Cancer stem cells: perspectives of new therapeutical approaches for breast cancer

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TABLE OF CONTENTS

1. ABSTRACT

Currently stem cells are hypothesized to play a central role in the origin, spread and resistance to treatment of breast cancer. Common anticancer therapy is effective but transient, with tumor relapse and metastatic disease often occurring. For therapy to be more effective, debulking of differentiated tumors must occur followed by targeting of the remaining surviving often quiescent tumor stem cells. New therapeutics aimed at cancer stem cells are achieved through non immunological and immunological methods. The former include elective ABC drug transporters or the heat shock protein 90 inhibition, targeting the self-renewal signalling pathways or the EMT program, differentiation therapy, or other interventions to eliminate BrCSCs. The latter include targeting specific antigens expressed on BrCSCs, dendritic cells (DCs) based vaccination and blockers of the extrinsic signals at CSC niche. Here all these novel approaches related to breast cancer stem cells are described.

2. INTRODUCTION

Stem cells are at the top of the cellular hierarchy and give rise to progenitors with more restricted lineage potential. They can generate daughter stem cells or can differentiate into a variety of mature cell types (1). The perpetual renewal of tissues and organs is driven by resident stem cells and progenitors that guarantee maintenance and regeneration after injury or involution. Therefore, most tissues and organs contain small populations of primitive stem cells and progenitors. Because of their special properties, in the last decade a large debate arose considering stem cells as potentially useful in many fields of human diseases. Also they have been hypothesized to play a central role in the origin of cancer, spread and resistance to treatments. In breast cancer, the recent better understanding of the mechanisms of resistance to pharmacological therapy related to stem cells has open new scenarios and perspectives for therapeutical approaches that here will be considered.
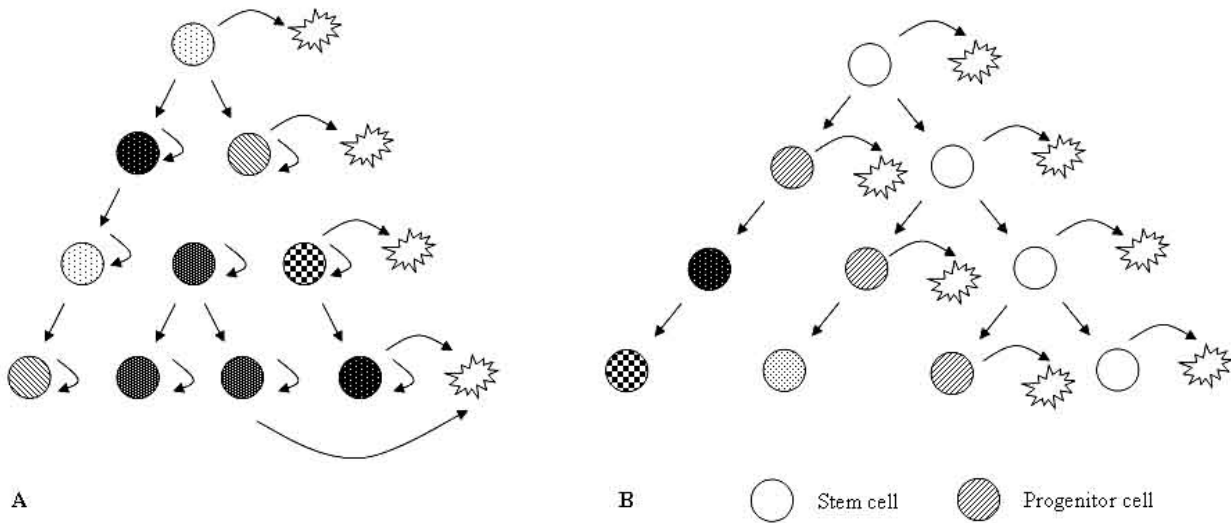
Figure 1. Two models of heterogeneity of the cancer cells. A, Heterogeneity is due to environmental factors or ongoing mutations in the cancer cells. In this model, all of the cancer cells have the intrinsic ability to form tumors. B, Cancer stem cells have the exclusive ability to self renew. As in normal tissues, the stem cells would give rise to more stem cells with the capacity to form new tumors, as well as the other heterogeneous populations of cancer cells that lack the ability to form new tumors (also see text).

3. IDENTIFICATION AND TUMOURIGENICITY OF BREAST CANCER STEM CELLS (BrCSCs)

The first evidence of a cancer stem cell origin for breast cancer was reported in 2003 by Al-Hajj *et al* (2). Using cells derived from primary breast tumors, they demonstrated that a subpopulation within the samples could generate palpable tumors in non-obese diabetic severe combined immunodeficient (NOD/SCID) immunocompromised mice. All of the tumorigenic cells expressed CD44, alone or in conjunction with ESA (epithelial specific antigen, EpCAM) but did not express CD24. The fact that these cells exhibited characteristics of stem cells and the similarities between the expression markers with those of multipotent epithelial progenitor cells lead to the proposal that these breast CSCs (BrCSCs) originated from a normal mammary stem cell (2). Two theories describe the origin of BrCSC. One is that CSC arise from somatic stem or progenitor cells with genetic alterations that lead to malignant behavior. The long lived nature of somatic stem cells means that they are potential targets for multiple accumulated mutations that lead to malignancy (3). Moreover, a number of adult stem cells have been shown to undergo spontaneous transformation *in vitro* to generate tumorigenic stem cells (4-6). The other theory proposes that BrCSCs arise from the dedifferentiation of a lineage committed cell that has acquired stem cell characteristics through mutation (7). Both theories incorporate the essential features of CSC: the retention of stem cell characteristics with the acquisition of malignant properties (8) (Figure 1).

