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Resistance to Endocrine Therapy: Are Breast Cancer Stem Cells the Culprits?

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Abstract From a developmental point of view, tumors can be seen as aberrant versions of their tissue of origin. For example, tumors often partially retain differentiation markers of their tissue of origin and there is evidence that they contain cancer stem cells (CSCs) that drive tumorigenesis. In this review, we summarise current evidence that breast CSCs may partly explain endocrine resistance in breast cancer. In normal breast, the stem cells are known to possess a basal phenotype and to be mainly ERα−. If the hierarchy in breast cancer reflects this, the breast CSC may be endocrine resistant because it expresses very little ERα and can only respond to treatment by virtue of paracrine influences of neighboring, differentiated ERα+ tumor cells. Normal breast epithelial stem cells are highly dependent on the EGFR and other growth factor receptors and it may be that the observed increased growth factor receptor expression in endocrine-resistant breast cancers reflects an increased proportion of CSCs selected by endocrine therapies. There is evidence from a number of studies that breast CSCs are ERα− and EGFR+/HER2+, which would support this view. CSCs also express mesenchymal genes which are suppressed by ERα expression, further indicating the mutual exclusion between ERα+ cells and the CSCs. As we learn more about CSCs, differentiation and the expression and functional activity of the ERα in these cells in diverse breast tumor sub-types, it is hoped that our understanding will lead to new modalities to overcome the problem of endocrine resistance in the clinic.

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Abbreviations

CSC	Cancer stem-like cells
ER	Estrogen receptorα
EGFR	Epidermal growth factor receptor
EMT	Epithelial mesenchymal transition
LN	Lymph node
ALDH1	Aldehyde dehydrogenase 1
HDAC	Histone deacetylase
DNMT	DNA methyl transferase

Introduction

From a developmental point of view, tumors can be seen as aberrant versions of their tissue of origin. Certainly, tumors often partially retain differentiation markers of their tissue of origin. In normal development, adult tissues such as the mammary epithelium are derived from tissue-specific stem cells, which can be identified by specific cell surface markers and enriched using antibodies and flow cytometry before transplantation into new host animals to confirm that they can regenerate mammary epithelial tissue [1, 2]. In human leukemia, an infrequent population of stem-like cells with a surface-marker phenotype similar to normal hematopoietic stem cells have been shown to transfer the disease into immune-deficient mice supporting the idea that these cancers contain their own stem cell population [3]. The concept that epithelial and other solid tumors are aberrantly developed tissues containing a developmental hierarchy including cancer stem-like cells (CSCs) and more differentiated progenitor cells is supported by accumulating evidence. The frequency of this CSC population has been

hotly disputed, ranging from very infrequent in leukemia (0.02%) to very frequent (10–25%) in some transgenic models of lymphoma and human melanomas [3–5]. CSC frequency in breast tumors may very well depend on grade and molecular sub-type. There is no doubt that the evidence that CSCs are responsible for tumorigenesis and cancer recurrence is becoming increasingly solid and needs to be considered for therapeutic decision-making in the clinic. In terms of clinical trials of novel therapies, it will be important to determine biomarkers for breast CSCs so that their successful targeting can be assessed. In this review, we will address the likely contribution of CSCs to resistance to breast cancer treatment, in particular endocrine therapies, and explore the potential for targeting CSCs in order to re-sensitise them to treatment.

Cancer Stem-like Cells (CSCs)

There is now a large body of evidence to show that leukaemia originates from an infrequent leukaemic stem-like cell. The first evidence for such CSCs described a small but variable proportion of human acute myeloid leukaemia (AML) cells which could be identified and purified with cell surface markers $CD34^+CD38^-$. These cells were found to be the only cells capable of transferring AML from human patients to NOD/SCID mice, providing evidence that not all AML cells have in vivo clonogenic capacity and only the small subset of CSCs was capable of regenerating the cancer [3]. Many groups have extrapolated the CSC hypothesis from the haematopoietic system to solid cancers and although the evidence for CSCs in solid cancers is in its infancy compared to the haematopoietic field, the body of supporting data is growing rapidly. Cells with CSC characteristics from human brain tumors (glioblastomas) were first isolated using clonogenic sphere culture technique to produce so-called neurospheres (NS) [6, 7]. These NS cells are highly enriched for cell surface marker CD133 and nestin (a neural stem cell marker), have a marked capacity for proliferation, self-renewal, and are capable of in vitro differentiation into phenotypes identical to the tumor in situ. CSC populations have also been found in prostate, colon and breast cancers [8–11].

