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REVIEW

Breast cancer stem cells: treatment resistance and therapeutic opportunities

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The clinical and pathologic heterogeneity of human breast cancer has long been recognized. Now, molecular profiling has enriched our understanding of breast cancer heterogeneity and yielded new prognostic and predictive information. Despite recent therapeutic advances, including the HER2-specific agent, trastuzumab, locoregional and systemic disease recurrence remain an ever-present threat to the health and well being of breast cancer survivors. By definition, disease recurrence originates from residual treatment-resistant cells, which regenerate at least the initial breast cancer phenotype. The discovery of the normal breast stem cell has reignited interest in the identity and properties of breast cancer stem-like cells and the relationship of these cells to the repopulating ability of treatment-resistant cells. The cancer stem cell model of breast cancer development contrasts with the clonal evolution model, whereas the mixed model draws on features of both. Although the origin and identity of breast cancer stem-like cells is contentious, treatment-resistant cells survive and propagate only because aberrant and potentially druggable signaling pathways are recruited. As a means to increase the rates of breast cancer cure, several approaches to specific targeting of the treatment-resistant cell population exist and include methods for addressing the problem of radioresistance in particular.

Introduction

Breast cancer is a heterogeneous disease but can be grouped into major subtypes by both traditional histopathological features (e.g. histological type, grade, estrogen receptor, progesterone receptor and HER2 status) used in diagnostic practice as well as the newer microarray-based molecular profiling (1,2). The new molecular taxonomy describes five major subtypes (Luminal-A, Luminal-B, Basal-like, HER2 and Normal) that overlap with different clinicopathological classification systems, correlate with clinical behavior and are vital for informing patient management.

Early breast cancers (stage I, II, IIIA and operable stage IIIC) are treated with curative intent using surgery followed by radiotherapy. To avoid recurrence from micrometastases, adjuvant treatments (hormonal agents, trastuzumab and cytotoxic chemotherapy in sequence and/or in combination) are often prescribed. The administration of systemic therapies is driven by assessment of clinico-pathologic features such as tumor size, nodal involvement, hormone receptor status and *Her2* gene amplification (3–5). Stage IIIB and inoperable stage IIIC breast cancer are treated with systemic chemotherapy or hormone therapy, in the neoadjuvant settings, to downstage locally advanced tumors followed by surgery and radiotherapy (6). Stage IV or metastatic breast cancer is treated with palliative intent using hormonal agents, trastuzumab or lapatinib, conventional cytotoxic drugs. These drugs tend to be employed in sequence often as single agents, although some of these agents may be used in two-drug combinations.

Although adjuvant therapy plays a crucial part in the management of early breast cancer, local relapse still occurs. A meta-analysis of 42 000 women in 78 clinical trials demonstrates that the 10-year local recurrence rate in patients who received lumpectomy and radiation was 13% compared with 47% for patients who did not receive radiation. In the case of patients receiving a mastectomy and radiation, the recurrence rate was 8% compared with 28% for those not receiving radiation (7). Despite the reduction of recurrence by the use of radiotherapy, the 15-year overall survival of these patients is marginally affected and mortality rates are 26% and 48% following breast lumpectomy and radiotherapy for lymph node-negative and positive breast disease, respectively. Even higher mortality rates of 31% and 55% are reported for patients receiving a mastectomy and radiation for lymph node-negative and positive disease, respectively (7). At present around 40% of all breast cancer patients suffer a recurrence; 10–20% of all recurrences are local and 60–70% are distant metastases (8).

Although several prognostic factors, depending on breast cancer type, can predict recurrence, the explanations for recurrences remain hypothetical. Undertreatment of breast cancer patients with adjuvant therapies due to borderline classification of the disease may contribute to some but not all recurrences. Local and metastatic recurrence after surgical treatment of the primary tumor may be due to local deposits of cancer cells that were not removed during surgery or early micrometastases that were resistant to adjuvant treatments. Recurrence and disease spread in locally advanced breast cancer may be explained by resistance to neoadjuvant systemic therapy and/or radiotherapy. Conventional therapeutic approaches (chemotherapy and radiotherapy) as well as most of current targeted therapies are based on an intention to target all cells similarly using maximum tolerated doses. Nevertheless, the relative failure of these therapies to cure most solid cancers as well as local and metastatic disease recurrence has revived interest in the controversial cancer stem cell (CSC) model as it described a therapy-resistant subpopulation of cells that are capable of tumor 'regeneration'.