The major signalling pathways implicated in stem cell self-renewal and carcinogenesis are the Wnt, Notch and Hedgehog signalling pathways (9). Tumourigenicity assays test the ability of a putative cancer stem cell population to generate a tumour that recapitulates the phenotype of the tumour from which it was sourced. While tumourigenicity is observed in the $CD44^+/CD24^-$ population, this characteristic may not be confined to $CD44^+/CD24^-$ cells, and therefore, the proportion of $CD44^+/CD24^-$ may not correlate with tumourigenicity (10). However, although larger numbers of cells with an alternative phenotype could induce tumours in NOD/SCID mice, only the $CD44^+/CD24^-/ESA^+$ cells could recapitulate the morphology and heterogeneity of the unsorted original tumour (11).

The gold standard of tumourigenicity is considered to be the ability to generate tumours in immunodeficient mice. However, these models are not immune to bias. The genetic background of the animal model used, as well as the number of cells and the site of injection, may influence the tumourigenic capacity of human cells (12-13). These studies highlight the importance of the microenvironment and using more than one mouse model to assess *in vivo* tumourigenic capacity.

4. BREAST CANCER STEM CELLS AS THERAPEUTIC TARGETS

In the past two decades, more than 30 new anticancer drugs have been introduced, but survival rates have improved only marginally for many forms of cancer (14). In contrast to most cancer cells, cancer stem cells are slow-dividing and have a lowered ability to undergo apoptosis and a higher ability of DNA repair, making them more resistant to traditional methods of cancer treatment such as radiation and chemotherapy (15-16). Current anti-cancer therapy is effective but transient, with tumour relapse and metastatic disease often occurring. For therapy to be more effective, debulking of differentiated tumours

must occur followed by targeting of the remaining surviving, often quiescent, tumour stem cells (17).

New therapeutics aimed at cancer stem cells are achieved through non immunological and immunological methods. The former include elective ABC drug transporters or the heat shock protein 90 inhibition, targeting the self-renewal signalling pathways or the EMT program, differentiation therapy, or other interventions to eliminate BrCSCs. The latter include targeting specific antigens expressed on BrCSCs, dendritic cells (DCs) based vaccination and blockers of the extrinsic signals at CSC niche.

4.1. Non immunological interventions
4.1.1. The inhibition of ABC drug transporters

The high expression of ATP-binding cassette (ABC) transporters such as breast cancer resistance protein (BRCP-ABCG2) and MDR-associated protein-1 (ABCB1/MDRR1) is a property of stem cells which is also a feature of cancer stem cells (18). These transporters provide a protective mechanism against xenobiotic toxins and also are partially responsible for the resistance of cancer stem cells to traditional therapies. Pheophorbide, a chlorophyll catabolite, is a specific probe for ABCG2 which causes inhibition of ABCG2 efflux properties (19). The combined use of ABC transporter inhibitors and chemotherapy could be used to increase the efficiency of chemotherapeutic drugs to kill cancer stem cells (16). ABC transporter inhibitors also cause inhibition of normal stem cells, leading to potential toxicity in the bone marrow, and play a role in the maintenance of the blood-brain barrier (20). An *in vitro* study has shown the benefit of gefitinib, a tyrosine kinase inhibitor, in reversing chemotherapy resistance in multidrug resistant breast cancer cells expressing ABC transporters (21). Also, gefitinib has been reported to successfully overcome SN-38 resistance in small cell lung cancer *in vitro* (22). Therefore, gefitinib in combination with current chemotherapeutic agents may be an alternative approach in eradicating breast CSCs (23).

4.1.2. The self-renewal signalling pathways
4.1.2.1. Hh, Notch, Wnt, Akt and mTOR signalling inhibitors

CSCs may be eliminated by selectively targeted therapies against various self-renewal signalling pathways including Shh, Notch, BMI-1 and Wnt signalling pathways; in fact, although normal stem cells and CSCs share some pathways to maintain their self-renewal, it appears that CSCs are more likely dependent on certain putative pathways (23-24).

The hedgehog (Hh) signalling, acting through BMI-1, regulates the self-renewal of normal and malignant human mammary stem cells. This process is blocked by specific inhibitors such as cyclopamine, a steroid-like molecule. The development of cyclopamine analogs and other Hh inhibitors is currently underway. It has shown some promise in inhibiting the growth of medulloblastoma and could be used in treatments of other tumours (25-26).