In the breast, Al Hajj et al. were the first to identify a subpopulation of human breast cancer cells which initiated tumors in immune-deficient NOD/SCID mice [8]. They reported using a set of cell surface markers to sort cells with an increased tumorigenic capacity. Cells which were $CD44^+$, $CD24^{lo}$, ESA^+ and $lineage^-$ (cells lacking markers CD2, CD3, CD10, CD16, CD18, CD31, CD64 and CD140b), isolated from one primary breast cancer and eight metastases were able to form heterogeneous tumors eight out of nine times. The tumors contained not only the $CD44^+$, $CD24^{lo}$, ESA^+ and $lineage^-$ tumor initiating cells but also the phenotypically diverse non-tumorigenic cells that comprise the bulk of tumors. As few as 200 $CD44^+/CD24^{lo}/ESA^+/lineage^-$ cells transplanted into NOD/SCID mice could form tumors with 100% efficiency, while no tumors formed using 200 cells from the $CD44^-/CD24^+/ESA^-$ cell population. A subsequent study by Ponti et al. carried out on 16 breast lesions (13 primary invasive carcinomas, one recurrent carcinoma, and two fibroadenomas) using the sphere culture technique resulted in the production of three long term primary cultures which had self renewing capacity and could differentiate into the different breast lineages [12]. Almost all sphere derived cells were found to be $CD44^+/CD24^{lo}$, however cells with self renewal capacity only accounted for 10–20% of the total cell number, showing that only a sub group within the $CD44^+/CD24^{lo}$ sorted cells had self renewal capacity. This is consistent with only one in 200 cells being capable of initiating a tumor in the previous study. Tumor initiating capacity was measured in a long term sphere culture of the MCF7 breast cancer cell line, termed MCF-S. $CD44^+/CD24^{lo}$ cells from parental MCF7s were implanted into the mammary fat pad of SCID mice, and only gave rise to tumors when at least 1 million cells were implanted. However, $CD44^+/CD24^{lo}$ MCF-S cells gave rise to tumors with smaller numbers of cells (10^5, 10^4 and 10^3) with at least a 60% success rate. Thus both the mammosphere culture system and the cell surface marker selection enriched for tumor initiating cells in this study.

These data indicate that sorting for a $CD44^+/CD24^{lo}$ population enriches for tumor initiating cells but highlights the need for additional markers to further enrich the de facto CSC. One such marker is aldehyde dehydrogenase (ALDH1), the cellular activity of which can be demonstrated using the fluorescent substrate Aldefluor and flow cytometric analysis [13]. ALDH1 activity has been shown to identify a stem/progenitor population in both human haematopoietic tissue and the normal mammary gland. Using primary human breast cancer samples cultivated as xenografts prior to disaggregation and sorting, Ginestier et al. demonstrated that only Aldefluor-positive cells could generate tumors in NOD/SCID mice. When combined with FACS analysis for CD44/24/lin the $Aldefluor^+/CD44^+/CD24^{lo}/lin^-$ population of cancer cells could reliably form tumors with as few as 20 cells in the inoculum, whereas 50,000 $Aldefluor^-/CD44^+/CD24^{lo}/lin^-$ cells failed to form tumors [14].

There is also emerging evidence that some breast cancer cell lines will provide valuable and reliable models of tumor hierarchies containing CSCs with both cell sorting and xenografting being demonstrated from infrequent cell populations expressing markers such as CD44 and cytokeratin 5 [15, 16]. A common theme of many investigations

into CSCs is that they have inherent resistance to chemo- and radiotherapy. This is proposed to be due to mechanisms such as more efficient DNA damage checkpoints and survival pathways compared to more differentiated tumor cell populations. These issues are discussed in a review by Wendy Woodward elsewhere in this issue and we focus in the next section on how breast CSCs may have inherent resistance to endocrine therapies for a variety of reasons including their basal-like phenotype and the pathways that determine their stem cell-like behaviour.

Estrogen Receptorα (ER) and the Cellular Hierarchy of the Normal Breast

Estrogen deprivation is a powerful treatment for breast cancers that express estrogen receptor-α (from this point referred to as ER). However, despite initial response to endocrine therapy, 25% of patients with early breast cancer and all patients with metastatic disease will eventually relapse [17].