The existence of a radiation-resistant subpopulations of tumor cells has been long proposed by radiobiologists (9,10), but whether these cells can be prospectively identified and targeted is an ongoing debate. A similar difficulty applies to the CSC hypothesis, which defines 'a small subset of cells within a cancer that constitutes a reservoir of self-sustaining cells with the exclusive ability to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor' (11). Preclinical data from cell lines and tumor models support the concept that breast cancer-derived tumor-initiating cells are relatively resistant to radiation and chemotherapy. The relationship between the CSC hypothesis and the normal breast epithelial hierarchy has fueled much speculation on breast cancer histogenesis, i.e. the normal cellular origins of specific breast cancer subtypes. How important understanding tumor cell origin will be in improving breast cancer outcomes is debatable. Therefore, we believe that studies concentrating on the treatment-resistant cells among the heterogeneous cell populations of human breast cancer could be helpful in not only

Abbreviations: BCSCs, breast cancer stem cells; CSC, cancer stem cell; DDR, DNA damage response; DPPE, N,N-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine; DSBs, double DNA strand breaks.

identifying patients requiring more aggressive treatment and monitoring but also in broadening the scope for identification of new therapeutic targets and approaches. Clear parallels can be drawn between these studies and the investigations of putative breast cancer stem cells (BCSCs). Is there sufficient preclinical and clinical evidence, however, for uniting the concepts of treatment-resistant cells and breast cancer stem-like cells? Here, we will review data for each concept with a focus on discussing new and improved methods of reducing breast cancer recurrence after therapy, particularly after radiotherapy by targeting the mechanisms of resistance.

Cancer models

Two models have been proposed to account for solid tumor heterogeneity: the 'clonal evolution' and the 'CSC' models (12). The conventional clonal evolution model is a non-hierarchical model that proposes all cells within a tumor have an equal chance of acquiring the genetic mutations necessary for driving tumor growth. In this model, cancer cells over time stochastically acquire a myriad of combinations of mutations over time in a by-chance fashion, so that by natural under selection pressures, the most aggressive cells drive the most aggressive cells drive tumor propagation progression and therapy resistance. The CSC model is a hierarchical model proposing that only a subset of cells can propagate the tumor by acting as multipotent progenitors, with the ability to recapitulate the molecular and phenotypic heterogeneity of the original tumor mimicking stem cells. The genetic basis for heterogeneity needs to be addressed in both of these cancer models (see A Mixed Model of Tumorigenesis), however, it is important to emphasize that CSCs are not necessarily the product of normal stem cell transformation; they may arise from restricted progenitor or differentiated cells by acquiring stem cell-like properties (11,13,14).

Breast cancer and stem cells: is there a link?

Normal stem and progenitor cells clearly play an active role in the human breast because they participate in the cyclical changes of this dynamic tissue, which is remodeled during ovulations and pregnancies throughout the reproductive lifespan of a woman (13,15). Breast stem cells have the capacity for self-renewal as well as generating the three major lineages that comprise the breast gland: myoepithelial cells forming the basal layer of ducts and alveoli, ductal epithelial cells lining the lumen of ducts and alveolar epithelial cells synthesizing milk proteins (16). In mice, mammary stem cells are localized in the cap cells of the terminal end buds and a single mammary stem cell has the capability to regenerate a complete mammary gland *in vivo* (17,18). In humans, stem cell zones were identified in the mammary ducts that are enriched for quiescent cells (19). In 3D cultures, $EpCAM^{high}/CD49f^{+}/Lin^{-}$ cells form terminal duct lobular unit-like structures that could both self-renew and give rise to stem cell like and lineage-restricted luminal and myoepithelial cells (19–21). The current model of normal mammary development (Figure 1) is that stem cells give rise to a common primitive progenitor that undergoes differentiation into committed luminal cells, mature luminal cells and myoepithelial cells (13,22–26).

Breast cancer stem cells

Recent identification, within several cancers, of subpopulations of cells that have some of the functional and phenotypic properties in common with stem cells: the capacity for self-renewal, the ability to differentiate, activate telomerase and anti-apoptotic pathways, increase membrane transporter activity and acquire anchorage independence, all support the CSC hypothesis (27–34). In addition to their tumorigenic role, carcinoma cells acquire the ability to migrate to niches and thus CSCs may play a role in metastasis (35). The inherent plasticity and pluripotency of CSCs makes them the likeliest candidates to thrive in foreign sites and to initiate and sustain cancer growth. If only a rare subpopulation of breast cancer cells can initiate tumors (28), then it is reasonable to propose that such rare plastic and tumorigenic cells would be responsible for initiating and propagating heterogeneous metastatic lesions (35).

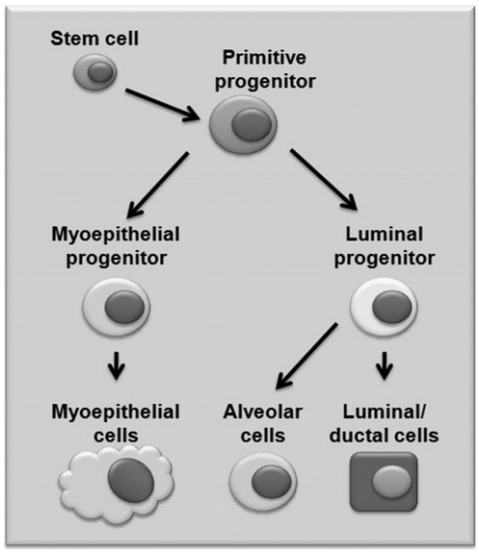

Fig. 1. Hierarchical organization in the normal breast. Breast stem cells give rise to primitive progenitor cells that differentiate into myoepithelial and luminal progenitors. The differentiated progenitors generate the three subtypes of cells that constitute the mammary gland: myoepithelial, alveolar and luminal/ductal cells.

