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Review

Implications of stemness-related signaling pathways in breast cancer response to therapy

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ABSTRACT

There is accumulating evidence that breast cancer may arise from a small subpopulation of transformed mammary stem/progenitor cells, termed breast cancer-initiating cells (BCICs), responsible for initiation and maintenance of cancer. BCICs have been identified in clinical specimens based on $CD44^{+}/CD24^{-/low}$ membrane expression and/or enzymatic activity of aldehyde dehydrogenase 1 (ALDH1+), or isolated and *in vitro* propagated as non-adherent spheres. This cell population has been demonstrated to be able to recreate, when injected in mice even at very low concentrations, the same histopathological features of the tumor they were derived from and to escape from current therapeutic strategies. Alterations in genes involved in stemness-related pathways, such as Wnt, Notch, and Sonic Hedgehog, have been proven to play a role in breast cancer progression. Targeting these key elements represents an attractive option, with a solid rationale, although possible concerns may derive from the poor knowledge of tolerance and efficacy of inhibiting these mechanisms without inducing severe side effects. In addition, efforts to develop alternative BCIC-targeted therapies against stemness markers (CD44 and ALDH1) and molecules involved in regulating EMT- and HER2-related pathways, or able to reverse the multi-drug resistance phenotype, or to induce differentiation and to control cell survival pathways are currently ongoing and encouraging results from pre-clinical studies have already been obtained using *in vitro* and *in vivo* models.

1. Introduction

Despite advances over the last decades in breast cancer management, this malignancy is still the most common cause of cancer-related mortality among women worldwide. The heterogeneity of breast cancer is now broadly proven and categorized by tumor segregation into different molecular subtypes, defined by gene expression profile, that correlate with clinical behavior and are used to refine therapeutic strategies [1,2]. Nevertheless, in the majority of breast cancer patients treatment failure still occurs and women continue to die, mainly due to an evolutionary process toward a metastatic and treatment-resistant disease. This might suggest the involvement of a subpopulation of tumor cells able to resist to treatment and to regenerate tumor, also at distant sites. This behavior seems to be ascribable to tumor cell subpopulation with stem-like characteristics, intrinsically chemotherapy and radiotherapy resistant [3–5]. These cells are, in theory, responsible for breast cancer initiation growth, and able to drive disease progression, due to their potential involvement also in tumor relapse [4,6–9]. Thus, to plan effective therapeutic strategies for tumor eradication and to counteract relapse, attempts have been done to identify alterations in stemness-related signaling pathways frequently endowed by stem-like tumor cells and which may represent therapeutic targets.

2. Breast cancer initiating cells

The existence of a small subpopulation of transformed self-renewing stem cells, the so-called cancer stem cells or, more properly defined, cancer initiating cells (CICs), responsible for initiation and maintenance of cancer, was postulated more than 50 years ago [10]. Since then, a growing body of evidence has shown that cancer cells with stem-like characteristics can be identified within a number of different malignancies. However, the definitive evidence of the existence of CICs, as well as their precise

Abbreviations: BCIC, breast cancer-initiating cell.

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characterization, remains to be obtained, at least in the majority of solid tumors.

By definition, CICs can be identified by three main features: (1) the expression of cell surface markers that can be used for their differential and reproducible isolation; (2) the ability to grow in non-adherent conditions; (3) the ability of both self-renewal and differentiation that allows these cells, when xenografted in immunocompromised mice, to rebuild the cell heterogeneity observed in the original tumor [11]. The presence of stem-like cells in human breast cancers was first demonstrated in 2003 [5]. Breast cancer cells are extremely heterogeneous and by subfractioning the parental population using different markers it was possible to isolate cells lineage negative (LIN^-, lacking the expression of CD2, CD3, CD10, CD16, CD18, CD31, CD64 and CD140b), positive for CD44 ($CD44^+$) and negative (or with a low expression) for CD24 ($CD24^{-/low}$). The subpopulation of cells characterized by high/low presence of CD44/CD24 (two adhesion molecules) was demonstrated to be highly tumorigenic since few hundreds of these cells were able to drive tumor formation when inoculated into NOD/SCID mice and to recreate the heterogeneity of the parental tumor [5]. In addition, this cell population was detected with high frequency in metastatic lesions [2] and in pleural effusions, as well as among the cytokeratin-positive fraction of cells disseminated in the bone marrow of breast cancer patients [9]. Another hallmark of stemness is associated with the cell capability to survive and proliferate in non-adherent culturing conditions in medium without serum and supplemented with growth factors [12]. This functional approach has been used to isolate and propagate as spheroids (mammospheres) cells from clinical tumors along several *in vitro* passages. Moreover, this population of breast cancer-initiating cells (BCIC) was also found to be enriched in $CD44^+/CD24^-$ cells and showed tumor-initiating capability when injected into NOD/SCID mice [13].

