both normal and malignant breast cells [93], which may translate into an upregulation in the CD44<sup>+</sup>CD24<sup>-</sup> CSC population. Additionally, CD44<sup>+</sup>CD49f<sup>hi</sup>CD133/2<sup>hi</sup> cells demonstrated upregulation of Sox2, Bmi-1, and Nanog (transcription factors known to play key roles in the stem cell self-renewal process) [75]. Unfortunately, due to the complex nature of stem cell self-renewal, it is unlikely that a single pathway will be shown to be responsible for CSC self-renewal.

## 2.6 The Role of CSCs in Metastasis

Breast cancer is a highly treatable disease if caught in the primary stage; however, once the disease metastasizes, patient prognosis becomes much worse [94, 95]. The stepwise process of metastasis is well established, whereby cells must first escape from the primary tumor into the bloodstream and/or the lymphatic system via intravasation. Once in the circulation, the cells must survive until they reach a secondary site where they arrest and enter the tissue (extravasate). Tumor cells able to initiate and maintain colony growth in the secondary sites form micrometastases, which, following angiogenesis, progress to macrometastases [94, 96, 97]. Although tumor cells may readily escape the primary tumor and enter circulation, production of sustainable metastatic lesions is a highly inefficient process (reviewed by Hunter et al. [98]). This was exemplified by an *in vivo* videomicroscopy study by Luzzi et al. which reported that only 0.02% of melanoma cells injected to target the liver could successfully complete the metastatic cascade [99]. Interestingly, this paper highlighted that not all metastatic stages are equally inefficient: the main inefficiencies occur during the initiation and maintenance of the metastatic lesions once tumor cells have reached

the secondary site. This observed inefficiency may be accounted for by the rarity of the CSC population and the lack of a conducive microenvironment for secondary growth. In an eloquent review, Croker and Allan [100] summarize that breast CSCs would be an ideal metastasis initiating cell, as they exhibit unlimited self-renewal, require a specific microenvironment to inhabit, use the SDF-1/CXCR4 axis to migrate, resist apoptosis, and are inherently resistant to many drugs.

Breast CSCs have been shown to demonstrate an increased metastatic propensity *in vitro* [56, 71, 101], *in vivo* [56, 57, 102], and in clinical observations [31, 103]. Although the mechanisms by which this occurs have yet to be identified, there are many theories about how CSCs contribute to breast cancer metastasis. The most common site of breast cancer metastasis is the bone, but metastatic lesions are also found in the lymph nodes, liver, lungs, and brain. Interestingly, both HA and osteopontin, common ligands for CD44, are expressed in the bone and other common sites of breast cancer metastasis [104], suggesting a possible adhesive interaction for circulating tumor cell arrest. Experimentally, CD44 has been shown to mediate the attachment of metastatic breast cancer cells to human bone marrow endothelial cells [105]. Additionally, breast cancer cell lines exhibit different levels of CXCR4, which appears to correlate with CSC proportions and the propensity to metastasize [56, 106]. Similar observations have been made in pancreatic cancer, where, within the identified CD133<sup>+</sup> CSC population, there existed two populations of CXCR4 expression, and only the CXCR4<sup>+</sup> population was capable of metastasizing [107]. Although the mechanisms have not yet been elucidated, there is much evidence to suggest that CSCs are not only tumor-initiating cells but also metastasis-initiating cells. This area requires further investigation, as it might reveal novel targets for therapy.

## 2.7 Breast CSCs and Therapy Resistance

Recent studies have indicated that breast CSCs [108] and other tumorigenic stem cells demonstrate resistance to chemotherapy and radiation therapy [4, 109, 110]. A study in human leukemia revealed that CSCs are often quiescent, and remain in the G<sub>0</sub> phase, conferring resistance to many chemotherapy agents as they often target actively replicating cells [111]. Clinical observations have noted an increase in CD44<sup>+</sup>CD24<sup>-</sup> breast cancer cells after neoadjuvant chemotherapy treatment, indicating they may be resistant to therapy [112]. Possible mechanisms for this include the expression of cell surface pumps, including ABCG2/BCRP1, capable of expelling chemotherapeutic drugs [113]. Interestingly, this same pump has been found to be highly expressed in normal hematopoietic stem cells [114]. Additionally, the presence and activity of ALDH allows CSCs to metabolize cytotoxics such as cyclophosphamide [29]. Other factors potentially prolonging the lifespan of CSCs include the increased expression of anti-apoptotic molecules such as BCL2 and survivin [115, 116].

There is evidence in glioma and leukemic stem cell populations that cell cycle checkpoints and DNA repair mechanisms play a role in both radiation and

chemotherapy resistance, and that these mechanisms may apply to breast CSCs [117–119]. Further, the observed radiotherapy resistance of CSCs may be due to the decreased levels of pro-oxidants in the CD44<sup>+</sup>CD24<sup>-</sup> population [120] or through Wnt/ $\beta$ -catenin pathway signaling [121].

These innate therapy resistance mechanisms make breast CSCs a difficult target to treat; however, their defined characteristics may provide the basis for new therapies. For example, deregulated pathways in breast cancer offer potential treatment options. However, the exact pathways responsible for the self-renewal of these cells have yet to be firmly established, and when they are, it is likely that they will heavily overlap with those used by normal stem cells, thus providing a barrier to treatment. Preclinical and Phase I clinical trials are underway targeting hedgehog, Notch, Akt, and CXCR1 [17]. Currently, high throughput screening is being used on cells sorted for CSC phenotypes, looking for small molecules, siRNA or lentiviral shRNA that target the CSC population. The effects of therapy may be analyzed in many ways including through changes in cellular growth [122], spheroid formation [123], migration [124], or through pathway-specific flow cytometry [125]. Until the biology of CSC therapy resistance is thoroughly understood, high throughput screening may provide the best hope of finding new therapies to target the CSC population.

## 2.8 Conclusions and Future Perspectives

While large steps have been made toward the absolute identification of the breast CSC, the definition still requires further refining. The CD44<sup>+</sup>CD24<sup>-</sup> and/or ALDH<sup>+</sup> phenotype has allowed for the establishment of the presence of a CSC population; indeed, gene expression profiling based on stem and differentiated cell markers indicates that the CD44<sup>+</sup> population is more stem-like and that the CD24<sup>+</sup> population is more differentiated [101, 126]. Unfortunately, due to the vast heterogeneity observed between breast cancers, this phenotype does not extend to all cases, thus further markers need to be established.

CSCs exist both in primary tumors and in metastatic lesions where they appear to play a role in the initiation and maintenance of both tumors. When an unambiguous definition of the CSC phenotype is elucidated, further research should be done to define the role of the CSCs in metastasis, and to identify unique therapy targets, either based on cell surface markers or based on a functional target. Before work targeting CSCs can move forward, it is essential that the functional and cell surface characterization of CSCs is completed. Once a pure population is identified, scientists will then be able to generate novel treatment strategies that aim to eradicate the cells postulated to be responsible for tumor initiation, recurrence, and metastasis.

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