The rudimentary mammary gland matures at puberty and functionally differentiates during pregnancy, lactation and menopause due to the influence of steroid hormones and epidermal growth factors [18–21]. This developmental plasticity at tissue level, suggests a stem cell population within the mammary gland which renews and differentiates to form a cellular hierarchy according to highly regulated functional cues. Human embryonic post mortem studies show absent expression of ER before 30 weeks gestation, although rudimentary mammary development commences from week 12 [22]. Moreover ER knockout mice show no development of the breast beyond the rudimentary ductal structures of early gestation [23]. By contrast in the mature human mammary gland 10–20% of luminal epithelial cells express ER [24, 25]. Interestingly ER+ cells in mature mammary glands of both mice and humans do not actively divide but are in close proximity to mitotic cells [24, 25]. This would suggest a model where ER expression in the normal mammary gland is closely linked to a differentiated cell phenotype with limited replicative capacity (Fig. 1).

The mouse mammary stem cell population characterised by expression of the markers $CD29^{hi}$(β1 integrin)/$CD24^{+}$/Lin^{-} [1] consists of less than 0.01% cells expressing ER [26]. Interestingly, epidermal growth factor receptor (EGFR) was found to be expressed in $CD29^{hi}$(β1 integrin)/$CD24^{+}$/Lin^{-} although expression of PR and erbB2/HER2 receptor was absent [26]. A further murine study defined the cellular hierarchy further, separating the luminal compartment by expression of Sca1, CD133, CD24 and ER [27]. ER rich $CD133^{+}$/$Sca1^{+}$/$CD24^{hi}$ cells were weakly proliferative whereas the milk-protein rich, ER low population of CD133−/Sca1−/$CD24^{hi}$ cells showed high proliferative capacity.

In an important recent study using normal human tissue derived from mammoplasties, Raouf et al. [28] defined bipotent progenitor cells, luminal committed progenitor cells and differentiated luminal cells by surface marker and subsequently assessed gene expression in each population. The cell sorting methods used enriched for primitive bipotent cells ($EpCAM^{+}$/$CD49f^{hi}$/CALLA $(CD10)^{+}$/$Thy1^{+}$/$CD133^{-}$) at a purity of 45±3% (containing 57% of all bipotent cells) and luminal-restricted progenitors ($EpCAM^{+}$/$CD49f^{+}$/$MUC1^{+}$/$CD133^{+}$/$CD10^{-}$/$Thy1^{-}$) at a purity of 32±3% (containing 96% of all luminal progenitor cells). Transcriptional profiling revealed ER^{lo}/PR^{hi} expression in the bipotent cell population compared to ER^{hi}/PR^{lo} expression in the luminal committed progenitor population of the normal human breast. These findings concur with the work of Shipitsin et al. [29] who determined ER^{lo} expression

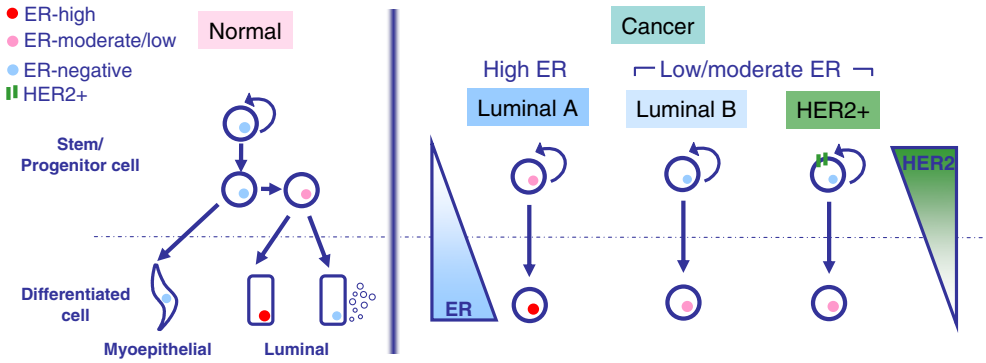

Figure 1 Hypothetical cellular hierarchy of normal and malignant breast illustrating putative differential estrogen receptor α (ER) expression. In the normal breast an ER− stem/progenitor cell either differentiates into an ER− myoepithelial cell lineage or via a ER moderate/low expressing progenitor will produce the luminal lineage which is either ER+ (non-milk secreting) or ER− (milk secreting). Three different breast cancers are illustrated showing the Luminal A high ER+ tumors differentiating from ER low/moderate stem/progenitor cells. The Luminal B and HER2+ low to moderate ER tumors both differentiate from an ER− stem progenitor population. In the HER2+ tumors the stem/progenitor populations are highly HER2+.

of the stem cell population albeit defined by an alternative cell marker methodology ($CD44^+/PROCR^+/CD24^{lo}$).