Even though the combination of $CD44^+/CD24^-$ has emerged as one of the most important marker for BCIC isolation, only a subpopulation of $CD44^+/CD24^-$ cells is truly responsible for xenograft tumor formation and, for this reason, additional markers have been and are currently being investigated. In this respect, elevated activity of the detoxifying enzyme aldehyde dehydrogenase (ALDH1) has been found associated to tumor-initiating characteristics of breast cancer cells [14]. Accordingly, this enzymatic activity, evaluable by ALDEFLUOR assay based on its ability to oxidate intracellular aldehydes, has been previously reported as a known stem cell marker in human hematopoietic cells and in other tumor types (such as colon and lung cancers) [15,16], However, it is important to highlight that even though the marker combination $CD44^+/CD24^-$ allows the isolation of breast cancer cells enriched for stem-like properties, this population showed only partial overlapping to the fraction of $ALDH1^+$ cells. The small subpopulation, identified by the combination of all these BCIC markers ($CD44^+/CD24^-$ and $ALDH1^+$), has been shown to be endowed with a higher tumorigenic ability than other subpopulations, being just 20 cells sufficient to rebuilt tumor when xenografted in NOD/SCID mice [14]. In our experience on mammosphere-derived cells, co-localization between CD44 and ALDH1, with the absent/low expression of CD24, accounted for a large intertumor diversity, with the fraction of $CD44^+/CD24^-/ALDH1^+$ cells varying from 0.7% to 19% (median value, 7%) among the different tumors. Furthermore, a recent study has shown that also in primary breast cancers $CD44^+/CD24^-$ and $ALDH1^+$ stainings are only partially overlapping [17]. Accordingly, $CD44^+/CD24^-$ markers usually stain cells located at the tumor edge, near the stroma counterpart, whereas ALDH1 marks more centered cells. These results are consistent with previously published data showing an increase of ALDH1 activity in hypoxic condition, as it could be in the center of the tumor [18]. In the same study, gene expression analysis showed that $CD44^+/CD24^-$ cells were enriched for mesenchymal-related genes, while $ALDH1^+$ cells appeared more epithelial-like. This evidence suggested the existence of two different subsets of BCICs: a subpopulation with mesenchymal-like phenotype responsible for the invasion potential and another one with epithelial-like features responsible for tumor growth. It is possible that the high plasticity of these cells could allow the interplay between these two phenotypes responsible for the identification of a population showing both $CD44^+/CD24^-$ and $ALDH1^+$ [17]. An important role in the control of this phenotype switching could be played by tumor microenvironment able to induce epithelial to mesenchymal transition (EMT) and its reverse MET by several stimuli. In support to this hypothesis, recent evidence has shown that BCICs displayed a very similar gene signature to cells that have undergone EMT [19,20]. Accordingly, it has been demonstrated that overexpression of two EMT master key regulators, TWIST1 and SNAIL1, not only induced breast epithelial cells to undergo EMT, but also to acquire self-renewal and tumor-initiating abilities as well as to display $CD44^+/CD24^-$ phenotype [19,21]. Interestingly, pathways involved in CIC maintenance and self-renewal, like Notch, Wnt and Hedgehog signaling pathways, are also able to induce EMT [22–24]. The capability of CICs to switch between epithelial-like and mesenchymal-like states and *vice versa* has been postulated and verified in several studies and could be responsible for their ability to colonize distant sites and to form metastasis [25,26]. Interestingly, it has been shown that BCICs and tumor bulk cells shared similar DNA alterations [27], suggesting that genetic diversity cannot account for BCIC high tumorigenicity. Consistent with this observation, differentiated mammary epithelial cells *in vitro* have been described to spontaneously acquire stem cell features without gain of genetic alterations [28,29]. Possibly confirmation of such evidence could indicate that a combination treatment, able to simultaneously target both tumor bulk and BCICs, may represent a successful therapeutic option.

3. Breast cancer initiating cell resistance to conventional anticancer therapies

Cytotoxic anticancer agents preferentially hit active proliferating cells while spare slowly dividing cells. However, chemotherapy-induced cytotoxic effects could be bypassed by several resistance mechanisms, such as a decreased drug uptake and/or increased drug extrusion. Two types of resistance to chemotherapy can occur: the intrinsic one (in which patients never respond due to the attitude of cancer cells to immediately escape from pathways directly hit by the treatment, or to their lack of drug uptaking) and the acquired one (in which patients initially respond to treatment and then become resistant). In both cases current anticancer therapies are not effective leading to an unfavorable patient outcome [2].

A link between intrinsic resistance to anticancer treatments and stem cell features has been suggested by the evidence of an increased number of $CD44^+/CD24^{-/low}$ cells in the residual tumor following chemotherapy [30,31]. Although the molecular players involved in such effect are still subjects of speculations, these observations can be explained on the basis of CIC features. In fact, CICs: (1) have a quiescent status and/or a low proliferation rate [32] that contrast the activity of antineoplastic agents which preferentially target rapidly dividing cells; (2) overexpress anti-apoptotic proteins, such as bcl-2 and survivin, that, at least in part, protect from apoptosis induction [33]; (3) express high levels of proteins involved in efflux pumping mechanisms, which decrease the cell capability to retain drugs [33].

BCIC resistance to chemotherapy has been studied both *in vitro*, taking advantage from the isolation of putative BCICs from patient tissues but also from established breast cancer cell lines [13], and *in vivo*, in breast cancer mouse models showing that conventional