The Notch pathway has also been investigated as a target. Notch signalling acts as a regulator of asymmetric cell fate decisions and affecs cell fate commitment (27); there are 4 Notch receptors (notch 1 to 4) interacting with surface-bound or secreted ligands (Delta-like 1, Delta-like 3, Delta-like 4, Jagged 1 and Jagged 2). On ligand binding, Notch receptors are activated by serial cleavage events involving gamma secretase. Because the enzyme gamma secretase is necessary for Notch processing, gamma secretase inhibitors are able to inhibit Notch signalling (28). Inhibition of the Notch pathway by the gamma secretase inhibitor DAPT or a Notch-4 neutralizing antibody significantly reduced mammosphere formation *in vitro* (29). Clinical trials utilizing gamma secretase inhibitors in combination with chemotherapy for women with advanced breast cancer are being initiated. An antibody capable of blocking Notch-4 has been used *ex vivo* to block the formation of mammospheres from primary human specimens (30). Notch3 signalling is particularly important for the proliferation of HER-2 negative breast cancer cells (31); Mammosphere formation in HER2 overexpressing cell lines was significantly reduced by trastuzumab, and the effect was ameliorated by antagonism of Notch pathway (32). The mechanism of this interaction between Notch and EGFR pathways remains to be elucidated; however, this cross talk appears to be relevant therapeutically, as a 2-6 fold increase in Notch-1 activity in cell lines after treatment with trastuzumab or lapatinib was demonstrated. Inhibition of the Notch pathway led to desensitization to trastuzumab, and combination of Notch antagonism and trastuzumb inhibited growth in both trastuzumab resistant and sensitive cell lines (33). Conversely, estrogen signalling down regulates Notch signaling. Estradiol reduced the expression and activation of Notch-4 and Notch-1 in cell lines; this reduction in Notch activity could be abrogated by tamoxifen and fulvestrant. In a mouse xenotransplantation assay using BT474 cell line, combination therapy with tamoxifene and a gamma secretase inhibitor (GSI) was significantly superior to tamoxifen alone and authors conclude that tamoxifen antagonism of the estrogen stimulus leads to the reactivation of the Notch signalling pathway promoting proliferation and survival (34). Further investigations are needed to determine whether this effect is on a cellular population level or specifically mediated by the CSC population.

Wnt signalling exerts an effect on the self-renewal and differentiation of stem cells. Transgenic mice expressing a MMTV-Wnt oncogene develop mammary tumours which strongly express stem cell markers (35). Activation of Wnt pathway increases cytoplasmic beta-catenin, which translocates to the nucleus, where it binds to transcription factors in the LEF1/TCF family. Targeting of beta-catenin has received a lot of attention as retinoic acid (RA) has been shown to inhibit beta-catenin activity (36) and tyrosine kinase inhibitors such as imatinib have been shown to down-regulate beta-catenin signalling (37). This indicates the potential to block the self-renewal capacity of BCSCs in the patient with this antibody and opens up the use of other antibody therapies in the elimination of BCSCs.

Another pathway which is frequently deregulated in cancer cells originates with the activity of phosphoinositide-3 kinase (PI3K) and results in the induction of the Akt kinase and mTOR. An intermediate pathway component is PTEN, a phosphatidylinositol phosphatase, an enzyme which is functionally impaired (gene deletion) in about 40% of the breast cancer cases (38). A correlative observation concerns the self-renewal of haematopoietic and neuronal stem cells. They are regulated by PTEN (39) and it is therefore reasonable to assume that inhibitors of Akt and mTOR may be able to target normal and malignant breast stem cells (35) and might become useful therapeutics in breast cancer treatment.

4.1.3. Epithelial to mesenchymal transition (EMT) program inhibition

Isolated human breast CSCs display a mesenchymal phenotype, similar to that in cells that have undergone EMT. The EMT program seems to give carcinoma cells the phenotypic traits necessary for invasion and metastasis as well as for resistance to cancer therapies including resistance to EGFR inhibitors. The role of EMT in resistance to EGFR-targeted therapies is likely to also have implications in breast cancer, because 15% to 35% of tumors have EGFR overexpression, which is associated with poor patient prognosis. Although EGFR-targeted therapies have shown promise for breast cancer in cell culture and xenograft studies, EMT may be one mechanism that explains why fewer than 10% of patients show responses in clinical trials of EGFR-targeted therapies (40). By targeting the molecular activators of EMT, such as TGF-beta, Wnt/beta-catenin, Notch, and Hedgehog signaling pathways and pathways activated by several tyrosine kinase receptors and downstream transcriptional regulators of EMT, including Twist, FOXC2, ZEB1/2, and Snail family members, carcinoma cells could be prevented from undergoing EMT and gaining an invasive phenotype. Therefore, pharmacologic inhibition of EMT or reversion of CSCs to a more-differentiated epithelial phenotype by inducing MET (i.e., redifferentiation) may induce cell death or sensitize CSCs to conventional therapies. Thus, the EMT program could be an attractive target for therapeutic intervention, as the inhibition of EMT may serve to not only inhibit tumor cell invasion and metastasis but also the formation of CSCs via EMT. Moreover, as many of the pathways found to regulate EMT in various human cancers also have roles in stem cell self-renewal (41-42), future therapies that target these common pathways are hypothesized to be a more effective way of treating cancer patients.

4.1.4. Selective inhibition of heat shock protein 90 (HSP90)

A possible target for a directed therapy against breast CSCs may be heat shock protein 90 (HSP90).