Putative CSC populations are often identified using *in vitro* sphere formation, differentiation and clonogenicity assays and *in vivo* xenograft tumuorigenicity assays. Fluorocytometric sorting steps that separate putative CSC phenotypes from more differentiated progeny and extraneous cell types often precede these assays. In the case of BCSCs, studies have identified HER2 (36,37), CD49f (38), epithelial cell adhesion molecule (28,39), acetaldehyde dehydrogenase activity (40–42) and phosphatase and tensin homolog (43) amongst others as important putative markers for BCSCs identification. To date, the $EpCAM^{+}CD44^{+}CD24^{-}$ putative BCSCs are the most investigated. This surface marker expression profile has been proposed as a BCSC phenotype based on evidence of increased tumorigenicity in a xenograft model of this population sorted from samples of human breast cancer metastasis (28) and have been the subject of many investigations (39,44). A meta-analysis based on published studies found that acetaldehyde dehydrogenase positive and $CD44^{+}/CD24^{-/low}$ tumor cells are prognostic factors in breast cancer and associate with poor overall survival (45).

Studies also support a role for $CD44^{+}CD24^{-}$ putative BCSCs in metastasis. As few as 10 cells of stably labeled $CD44^{+}$ BCSCs from primary and metastatic human tumors, tracked *in vivo* using non-invasive imaging approaches, were shown to spontaneously metastasize from primary tumors in mice (46). Fifty bone marrow specimens from early breast cancer patients, shown to express cytokeratin, also displayed the $CD44^{+}CD24^{-}$ phenotype in all specimens. The mean prevalence of putative stem/progenitor cells among marrow samples was 72% compared with a mean prevalence of $<10\%$ of the primary tumor samples (47). Furthermore, another study reported that 35% of circulating tumor cells from breast cancer patients had the $CD44^{+}/CD24^{-/low}$ stem/tumorigenic phenotype (48). An expression profile of an 11-gene stem cell-like signature in primary breast tumors is a consistently powerful predictor of a short interval to disease recurrence, distant metastasis and death after therapy (49). Genes differentially expressed as an invasiveness gene signature in $CD44^{+}/CD24^{-}$ cells compared with normal breast epithelium showed a significant association with both metastasis-free and overall survival in patients with breast cancer independently of established clinico-pathologic variables (50). Interestingly, this $CD44^{+}$ cell signature in primary invasive tumors was associated with a higher risk of distant metastasis, but distant metastases were enriched for more luminal epithelial $CD24^{+}$ cells, implying a phenotypic switch during tumor progression (51,52). Altogether, these

studies suggest that BCSCs are present in disseminating circulating tumor cells (48), enriched in early metastatic lesions (47) and can recapitulate the molecular signature of the primary tumor (28), where expression of 'stemness' genes is associates with recurrence and metastasis (49,53).

Breast cancer subtypes, BCSCs and the normal cell of origin

The identification of distinct intrinsic subtypes of breast cancer begs the question as to whether the different subtypes contain different CSC phenotypes as well as the cell of origin from which each subtype arises (13).

Different cancer subtypes; different BCSCs?

A study of 321 node-negative and 318 node-positive breast cancers concluded that identification of putative CSCs *in situ* identified high-risk breast cancer patients (54). Recently, in a 275-patient study, Park *et al.* (55) investigated the $CD44^+CD24^-$ putative stem cell marker as well as others (vimentin, osteonectin, connexin 43, acetaldehyde dehydrogenase, CK18, GATA3 and MUC1) in primary breast cancers of different subtypes and histologic stage. Generally, this study revealed a high degree of diversity in the expression of several of the selected markers in different tumor subtypes and histologic stages. $CD44^+CD24^-$ cells were most common in basal-like tumors, $ALDH1^+$ cells were the highest in $HER2^+$ and basal-like tumors, whereas CK18, GATA3 and MUC1 expression was more common in luminal subtypes (55). In addition to these clinical data and based on experimental data, Campbell *et al.* proposed that $CD44^+CD24^-$ and $CD44^-CD24^+$ cells may be cancer cell subpopulations competing for dominance as in the clonal evolution model (56). This notion is supported by studies that failed to correlate $CD44^+CD24^-$ cells with breast cancer progression or prognosis but negatively correlated $CD24^+$ cells with outcome (51,56–60). The $CD44^+/CD24^-$ profile has been suggested to be more relevant to breast cancer of basal origin and CD44 expression may indicate enhanced engraftment regardless of any CSC characteristics (61). It is noteworthy that CSCs specific for luminal type A and type B breast cancers are yet to be reported.

Cell of origin and breast cancer histogenesis

The CSC hypothesis does not necessarily imply origin from adult stem cells although self-renewal and multilineage potential are cardinal features of both. Better understanding of the differentiation hierarchy of normal breast tissue may yield insights into understanding histogenesis, hierarchical organization and heterogeneity of breast tumors. However, caution must be exercised in overinterpreting similarities in these features to mean they indicate similarities in cell of origin between normal and malignant breast tissue.