Breast Cancer Stem Cells and Endocrine Resistance

In contrast to the normal mammary gland, actively dividing ER+ cells are prominent in breast hyperplasia and breast cancers. The levels of ER and PR expression are predictive of treatment response rates to endocrine therapy and distinguish Luminal A tumors, which are highly ER+ and PR+, from luminal B and erbB2/HER2 tumors which have lower ER expression, do not express PR and co-express other growth factor receptors such as EGFR and erbB2/HER2 [30, 31]. Intrinsic and acquired resistance to endocrine therapy remains a significant cause of disease relapse and mortality in ER+ breast cancers [32, 33].

EGFR Pathway

Enhanced interaction between estrogen receptor signalling and growth factor tyrosine kinase pathways such as EGFR, HER2/erbB2 and IGFR mediates resistance to endocrine therapy. For example EGFR1 expression is inversely correlated with that of the ER and co-expression of both receptors confers relative resistance to endocrine therapy compared with tumours not expressing EGFR1 [31, 34]. A similar inverse expression relationship occurs between ER and erbB2/HER2. Tamoxifen resistant MCF7 breast cancer cells show a 5–10 fold increase in mRNA and protein expression of erbB2/HER2 and the EGFR receptor compared to sensitive MCF7 cells [35]. Similarly, resistance to fulvestrant and aromatase inhibitors can also be mediated by upregulation of the erbB2/HER2 pathway [36]. Long term stimulation of the EGFR and HER2/erbB2 pathways in endocrine resistant cancer cells down-regulates the ER. Ligand-independent activation of ER may be mediated by growth factor or intracellular kinase phosphorylation of the AF1 domain of ER, for example at serine 118 or 167 [37, 38] by MAPK, PI3K, akt and src-K [37, 39, 40] thus allowing expression of estrogen regulated gene products despite endocrine therapy.

The acquisition of enhanced EGFR/erbB2 pathway signalling in ER+ breast cancer with tamoxifen resistance potentially results from selection of a more stem-like phenotype. Expression of EGFR has been demonstrated in stem cells of the normal mammary gland in mice and humans [26, 41]. This is in contrast to ER which is predominately expressed in more differentiated luminal cells [25, 27, 29]. In malignant CSCs, Farnie et al. [42] showed activation of the EGFR pathway in ductal carcinoma in-situ (DCIS) of the breast. Inhibition with gefitinib, an EGFR pathway inhibitor, significantly reduced mammosphere formation in vitro.

There is also emerging evidence for a role of the HER-2 pathway in the function of CSCs. In one series of 491 breast cancer patients, expression of erbB2/HER2 and presence of ALDH1+ CSCs were positively correlated [43]. Recently, a report showed erbB2/HER2 over-expression enriched for normal and malignant stem cells in mammosphere and Aldefluor assays and increased in vitro clonogenicity and tumorigenicity in immunocompromised mice [44]. Separately, the CSC population of four HER2+ breast cancer cell lines have been demonstrated to express more HER-2 m-RNA and protein compared with the non-CSC cell population, regulated at the level of transcription. Furthermore, trastuzumab (Herceptin), reduced mammosphere-forming capability and tumorigenicity on serial xenotransplantation [45]. In a clinical study in HER2 over-expressing large primary breast cancers, lapatinib (Tykerb; a dual EGFR/HER-2 tyrosine kinase inhibitor) reduced the $CD44^+/CD24^{lo}$ CSC fraction and mammosphere forming efficiency of the residual tumor, although this did not reach formal statistical significance [46]. Notably treatment with chemotherapy alone increased the proportion of CSCs in the residual breast cancer [46, 47]. Thus the well-described upregulation of the EGFR/HER2 pathway in endocrine resistant breast cancer may in fact reflect an enrichment of a CSC phenotype (Fig. 1).