Hsp90 is a member of a group of proteins known as molecular chaperones, which act as facilitators and quality control factors for the correct folding, assembly and transport of proteins within the cell. Higher levels of Hsp90 expression have been observed in cancer cells and inhibition of Hsp90 has been linked to cancer and stem cell differentiation (43). However, there is relatively little

known about the difference in the Hsp90-mediated signalling pathways in CSC (43) compared to cancer cells or indeed, normal stem cells. If there is no sufficient difference between Hsp90 expression and function in normal stem cells compared with CSC, there is a risk that anti-Hsp90 drugs may deplete the normal stem cell population that are essential for correct organ homeostasis. Comparative analysis of the biochemistry and cell biology of somatic and CSC populations should be an essential component of these drug development programmes. The most important features of Hsp90 as an anti-cancer target are the ability to simultaneously inhibit multiple-signalling pathways, and the drug selectivity (44-45). The selectivity of the drug is achieved by the specific characteristics of Hsp90 function in cancer cells and stem cells (46). Hsp90 functions to exclusively mediate the folding and stability of numerous (over 300 interactions have been determined) transcription factors and signalling intermediates *in vivo*, including some key regulators of breast cancer, such as HER2 and Akt (47-48). This makes Hsp90 an attractive target to simultaneously inhibit multiple cellular pathways in cancer (44). Many Hsp90 client proteins control fundamental cellular processes, such as apoptosis, cell cycle control and cell proliferation and differentiation (49-51), many of which are essential to the establishment and survival of cancer and CSC (Figure 2). In most cases, the drugs targeting Hsp90 are selective for cancer cells, due to the fact that Hsp90 is over-expressed and highly complexed in cancerous cells compared to normal cells (52). A number of anti-Hsp90 drugs are currently being developed as anti-cancer agents (53). The most advanced of these compounds is 17-AAG (17-(Allylamino)-17-demethoxygeldanamycin), which is based on the natural Hsp90 inhibitor geldanamycin (54) and currently undergoing Phase II clinical trials. Data from a recent paper support a role for 17-AAG in the removal of CSC. In particular, 17-AAG was effective at inhibiting growth of both glioma cells and glioma CSC. The drug also was shown to synergise with radiation therapy (55). Tanespimycin is an intravenous HSP90 inhibitor with a reported response rate of 26% when combined with trastuzumab in heavily pretreated HER2 positive patients with progressive disease on trastuzumab (56-57). The PI3K/Akt (58-60) and Ras/Raf/MEK/ERK (61-62) signalling pathways are chaperoned by Hsp90.

The PI3K/PDK/Akt pathway induces the expression of genes that regulate cell proliferation and over-expression of Akt has been shown to be responsible (at least in part) for drug resistance in tumours (60). The mitogen-activated protein kinase (MAPK) Ras/Raf/MEK/ERK pathway, of which Raf-1 is an Hsp90 client (63) leads to the induction of genes that control cell differentiation. Mutations of Ras and Raf are commonly observed in cancers. In a study, where both the PI3K/Akt and MEK/ERK pathways were activated by mutant Ras, astrocytes transformed into cells resembling brain CSCs in mice deficient in p53. These cells were capable of self-renewal and were aberrantly differentiated. (64). The dual targeting of both the PI3K pathway (controlling proliferation) and the MAPK pathway (controlling differentiation) by selective inhibition of Hsp90 could represent an efficient treatment (8).
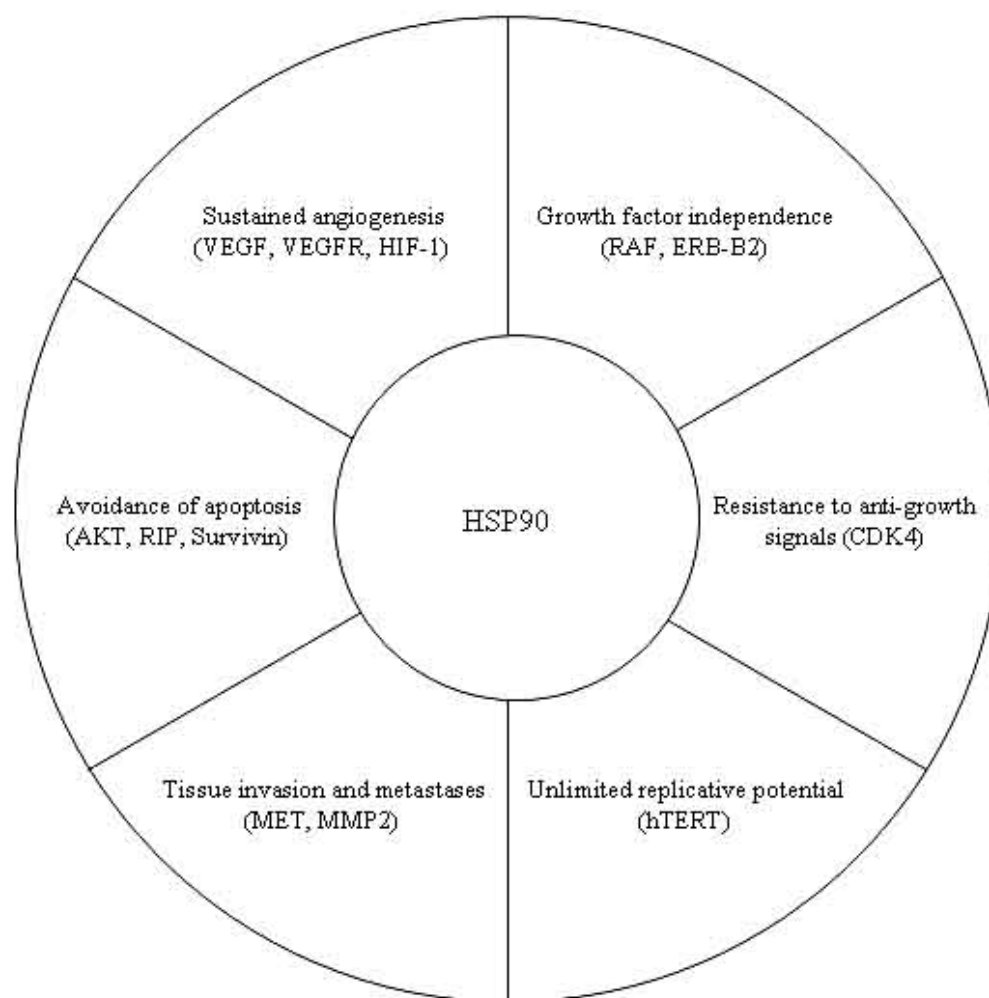
Figure 2. How HSP90 inhibition may interfere with the principal molecular pathways sustaining cancer growth. Client proteins involved in the various phenotypic aspects of malignancy are shown.