The more frequent expression of luminal markers such ERα and GATA3 in luminal tumors may indicate origin from the normal luminal compartment. Similarly, CK5 and CK17 expression in basal tumors led to their nomenclature after the myoepithelial/basal compartment. For several years, many groups speculated that this tumor type had a basal cell origin and, particularly, a stem cell origin (62), only to have this confronted by the discovery of a luminal progenitor as the target population for basal-tumor development (20). A direct role for luminal progenitors as the cells of origin for *brca1*-mutant basal breast cancers was demonstrated in a mouse model. Targeted deletion of the *brca1* gene in the basal cell layer resulted in the development of aggressive malignant adenomyoepitheliomas, whereas *brca1* gene in luminal progenitors preferentially generated carcinomas that phenocopied human *brca1*-mutant and sporadic basal-like breast cancers (63). These findings lend further support to the emerging theme that the molecular classification, or currently accepted nomenclature, of cancer does not always reflect the nature of the cell of origin (64).

Criticisms of the CSC hypothesis

The CSC model is not universally accepted and some of the properties of CSCs can be explained by the clonal evolution model (51,65–67). One of the main criticisms is the use of xenotransplantations, where the microenvironment in mice is not suited to supporting the growth of tumors from human cancer cells, which would otherwise be tumorigenic. However, this criticism has been addressed using a syngeneic p53-null mouse mammary tumor model, which provided direct evidence for the existence of a tumor-initiating subpopulation of CSCs (68).

Another criticism is an assumption that CSCs express a stable phenotype in a disease marked by genetic divergence and instability. The relationships between the different abovementioned populations of BCSCs identified from *in vitro* and animal models are not well understood. It is unclear whether all of the different BCSCs phenotypes identified thus far represent similar primitive multipotent cells or whether some are lineage-restricted progenitors. The BCSC question is further complicated by the notion of altered 'states of stemness' and 'phenotypic plasticity'. Tumor cells can acquire or lose molecular markers throughout their progression as well as hijack normal mechanisms of phenotypic transition. Plasticity and genetic divergence among cancer cells are not necessarily incompatible with the CSC hypothesis, however, it would be difficult to define hierarchical structures in malignant tissues should transdifferentiation and dedifferentiation from more differentiated populations is possible [phenotypic switch (69)]. A recent study found that the expression of an embryonic stem cell-like signature is upregulated in all tumor cells in primary breast cancers (70), and behooves us to consider carefully the design of studies of CSCs and their role in malignancy. Nonetheless, this study also found that a tissue-specific stem cell transcriptional program is upregulated specifically in the CSC population versus non-tumorigenic cells (70). In fact, breast primary tumors with CSC molecular traces, which correlate to tissue-specific stem cell signatures, are significantly associated with higher risk of death for the patient (70). Although this study supports the concept of a CSC phenotype and its correlation with patient outcomes, tumor cells that adopt an embryonic stem cell-like dedifferentiated signature may have sufficient plasticity to adopt a tissue-specific stem cell signature and thus behave as CSCs via transdifferentiation. Programs for the plasticity of epithelial cells and the acquisition of stemness have been described: epithelial–mesenchymal transition or the reverse mesenchymal–epithelial transition. Several reviews have addressed studies linking epithelial–mesenchymal transition/mesenchymal–epithelial transition to BCSCs/tumor-initiating cells, cancer progression, metastasis and therapy resistance and readers are referred to an excellent treatise on this topic (71–75).

Not this CSC, the other one: clonal evolution of BCSCs?

Despite the pessimistic discussion of the CSC hypothesis, positive identification of the most primitive normal or malignant stem cells remains the key challenge in the field. In normal breast tissue from women and mice, it appears that primitive normal stem cells with bi/multipotent potential can be identified (17–19). The relevance of such bipotent cells to malignancy has been recently demonstrated. Pece *et al.* (76) isolated normal human mammary stem cells to near purity that shared features with both epithelial ($CD24^+/EpCAM^+$) and myoepithelial ($CD49f^+/CK5^+/TP63^+$) cells based on their quiescent state (retention of the lipophilic dye PKH26). The transcriptional profile of these bipotent $PKH26^{pos}$ stem cells could prospectively isolate stem cells from normal breast and breast tumors and predict biological and molecular features of breast cancer subtypes. More interestingly, the heterogeneity and molecular profile of human breast cancers were correlated with their CSC content (76). This study suggests that identifying a more primitive progenitor/stem cell type rather than a more differentiated progenitor cancer cell type would provide a more clinically meaningful measure for a particular breast cancer subtype. Thus, although the $CD44^+/CD24^-$ profile may be more relevant to basal breast cancer (61), the above observations suggest that all breast cancer subtypes have the same CSCs, but which differ in number (76) and thus lead to differences in disease progression and posttreatment relapse.