Notch Pathway

An intriguing interaction is emerging between the Notch pathway, CSCs and endocrine treatment in breast cancer. The Notch pathway has been implicated in cell fate delineation in the normal human mammary gland [28] and regulation of CSCs in ductal carcinoma in situ (DCIS) [42] and invasive carcinoma of the breast [48, 49]. For example, Farnie et al. observed that inhibition of the Notch pathway by the gamma secretase inhibitor DAPT or a Notch 4 neutralizing antibody significantly reduced mammosphere formation in primary human DCIS in vitro [42]. By comparison, breast cancer of luminal type has been shown to express low levels of Notch and ErbB2 but high levels of ER compared to basal breast cancers, which show the opposite pattern [50]. This inverse relationship between the expression of ER, ErbB2 and Notch activity in breast cancer may provide clues to the regulation of CSCs and endocrine resistance.

While Magnifico et al. demonstrated that mammosphere formation in HER-2 overexpressing cell lines was significantly reduced by trastuzumab, the effect was ameliorated by antagonism of the Notch pathway [45]. The mechanism for this interaction between the Notch and EGFR pathway remains to be elucidated. However, this cross talk appears to be relevant therapeutically as Osipo et al. recently demon-

strated a 2–6 fold increase in Notch 1 activity in MCF7, BT474 and SKBr3 cell lines after treatment with trastuzumab or lapatinib. Such treatment induced nuclear accumulation of Notch 1 intracellular domain and increased expression of Notch downstream targets including Hes 1, 5 and Hey 1. Inhibition of the Notch pathway led to re-sensitisation to trastuzumab, and the combination of Notch antagonism and trastuzumab inhibited growth in both trastuzumab sensitive and resistant cell lines [51].

Estrogen signaling conversely down-regulates Notch signalling. Rizzo et al. [50] demonstrated oestradiol induced reduction in the expression and activation of Notch 4 and Notch 1 in T47D and MCF7 cell lines. This reduction in Notch activity could be abrogated by tamoxifen and fulvestrant [50]. In a mouse xenotransplantation assay using the BT474 cell line, tumors were treated with tamoxifen alone or in combination with a gamma secretase inhibitor (GSI). Combination therapy was significantly superior to the use of tamoxifen alone and the authors conclude that tamoxifen antagonism of the estrogen stimulus leads to the reactivation of the Notch signalling pathway promoting proliferation and survival. However further investigations will have to be carried out to determine whether this effect is on a cellular population level or specifically mediated by the CSC population.

Cellular Hierarchy of Breast Cancer and ER Expression

One mechanism of resistance to ER targeted endocrine therapy may be the presence of an ER−, treatment-resistant CSC population, with the capacity to differentiate and produce treatment sensitive ER+ luminal cancer cells. One prediction that follows from this proposed mechanism is that after endocrine treatment, there would remain a resistant population of ER−/lo stem-like cells to seed relapse and metastases despite endocrine therapy.

In primary human breast cancer samples Shipitsin et al. [29] used transcriptional profiling to characterise $CD44^+$/$PROCR^+$ stem cells and $CD24^+$ luminal type cells from the same donor. This group showed that $CD44^+$/$PROCR^+$ cells in breast cancers were enriched for stem cell markers and for gene expression related to cell motility and angiogenesis. Interestingly, malignant CD44+ cells were ER^{lo} in a similar manner to $CD44^+$ cells from normal mammoplasty specimens in this report and in a study by Fillmore and Kupperwasser [15, 29].

A recent paper has also demonstrated the presence of rare steroid receptor negative $CD44^+$ cells present in the ER+ breast cancer cell line T47D [16]. The size of this $CD44^+$/$CK5^+$/ER^-/PR^- did not proportionally increase with expansion of the rest of the tumor population and this infrequent ER− cell type was observed in both in vitro clonogenic and in vivo tumorigenic assays, whereas the bulk of the tumor consisted of proliferative $CD44^-$/$CK5^-$/ER^+/PR^+ cells. Notably an intermediate $CK5^-$/ER^-/PR^+ cell population was demonstrable in vitro colony assays when treated with progesterone. These defined populations within an ER+ cell line appears to mimic the cellular hierarchy of steroid receptor transcript expression in the normal breast as shown by Connie Eaves' group [28].

Such findings might be consistent with a model in which an ER− stem cell generates a cellular hierarchy at a metastatic site comparable to the hierarchy of the primary tumor (Fig. 1). Endocrine therapy in resistant patients, may enrich for the CSC population, in an analogous manner to the effects of chemotherapy or radiotherapy [46, 47, 52] leading to eventual relapse. However this hypothesis, if the malignant cellular hierarchy is rigidly maintained, would require the CSCs to both rapidly self-renew and differentiate to generate ER+/$CD24^+$ cells to maintain tumor growth despite the inhibitory effects of endocrine therapy on the $CD24^+$ population.