### 4.1.5. Cell differentiation therapy

Targeting the cancer stem cell pool to differentiate results in the loss of the ability for self-renewal, a hallmark of the cancer stem cell phenotype and the reason behind maintenance of the cancer stem cells. One differentiation agent used in the clinic is retinoid acid (RA) (vitamin A) (26). RA and vitamin A analogues can promote differentiation of epithelial cells and reverse tumour progression through modulation of signal transduction. RA-based therapy followed by chemotherapy has found use in acute promyeloctyic leukaemia and could also find use in solid tumour therapy (65). Recently, the use of bone morpho-genetic protein (BMP)-4 has been described as a non-cytotoxic effector capable of blocking the tumourigenic potential of human glioblastoma cells (66). This therapeutic agent is able to work by reducing proliferation and inducing expression of neural differentiation markers in stem-like tumour-initiating precursors. These findings are intriguing in light of the role that BMP-4 may play in some breast tumours (67).

Recently, promising results have been reported *in vitro* (68) and *in vivo* (69) using zebrafish embryonic extracts as stem cell differentiation stage factors (SCDSF). Finding ways to specifically target BCSCs via differentiation therapy, alone or in combination with others, is an application that needs to be further defined. Figure 3 summarises the described therapeutical interventions.

4.1.6. BrCSCs elimination through other interventions
4.1.6.1. Metformin

Recently, it has been reported that a standard drug for diabetes, metformin, selectively kills CSCs in 4 genetically different types of breast cancer cell lines. The combination of metformin with doxorubicin, a well defined chemotherapeutic agent, kills both cancer stem cells and non-stem cancer cells in culture, and reduces tumor mass much more effectively than either drug alone in a xenograft mouse model. Authors conclude that the results provide a rationale and experimental basis for using the combination of metformin and chemotherapeutic drugs to improve treatment of breast cancer patients (70).
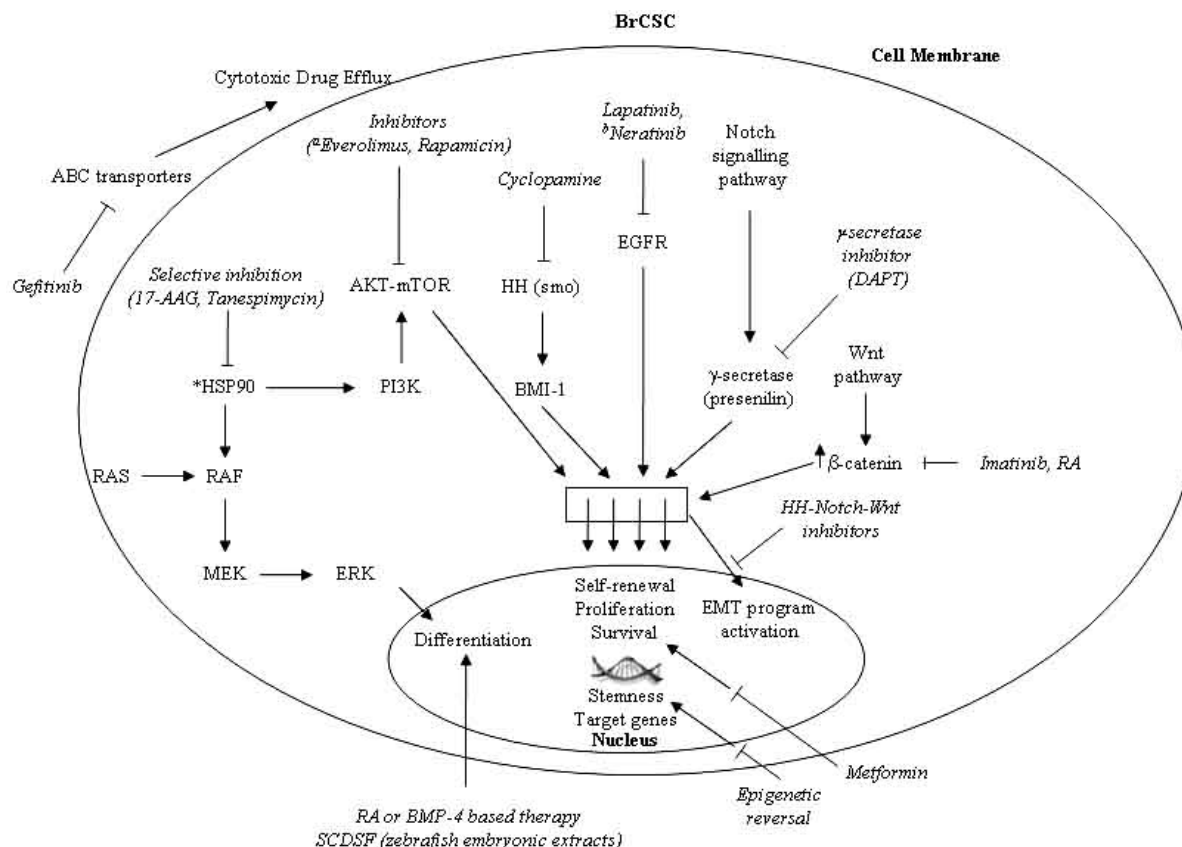
Figure 3. Proposed non immunotherapeutical interventions on breast cancer stem cells (BrCSCs); ABC transporters: ATP binding cassette transporters; 17-AAG: 17-(allylamino)-17-demetoxygeldanamycin; HH (smo): Hedgehog (smoothened); RA: retinoic acid; EMT: epithelial-mesenchymal transition; BMP-4: bone morpho-genetic protein-4; SCDSF: stemm cells differentiation stage factors; DAPT: *N*-[*N*-(3,5-Difluorophenacetyl)-l-alanyl]-S-phenylglycine *t*-butyl ester; HSP90: .*Folding and stability of many other signaling intermediates and transcription factors depend on heat schock protein 90 (HSP90). Client proteins of HSP90 control apoptosis, cell cycle, proliferation, differentiation (see Figure 2); [a]preliminary data suggest a cross talk between estrogen receptor and the PI3K-AKT pathway; [b]is an irreversible pan-ERB-B TKI targeting HER1, HER2 and HER4; (also see text).