Current CSC research lacks investigations into the contribution of CSCs to intratumoral genetic divergence and into the divergence

between the primary tumor and metastases of breast cancer [e.g. (77)]. Although different oncogenes and their mutations can transform different normal differentiated stem or progenitor cells to varying degrees of stemness, data concerning the intra-clonal genetic heterogeneity of CSC populations are missing (78). In the study by Pece *et al.*, the authors propose a model for mammary tumorigenesis, where oncogenic transformations determine the frequency at which CSCs will skip asymmetric divisions, thus influencing the number of CSCs within a tumor that in turn determine some biological and clinical features of breast cancer subtypes (76). Additional transformations and epigenetic changes may also restrict differentiation toward certain lineages (luminal versus basal) (76).

A mixed model of tumorigenesis

More recently, Greaves (78) proposed a mixed model for tumorigenesis to address the high genetic instability and plasticity of tumor cells. In this model, cancer-initiating cells with differences in self-renewal potency, phenotypic properties or numbers represent genetically diverse substrates for selection during cancer progression (78) (Figure 2). Such a model may account for the heterogeneity within the distinct subtypes of breast cancer as well as the genetic divergence within a tumor and among the metastases, which arise from CSCs within the primary tumor but which accumulate additional mutations in their new environment.

Although we seek to understand different models of breast cancer initiation and progression (CSC, clonal evolution or mixed), disease recurrence and spread from cancer cells that resist therapeutic intervention remains a clinical reality. The remainder of this review addresses the implications of resistant 'CSCs' and 'clones' for breast cancer therapy and the possible therapeutic strategies that may be used to enhance patient outcomes.

Implication of CSC on breast cancer therapy

Cytotoxic drugs principally target rapidly dividing cells, thus a self-renewing, long-lived and relatively quiescent CSC population may be more resistant to therapy. Practically, the definition of CSCs implies that recurrence after anticancer treatments is associated with the survival of these cells. This concept has gained much prominence in the field of cancer research with several studies reporting the enrichment of CSCs after conventional treatments (Figure 3).

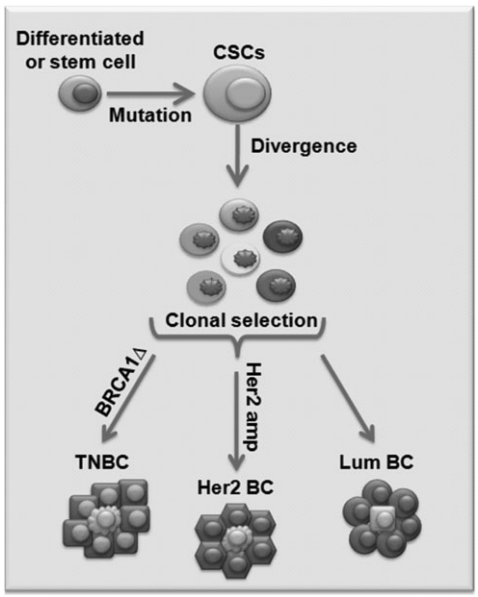

Fig. 2. Mixed model of clonal evolution of CSCs to account for tumor heterogeneity. Mutations transform differentiated normal cells, progenitor or stem cells to generate CSCs. CSCs may accumulate additional mutations (genetic divergence) that give rise to different clones of CSCs, which undergo clonal selection. Dominant clones determine the subtype of breast cancer [triple-negative breast cancer (TNBC); *Her2*-gene amplified and Luminal breast cancers].

The $CD44^{+}/CD24^{-/low}$ stem cells are relatively resistant to ionizing radiation (79–81). Similarly, side populations, which initiated tumors *in vivo* are more resistant to ionizing radiation than the non-side population (82). Furthermore, CSCs increase during the course of fractionated radiation (79,83). The enrichment of CSCs has been described following epirubicin treatment of SKBR3 breast tumor *in vivo* (84) and CSCs contribute to cisplatin resistance and tumor propagation in a BRCA1/p53 mammary tumor model (85). Finally, Li *et al.* have shown that conventional chemotherapy delivered in a neoadjuvant setting may enrich for putative $CD44^{+}CD24^{-}$ CSCs, whereas neoadjuvant lapatinib administered to patients with HER2-positive breast tumors might decrease putative CSC frequency (86), suggesting that conventional treatments may be selecting for resistant clones that can be candidates for disease recurrence.

Data are emerging to support the concept that BCSCs are responsible for some breast cancer recurrences posttreatment and perhaps after targeted anticancer therapies (86–90). Mechanisms underlying the therapeutic resistance of CSCs have not been fully elucidated. However, several mechanisms have been suggested to explain the response of these cells to therapies, including, amongst others: (i) DNA break repair; (ii) activation of cell cycle checkpoint proteins; (iii) activation of self-renewal pathways and self-renewal itself and (iv) evasion of senescence or apoptosis by CSCs.