Cancer Stem Cells, Mesenchymal Phenotype and Endocrine Resistance

Recent work by Weinberg's group [53] has linked the mesenchymal cell phenotype to stem cells in normal tissue and to CSCs. Immortalised Human mammary epithelial cells (HMECs) induced to undergo epithelial mesenchymal transition (EMT) exhibited stem cell markers and had increased capacity to form mammospheres enriched in stem cells. Similarly stem cells isolated from normal and cancerous human and mouse mammary glands demonstrated markers of mesenchymal phenotype normally apparent in EMT. This included up-regulation of the transcription factors Snail and Slug and also the TGFβ signalling pathway which has been previously implicated in stem cell function [29].

Metastatic potential has long been associated with the loss of markers of the epithelial cell phenotype and the acquisition of basal/mesenchymal properties. Interestingly, recent analysis of a panel of breast cancer cell lines of luminal, intermediate and basal phenotypes has showed a significantly increased fraction of CSCs (defined by $CD44^+$/$CD24^{lo}$/ESA^+ expression) in basal type breast cancers compared to hormone sensitive luminal cancers (2.5% vs 0.5% $p<0.0001$) [15]. Furthermore a positive correlation was shown between CSC number and cell line tumorigenicity in in vivo models [15].

Endocrine resistant ER+ breast cancers are reported to gain a more basal phenotype, for example reduction in E-cadherin expression [54] and enhanced motility and invasion by upregulation of src kinase [55, 56], NF-κB [57] and CD44 [58, 59] (S. Hiscox unpublished observa-

tions). As ER negatively regulates the expression of the key transcription factors regulating EMT such as Snail and Slug [60, 61] a functionally redundant ER in endocrine resistant breast cancer might therefore promote a more mesenchymal stem-cell-like phenotype. In an MCF7 model of tamoxifen resistance, tamoxifen resistant cells show enhanced mammosphere forming capacity compared to Tamoxifen sensitive cells, suggesting an increased CSC fraction (C S O'Brien unpublished data).

Epigenetic Regulation of the Cellular Hierarchy

Gene-expression profiling of breast cancer has demonstrated at least five distinct molecular subtypes; basal, erbB2/HER2, Luminal B, Luminal A and normal-like [30, 62]. These subtypes probably represent a differentiation spectrum comparable to the developmental hierarchy of the breast; with poorly differentiated ER-negative basal type at one extreme to well differentiated Luminal A type at the other. As such, these subtypes may derive from a cell of origin at a different stage of the developmental hierarchy [63] and reflect the hormone and growth factor sensitivity of that distinct cell. Prolonged endocrine therapy may lead to the re-acquisition of a more primitive cancer cellular phenotype with intrinsic resistance to hormone manipulation.

A recent study elegantly demonstrates that targeted epigenetic modification of the genome has an important role to play in cell-fate determination in the cellular hierarchy of the human mammary gland and breast cancer [64]. Using MDSK (methylation specific digital karyotyping) and SAGE (serial analysis of gene expression) techniques, adult mammary stem cells ($CD44^+$) and breast cancer stem cells (CSCs—$CD44^+$) were compared and contrasted to more lineage committed ($CD24^+$) cells. Normal adult mammary SCs and CSCs showed comparable genomic hypo-methylation of transcription factors implicated in stem cell function such as HOXA10, FOXC1 and TCF3 compared to the more highly methylated progenitor and lineage committed cells. Forced expression of FOXC1 in differentiated mammary cells, where FOXC1 is normally methylated, led to the re-acquisition of a progenitor like phenotype. This suggests an important role for epigenetic modification in cell fate specification and function of normal and cancer stem cells, which in the future may be amenable to therapeutic targeting.