4.1.6.2. Pro-apoptotic to anti-apoptotic proteins increased ratio

Chemotherapeutic-induced cell death is generally programmed by apoptosis; the elimination of CSCs may be feasible by increasing the ratio of pro-apoptotic to anti-apoptotic protein and signal pathways, for example targeting at the pro-apoptotic members of bcl-2 family. Akt through m-TOR maintains nutrient uptake to the mitochondrion. This nutrient uptake sustains cellular processes constitutively activating Akt itself. Moreover, the activated Akt blocks the pro-apoptotic bad protein. Rapamicin is m-TOR inhibitor that markedly decreases these Akt effects on nutrient uptake and apoptosis. This agent and its analogs are promising in tumors as breast cancer in which Akt has been activated. (71).

4.1.6.3. EGFR, HER2/neu (erbB2) and mTOR inhibitors

Treatment of patients with HER-2-positive tumours with lapatinib, an EGFR and HER-2/neu (ErbB-2)

dual-tyrosine kinase inhibitor, resulted in non statistically significant decreases in the percentage of CD44+/CD24low population and in the ability for self-renewal as assessed by mammosphere formation (72). Moreover, a significant increased pathological complete response rate occurred. Because HER2 was recently shown to drive the mammary stem cell pool (73), agents that directly target EGFR/HER2, such as lapatinib, may be effective therapeutic options for targeting the CSC population in HER2-positive breast cancer. New HER2 agents, such as pertuzumab and neratinib have shown promise in HER2 positive, trastuzumab refractory breast cancer (57). Giving the importance of the PI3K pathway in mediating resistance to trastuzumab, inhibition of the downstream mTOR kinase appears to restore sensitivity to trastuzumab in preclinical models (74). Early data from phase I clinical trials with the combination of everolimus, an oral mTOR inhibitor, with trastuzumab and cytotoxic chemotherapy indicate response rates of 46% and 14% respectively in heavily pretreated populations (57, 75-76). Figure 4 summarises the described immunotherapeutical interventions.
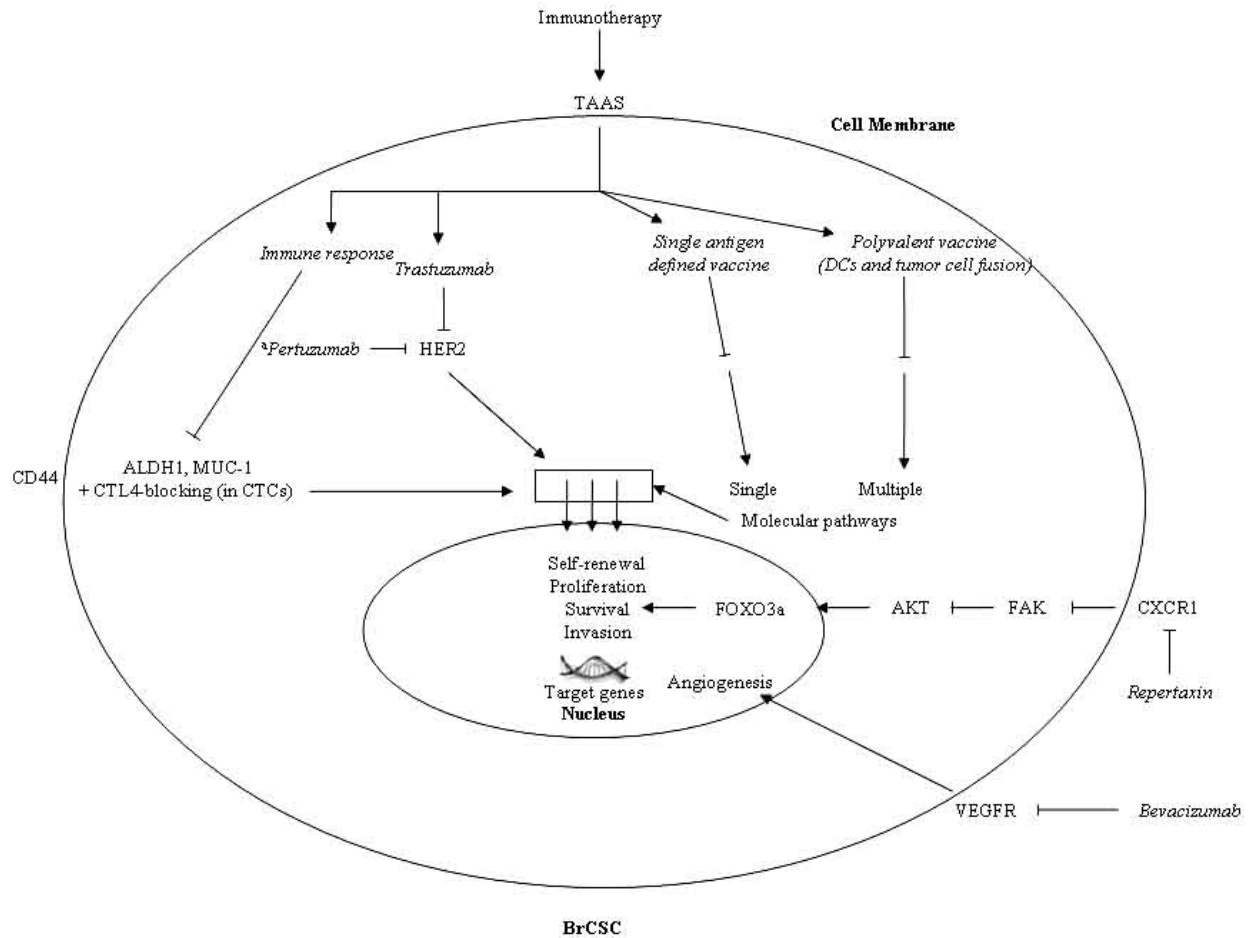
Figure 4. Proposed immunotherapeutical interventions on breast cancer stem cells (BrCSCs); TAAS: tumor-associated antigens; DCs: dendritic cells; CXCR1: chemokine receptor-1; VEGFR: vascular entothelial growth factor receptor; ALDH1: aldehyde dehydrogenase-1; CTL4: cytotoxic T-lymphocyte antigen (CTL)-4; CTCs: circulating tumor cells; [a]the epitope recognized by pertuzumab might be different than that trastuzumab binds on the extracellular portion of HER2. It blocks the ability of HER2 to heterodimerize with other members of the EGFR family (also see text).