DNA damage response

A delicate balance exists between DNA replication and repair in cell proliferation, self-renewal and quiescence in stem cell maintenance. Levels of DNA repair in human embryonic and adult stem cells are elevated (91–93), thus providing a mechanism for enhanced survival. DNA damage elicits various response pathways, which aim to repair the damage or eliminate cells if the damage is beyond repair. The DNA damage response (DDR) is a signaling cascade of proteins that interact upstream with damaged DNA and downstream with regulators of cell cycle progression and cell survival. The types of DNA lesions that activate the DDR pathways and subsequent cell cycle checkpoints have been the subject of many reviews (94–97). Cytotoxics induce single and double DNA strand breaks (DSBs); DSBs are generally considered as the more cytotoxic of the two DNA lesions (98). It is not possible to summarize all aspects of the DDR pathways here; instead, we will summarize findings relating to the DDR pathways and radiation resistance of BCSCs.

BCSCs, DDR pathways and radiation resistance as an example

Preclinically, putative BCSCs populations were found to be radioresistant compared with tumor cells with a non-stem cell-like phenotype (80,81). Future studies will have to investigate these differences more closely. *In vitro* studies are providing mechanistic insights to radioresistance of CSCs. DSBs identified by staining of γ-H2AX and reactive oxygen species levels were lower in $CD44^{+}CD24^{-}$ mammospheres compared with adherent and monolayer cultures following ionizing radiation (79). In agreement, Karimi-Busheri *et al.* (99) reported a reduced level of reactive oxygen species, a reduced number of DSBs identified by γ-H2AX staining and more active single DNA strand break repair pathway in mammospheres when compared with adherent monolayer cultures. However, the authors conclude that mammospheres exhibit a similar DSB response after irradiation, due to the formation of 53BP1 and Rad51 foci suggesting that both the non-homologous end joining and the homologous recombination pathways can be initiated (99). Nevertheless, it is difficult to comprehend from this study how DSB repair can be normal in mammospheres when the ataxia telangiectasia mutated activation (S1981 phosphorylation), γH2AX, ataxia telangiectasia mutated downstream activity (p53 Serine 15 phosphorylation) and retinoblastoma protein responses are abnormal. Future experiments therefore are required to

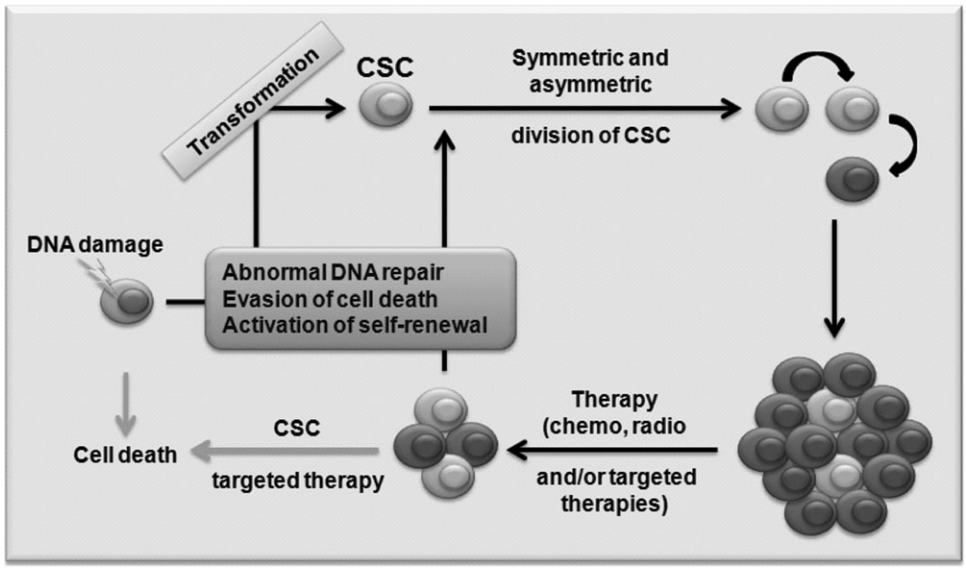

Fig. 3. Persistence of treatment-resistant CSCs produces disease recurrence but also new therapeutic opportunities. CSCs arising by transformation lead to tumor initiation. Upon treatment with conventional and targeted anticancer agents, resistant cells and/or CSCs may survive and cause recurrence. Survival of these cells is enhanced by the aberrant activation of DNA damage repair, anti-apoptotic and self-renewal signaling pathways. Specific targeting of these cells (perhaps after primary therapy) may result in their eradication and thus prevent disease recurrence.

study the upstream activation of the Mre11, Rad50 and Nbs1 complexes as well as altered chromatin states in mammospheres before and after ionizing radiation. Notably, the enhanced survival of mammospheres post-radiation was proposed to be due to downregulation of the senescence pathway associated with increased telomerase activity (99). Similarly, $CD44^{+}CD24^{-/low}$ $proteosome^{low}PKH26^{+}$ BCSCs have been shown to survive fractionated radiation, which mobilized them into cell cycle. Furthermore, BCSCs were resistant to radiation-induced apoptosis and were arrested in the G_2 phase of the cell cycle, although non-CSCs were prone to radiation-induced apoptosis and were driven into senescence (100). At present, it is difficult to extrapolate a differential capacity for DDR in sphere-forming tumor-initiating cells to a globally resistant tumor cell phenotype in the absence of quantitative preclinical studies because intratumoral heterogeneity may complicate this relationship. Some of these *in vitro* findings have been corroborated in cancer biopsies and carcinoma cell lines (including breast) by demonstrating a stem cell-like subset of $CD44^{high}$ cells with an increased frequency of cells in G_2, increased clonogenicity and decreased apoptosis (101). The extended G_2 phase may be used by these cells as a mechanism to prolong repair of DNA damage. These observations suggest that drugs targeting G_2 checkpoint proteins should be assessed because removing the G_2 block could make these cells more sensitive to apoptosis-inducing treatment.