Acquired endocrine resistance may thus result from an alteration in cancer phenotype between the primary tumor and the metastases, to a more stem-like hormone insensitive cellular identity but the evidence for this remains circumstantial. In one series of 200 patients, 19.5% of metastases were found to be ER− in the presence of an ER+ primary breast cancer and these findings have been replicated in another smaller study [65]. Fehm et al. have shown that in 88 patients with ER+ primary breast cancers, 76 had only ER− disseminated tumor cells (DTC) in the bone marrow [66]. These data raise the possibility that the ER− CSC is responsible for tumor metastasis and that cell surface phenotype of such cells facilitates communication with a stromal niche to enable intravasation and metastatic growth. It is worth noting that ER is lost completely in only 20% of metastases from ER+ primary cancers, suggesting that the ER− DTCs isolated by Fehm et al. undergo differentiation into tumors that can subsequently defined as ER+ [65]. Up to half of metastatic tumors which continue to express ER show no functional inhibition by endocrine agents. Interestingly, aberrant methylation of ER and PR promoters has been observed in up to 40% of hormone receptor negative breast cancers [67, 68] and epigenetic modifications have been shown in tamoxifen resistance [69]. Forced re-expression of ER by therapeutic demethylation may thus lead to the intriguing possibility of re-acquisition of endocrine sensitivity in these malignancies and we will discuss this possibility further later in this review.

The Stem Cell Niche and its Influence on Resistance to Endocrine Therapy

CSCs are associated with increased invasive and metastatic/migratory phenotype [70–72]. Cells isolated as CSC by virtue of $ALDH1^+$ and or $CD44^+/CD24^{lo}$ demonstrated increased metastasis from primary subcutaneous tumors in NOD/SCID/IL2γ receptor null mice. Such augmented invasive and metastatic phenotypes are also seen in endocrine resistant breast cancer cell lines [39, 73, 74]. These cell lines exhibit over-expression of EGFR and the c-MET receptor through which they derive proliferative and migratory/invasive signals from stromal derived ligand secretion. Significantly, such resistant cells also overexpress CD44, the adhesion of which to bone marrow derived endothelial cells is enhanced by stromal derived HGF in vitro [59, 75]. Thus adaptive endocrine resistance in cell lines is associated with a metastatic and stem cell-like phenotype.

Using human breast cancer cell lines in a murine model, it was demonstrated that CD44 was sparsely expressed in primary tumor cells but homogeneously over-expressed in cells transiting the lymphatics and populating lymph nodes (LN) [76]. The authors hypothesized that CD44 expression targeted tumor cells for metastasis to, and uptake in the LN although induction of CD44 expression by interaction of the epithelial cells with the LN stromal cells is also a possibility. The CD44 expressing cells were relatively insensitive to the effects of estradiol and estradiol withdrawal despite ER expression levels comparable to those in

the seen in the primary tumors [76, 77]. The same group have also recently shown that a small sub-population of the cells expressing CD44 express CK5 but not ER or PR and are resistant to both endocrine and chemotherapy [78]. Thus the LN and stromal microenvironments may be responsible for maintenance of the CSC phenotype and suppression of estrogen sensitivity in such cells.

Supporting the former hypothesis are recent data from Farmer et al. demonstrating a stroma-related gene signature in primary breast cancers [79]. This signature was associated with the presence of a reactive stroma and predicted for resistance to neo-adjuvant chemotherapy. Importantly the stroma-related signature demonstrated a pattern of expression similar to that of mammospheres suggesting that the stroma may support the CSC phenotype and promote resistance to therapy. As the signature was only tested in ER negative tumours, the relevance to luminal tumours and endocrine resistance is unknown but such analyses are eagerly awaited [79].

Another emerging target that is likely to impact on CSCs is antiangiogenic therapy since evidence is accumulating that both tissue stem cells and CSCs preferentially associate with blood vessels. For example, in oligodendrogliomas and glioblastomas, there is a direct correlation between nestin positive CSCs and microvessel density (MVD) [80]. This study also reported that the CSCs preferentially associated with the $CD34^{+}$ capillaries in vivo (in tumor sections) and endothelial vascular tubes in a basement membrane (Matrigel) culture assay in vitro compared with non-CSCs. In a prior report, this association had been shown to be secondary to CSC secretion of vascular endothelial growth factor (VEGF), which directly stimulates endothelial cell growth [81]. Currently there is little data to support or refute the existence of a vascular niche for the breast CSC and further investigation is required.

Differentiation Agents and Endocrine Treatment

There is evidence to show that histone deacetylation and DNA methylation plays a key role in inactivation of ER gene expression. In ER− breast cancer cells studies have demonstrated that the ER CpG promoter is occupied by abundant HDAC1 and HDAC2 [82, 83]. Similarly DNA methylation has also been reported to be up regulated in ER− breast cancer cells [84]. Investigation of de novo ER gene methylation in vitro showed DNA methyltransferase 1 (DNMT1) levels were significantly elevated in ER− breast cancer cell lines compared with their ER+ counterparts [68]. Furthermore recent research into cell type specific DNA methylation patterns revealed that progenitors were hypomethylated compared to differentiated cells in the human normal breast and breast cancer [64]. The role of epigenetic modification in regulation of ER expression and the cell fate breast cancer may provide a therapeutic targeting strategy for ER− breast cancer patients.