4.1.6.4. Reversal of epigenetics

Tumours may be driven by mutated proteins and inappropriate signalling, but also epigenetic mechanisms of gene expression of genes involved in "stem-ness" such as Oct4, Nanog, and Sox2 could be behind tumour formation. Epigenetically altered genes have been reported in breast cancer (77). Epigenetic abnormalities include both losses and gains of DNA methylation. Particularly, DNA methyltransferases (DNMTs) are considered to be responsible for the DNA methylation abnormalities in cancer. Studies of the cancer epigenome are already beginning and reversal of tumor-associated silencing of tumor suppressor genes is increasingly being targeted for cancer treatment (77).

4.2. Immunological interventions
4.2.1. Immunotherapy targeting breast cancer stem cells

Immunotherapy aimed at stimulating the immune system to recognise and eliminate tumours has been explored for many years but recently has gained renewed interest. Many vaccines targeting solid tumours have been employed with varying success both preclinically and clinically in the treatment of cancer (78). Interest in these vaccines has been bolstered by increased understanding of the role that the immune system plays in cancer and by the molecular identification of tumour-associated antigens (TAAs) that can be used as targets for therapy. In the case of breast cancer, evidence is now coming to light that the immune system is involved in the surveillance of cancer, is impaired by tumours during the progression of cancer, and can recognise and eliminate cancer. Dendritic cells (DCs) are central to these processes as a result of their role in innate immunity and in generating humoral and cellular immune responses. DCs are professional antigen-presenting cells and initiators of adaptive immunity through processing antigens and presenting epitopes in the context of major histocompatibility complex (MHC) to T cells. They are capable of stimulating cyto-lytic T-cell responses (CTLs) to TAAs on tumours and are equipped with all of

the necessary co-stimulatory and cytokine signals needed to drive an effective immune response to tumours (79).