Signaling in BCSCs: therapeutic opportunity

Notwithstanding the controversy surrounding phenotypic identity of BCSCs, the finding of a distinct subpopulation of breast cancer cells that contribute to chemoresistance and radioresistance and organ-specific metastasis helps to set a direction for future therapeutic research (102). Many reports and reviews address the idea of targeting CSCs based on such characteristics (103–105) as microenvironment (niches) (93,106) and the developmental signaling pathways related to renewal and differentiation: Hedgehog (107–110); Notch (111,112) and Wnt (113–115). Importantly, interest grows in combining treatments that target these pathways with conventional anticancer treatments (chemo/radiotherapy) and/or with non-developmental pathways exploited by CSCs (116,117).

Mechanisms that BCSCs use to evade therapy-induced damage remain to be elucidated, and new targets that can be used to sensitize BCSCs to therapy remain to be discovered. Several examples of such targets have been described. For example, in line with the important role for the phosphatase and tensin homolog/phosphoinositide 3-kinase/Akt/β-catenin pathway in the regulation of breast cancer stem/progenitor cells (43), small-molecule inhibitors that specifically target components of Ras/PI3K/phosphatase and tensin homolog/mammalian target of rapamycin as well as Ca2+/calmodulin-dependent protein kinase, Ras/Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase pathways with conventional therapy, showed synergistic effects in the induction of death in drug-resistant breast cancer cells (118). Inhibition of the Akt pathway inhibits canonical Wnt signaling as well as selectively inhibiting repair of DNA damage in breast tumor-initiating cells thus sensitizing them to ionizing radiation (119).

Other agents have shown interesting anti-CSC activity that may be useful for sensitizing BCSCs to chemotherapy and radiotherapy. For example, although the exact mechanism of action of the tamoxifen analogue *N*,*N*-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine (DPPE; tesmilifene) is not known, treatment with DPPE alone reduced mammosphere formation and viability of $CD44^{+}/CD24^{-}$ breast cancer cells and DPPE further cooperated with doxorubicin to completely eradicate tumorigenic cells (120). Epidermal growth factor receptor (EGFR) signaling is positively linked to stemness in human breast cancer (121). Inhibition of EGFR signaling disrupts mammosphere formation (122) and, unlike chemotherapy, lapatinib (an EGFR1/HER2 tyrosine kinase inhibitor) does not lead to an increase $CD44^{+}/CD24^{-}/Lin^{-}$ BCSCs (86). An anti-EGFR monoclonal antibody disrupted mammosphere formation and decreased the percentage of $CD44^{+}/CD24^{-}$ cells in mammospheres (123). Another target may be heat shock protein-90 (HSP90) despite the lack of understanding of its role in CSCs. Experiments using tumorigenic glioma stem cells supported a role for the use of the HSP90 inhibitor, 17-N-allylamino-17-demethoxygeldanamycin, in the removal of CSC (124). Although HSP90 inhibitors have not been investigated in BCSCs, ectopic expression of Engrailed-1 (En-1) is associated with a stem cell phenotype that is inhibited by 17-N-allylamino-17-demethoxygeldanamycin (125). These data suggest that HSP90 inhibitors may be active against BCSCs, which would be advantageous due to their radiosensitizing effect (126).

Other interesting agents with emerging anti-CSC activity are tocotrienols; naturally occurring forms of vitamin E. Dietary delivery of γ-tocotrienol (γ-T3) suppressed tumor growth in a syngeneic

implantation mouse mammary cancer model by inhibiting cell proliferation and inducing apoptosis (127). Recently, Luk *et al.* (128) reported that γ-T3 may be an effective agent against prostate CSCs thus accounting for its anticancer and chemosensitizing effects. It seems feasible that γ-T3 might also be active against BCSCs and is worthy of investigation. Differentiation of CSCs presents another possible therapeutic strategy. Histone deacetylase inhibitors (HDACi) and other epigenetic drugs are promising CSC targets (129,130). Apoptosis is yet another major pathway abrogated in CSCs. Small-molecule inhibitors targeting key proteins in the intrinsic apoptotic pathway are an effective therapy in refractory malignancies (131–134).

Novel drug discovery programs for CSCs are being pursued. For example, using a high throughput screen of selective inhibitors of CSCs, Gupta *et al.* (135) identified that salinomycin significantly inhibited the expression of BCSC markers as well as the growth of mammospheres *in vitro* and mammary tumors and metastasis *in vivo*. Salinomycin is a *p*-glycoprotein inhibitor (136) that selectively induced apoptosis in apoptosis-resistant cancer cells via non-conventional apoptotic pathways (137) and thus represents a potentially novel and effective anticancer drug.