Epigenetic therapies such as HDAC and DNMT inhibitors have shown considerable promise in the treatment of haematological malignancies [85] and trials are ongoing in solid cancers. Cell line studies have shown that functional ER gene expression can be induced by pharmacological administration of a DNMT inhibitor 5-aza-2′-deoxycytidine (AZA) and a HDAC inhibitor trichostatin A (TSA) [86–88]. Furthermore, combination AZA and TSA treatment acted synergistically to induce re-expression of ER in ER− breast cancer cells [89]. A recent pre-clinical xenograft model has demonstrated that ER- MDA-MB-435 cells treated with AZA and TSA re-expressed functional ER, which by itself caused a significant reduction in tumor growth. In addition, after ovarian ablation to mimic endocrine treatment, there was a further reduction in tumor growth [90].

An inverse relationship between ER and EGFR expression has been well documented in breast cancer cell lines. Using the HDAC inhibitor vorinostat, two ER− cell lines, MDA-MB-231 and MDA-MB-468 cells, exhibit ER gene expression and reduced EGFR expression. Reduction in EGFR expression led to reduced EGFR signaling and PAK1 expression levels [91]. Interestingly immuno-histochemical analysis of PAK1 shows significantly increased expression in breast cancers from hormone resistant patients [92–94].

HDAC inhibitors are being used in a number of on going clinical trials including a phase II trial evaluating vorinostat in ER positive patients with metastatic breast cancer who failed prior aromatase inhibitor therapy and up to three chemotherapy regimes [95]. A report of preliminary findings presented at ASCO 2008 showed that out of the 17 enrolled patients 21% had a partial response and 29% had stable disease after treatment with vorinostat 400 mg daily for 3 of 4 weeks and tamoxifen 20 mg daily, continuously. These findings suggest that the addition of an HDAC inhibitor to tamoxifen in patients who have failed prior aromatase inhibitors or adjuvant tamoxifen may restore hormone sensitivity. The in vitro studies would also suggest that HDAC inhibitors in combination with endocrine inhibitors may be highly applicable to ER− breast cancers as well.

Concluding Remarks

In this review, we have summarised current evidence that supporting improving our understanding of CSCs in order to explain endocrine resistance in breast cancer. The biology of breast CSCs is becoming better characterized and the data suggest that they may be resistant to several forms of cancer therapy through diverse mechanisms. In

terms of responsiveness to endocrine therapy, we can learn about CSC biology and hierarchies in breast cancer by examining what is known about the developmental hierarchy of the normal breast epithelium (Fig. 1). In normal breast, the stem cells are known to possess a basal phenotype and to be mainly ER−. If the hierarchy in breast cancer reflects this, the breast CSC may be endocrine resistant because it expresses very little ER and can only respond to treatment by virtue of paracrine influences of neighboring, differentiated ER+ tumor cells. Normal breast epithelial stem cells are highly dependent on the EGFR and other growth factor receptors and it may be that the observed increased growth factor receptor expression in resistant breast cancers reflects an increased proportion of stem-like cells selected by endocrine therapies. There is evidence from a number of studies that breast CSCs are ER− which would support this view. CSCs also express mesenchymal proteins which are suppressed by ER expression, further indicating the mutual exclusion between ER+ cells and the CSCs. It is likely that this is regulated at the epigenetic level, and differences in DNA methylation and chromatin organization can be observed between breast CSCs and more differentiated populations. This may in turn be regulated extrinsically by the influence of stromal elements including the stem cell niche microenvironment associated with the vasculature, the lymph nodes and the bone marrow to which breast cancer cells often metastasise. It is known that the epigenetic programming can be remodeled by using drugs, particularly those that change the methylation and chromatin patterns of the DNA. Such drugs can effectively differentiate the cells, including potentially the CSCs, leading to a reduction in growth factor receptors and an increase in ER+ cells, which may overcome resistance to endocrine agents in combination therapy. Such combinations are currently in clinical trials and their outcome is eagerly anticipated. As we learn more about CSCs, differentiation and the expression and functional activity of the ER in these cells in diverse tumor sub-types, it is hoped that our understanding will lead to new modalities to overcome the problem of endocrine resistance in the clinic.

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