DNA repair and checkpoint inhibitors: targets in CSC therapy?

It is becoming increasingly clear that the pathways of DNA damage repair are qualitatively and/or quantitatively different between normal cells and cancer cells, and hence these pathways offer targets for new cancer therapies (95). Potent inhibitors of non-homologous end joining (138–140), base excision repair (141–145) and homologous recombination (146,147) DNA repair pathway have been characterized. The rationale and effect of checkpoint abrogation, specifically the G_2 checkpoint, on anticancer treatments have been reviewed (148–150). Inhibiting the checkpoint kinases, especially Chk1, may hold promise in BCSCs since these cells show prolonged G_2 arrest (100,101). Checkpoint inhibitors were shown to restore the sensitivity of glioblastoma CSCs to ionizing radiation (151). Nonetheless, and to our knowledge, the effect of molecular inhibitors of the DDR and checkpoints on CSCs specifically, at least in breast cancer, has not been investigated.

Old drugs, new tricks?

Most, if not all, of the drugs discussed above, including those that directly target developmental pathways [e.g. cyclopamine against hedgehog signaling (152)], have been in preclinical and clinical testing for some years. Despite some promising results, treatment failure in advanced and metastatic disease raises questions about therapy planning. The utility of drugs against CSCs *in vivo* either alone or in combination with conventional chemotherapy/radiotherapy should address a very important yet simple factor; the ultimate probability of therapeutic success. Are combinations that show synergistic activity against the bulk of the tumor mass relevant to the CSC population? When in a treatment schedule should a drug that targets CSC be applied? Do CSC progeny and differentiated cancer cells represent a frontline defense for the rare and niche-hosted CSCs? Are CSCs remaining after chemotherapy and radiotherapy the same as those remaining after targeted therapy?

Using primary tumor explants to discover appropriate markers for the tumor-initiating cells/CSCs has been problematic. Simply targeting these cells in their microenvironment may not be easy. If CSCs represent a rare subpopulation (0.1–10% of the tumor mass) and show further deregulation of DNA repair/response and apoptotic pathways than their differentiated progeny, then this subpopulation will ultimately limit the success of the therapy. In this sense, assaulting the treatment-responsive and differentiated cancer cell clones risks clonal selection of the most resistant and aggressive CSCs, which are left behind in the tumor mass as a 'Trojan Horse'. In the final analysis, the most effective and rational approach may be first to 'debulk' the tumor mass with conventional agents before specifically targeting this therapy-limiting CSC population with newer and yet to be discovered agents.

Here, we illustrate this approach by drawing on the example of the CSC developmental marker, Notch-1. Activation of Notch-1 post-radiotherapy might be a mechanism for accelerated repopulation of tumors by renewal of stem cells and their progeny [including BCSCs (111,153)] during planned radiotherapy treatment intervals (79). Selective immunoblockade of Notch-1 in established tumors showed potent inhibition of tumor growth in preclinical models for a 14-day period (154). It is not clear whether long-term monitoring would detect recurrence, a common observation in most preclinical studies mimicking clinical experience. Better outcomes might be expected if this Notch1 targeting strategy were initiated after initial debulking treatment that reduced the number of non-CSCs thus increasing the probability of CSC targeting. Furthermore, such antibody could be used to target alpha particles to the CSCs since it is estimated that carcinoma cells, including CSCs, are unable to survive one or two hits from an α-particle (155). Furthermore, such immunotargeting of nanoparticles may enable specific delivery of larger cytotoxic payloads loads to the CSC population (156,157). A similar rationale may be proposed for the immunotargeting of drugs or use of inhibitors [such as Notch-1 inhibitor (158)] with anti-CSC activity or chemosensitizing and/or radiosensitizing activity to increase the probability of killing these rare treatment-resistant populations after first removing the bulk of non-CSCs.

Concluding remarks

Although the question of cell of origin and histogenesis is biologically relevant, CSCs as a treatment-resistant subpopulation of tumor cells is of greater therapeutic relevance. Additional prospective clinical investigations to evaluate CSC phenotypes using different markers and/or gene expression signatures should be done. These studies should not only focus on the prognostic significance of these measurements but also on characterizing the changes in the level of the particular CSC phenotype before, during and after therapy as well as its relationship to disease relapse. Inherently, such studies may provide new targets with therapeutic potential. Urgently needed also are preclinical studies that characterize the plasticity and heterogeneity of CSCs and treatment-resistant cells and address their impact on the rates of tumor regrowth and cure after conventional treatments. Although surgery and adjuvant therapies remain the first treatment option for early breast cancer, agents that target the quiescent CSCs remaining locally or as micrometastases should be incorporated into preclinical models to aid in the design of appropriate clinical trials. These considerations may apply particularly to breast cancer patients with HER2-positive or triple-negative disease in whom survival is poorest. In the neoadjuvant setting, treatments that target CSCs after first-line therapies may reduce locoregional recurrence and distant relapse if CSCs and treatment-resistant cells drive disease recurrence.

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