4.2.1.1. Specific antigens expressed on the cancer stem cells: ALDH1, MUC-1, ESA, CK5, CD49F

Cancer stem cells need to be further defined in terms of gene expression that determines stem-ness and identifying molecules that are involved in regulating stem cell qualities. Gene expression comparisons have been conducted and can be used to identify what genes are expressed by the cancer stem cell compartment compared with normal stem cells (80). A recent work has demonstrated that the expression profile of BCSCs more closely resembles that of embryonic stem cells than that of adult stem cells. An embryonic stem cell-like gene expression pattern was found to be upregulated in the CD44+/CD24low tumourigenic fraction of cancer cells (81). Additionally, mapping the transcriptional profile of embryonic stem cell-like genes in primary human breast cancer has revealed two classes of tumours: those with an embryonic stem cell-like activated program and those with an embryonic stem cell-like repressed program. Those tumours with an embryonic stem cell-like activated program were associated with poorer differentiated tumours that were more likely to progress to metastasis and death. The CD44+/CD24low phenotype in human breast tumours has been found to be associated with basal-like tumours, and particularly BRCA1 hereditary breast cancer, and has been linked to expression of CD49f, elevated expression of CK5/14 and EGFR, and low expression of ER, PgR, and HER-2 (10). Basal-like tumours often have been linked to poorer prognosis. The occurrence of the CD44+/CD24low phenotype was found to be lower in tumours of luminal type, and particularly HER-2+ tumours, irrespective of ER status.

Mammospheres have shown expression of markers ESA, CK5, and CD49f (alpha6-integrin) among many others, which potentially could be used to identify or target BCSCs (82). High activity of ALDH1 identified the cells capable of self-renewal and high tumourigenicity in NOD/SCID xenografts. Another molecule used to identify or target BCSCs is CD44, which is a membrane receptor involved in cell adhesion, motility, and metastases and which along with P-glycoprotein (the product of the MDR1 [ABCB1] gene of drug transporters) has been linked to MDR (83). CD29 (beta1-integrin) and CD49f (alpha6-integrin) expression has also been associated with murine mammary stem cells with a Lin-CD24+ phenotype (84). Additionally, neither Sca-1 (stem cell antigen) expression nor the SP phenotype was found to be expressed in the mammary reconstituting Lin-CD29hiCD24+cell population (85). However, one report using the human breast cancer line MCF7 has shown that the SP phenotype can be used to identify cells with characteristics of cancer stem cells that express the tumour antigen MUC1, supporting a role for the SP in further analysis of human BCSCs (86). BRCA1-deficient murine breast tumours contain heterogeneous (CD44+/CD24low and CD133+ cell phenotypes) cancer stem cell populations (87). However, both populations of cells expressed the stem cell-associated genes Oct4, Notch1, Aldh1, Fgfr1, and Sox1. That study shows that

cancer stem cell populations share a common set of characteristics which may be exploited for targeting cancer stem cells (88). Determining what mutations are present in cancer stem cells, how these mutations aid either the stem cell-like phenotype or the tumourigenic phenotype of these cells, and how to best target these mutations is going to be a critical component of immunotherapy. It is preferable that any therapy developed is able to target metastatic disease before metastases occur. Circulating tumour cells have been detected in the blood of patients with metastatic and primary tumours and have been linked with a decrease in survival times (89). The identification of molecular targets on disseminated tumour cells, targets that might also occur on BCSCs, could lead to better treatments. Another critical component of immunotherapy targeting BCSCs is the determination of the number of BCSCs residing in the tumour and the ability to eradicate them with the treatment, as tumour regression will be dictated by any escape of BCSCs. Additionally, any immunotherapy approach will likely require additional therapies such as cytotoxic T-lymphocyte antigen (CTLA)-4 blocking of the T-cell regulation to overcome tolerance of the immune system to cancer (90). Finally, the identification of appropriate antigens expressed on BCSCs needs to be conducted along with identifying the best way to stimulate an immune response using these antigens (17).

4.2.1.2. HER2 receptor

The HER2 receptor is overexpressed in about 25% of human breast cancers. HER2 overexpressing tumors are more aggressive, are frequently associated with metastasis and have an unfavorable prognosis. Recent evidence indicates that HER2 pathway may play an important role in the maintenance of CSCs. HER2 overexpression is significantly correlated with the expression of stem cell marker ALDH1 and increases the proportion of stem cells (91). HER2 may regulate the stem cell population in breast tumors by preventing the progression of stem cells into ER+ progenitor cells (1). Trastuzumab, a recombinant humanized antibody against the HER2 receptor, reduces the stem cell population in trastuzumab sensitive breast cancer cell lines (73) So, the clinical efficacy of HER2 inibitors may be a result of the ability of these agents to directly target breast cancer stem cells (35).

Trastuzumab in particular has been used successfully to treat to HER2-positive, early and metastatic breast cancer. When used in combination with conventional chemotherapeutic drugs, trastuzumab results in a response rate of between 50 and 84%. However, the majority of patients develop resistance within 1 year of the start of treatment (92-94). Resistance is thought to be, at least in part, due to the role of the CSC population (57). The interaction between CD44 and hyaluron has been implicated in resistance to trastuzumab by masking the interaction of the antibody with the Her2 receptor on the cell surface (95). Recently it has been shown that trastuzumab resistance in a cell line isolated from the pleural fluid of a HER2 positive patient with progressive disease on trastuzumab (JIMT-1) is marked by an increase of adefluor-positive cells in culture, an effect

mediated by activation of the PI3K/Akt pathway (73). There is a growing literature documenting a variety of possible mechanisms of escape from trastuzumab, involving many of the same markers that have been implicated in the biology of CSCs: 1) loss or blockade of extracellular trastuzumab binding site, mediated by CD44; 2) signalling through alternative receptor pathways, mediated by CXCR4 and beta1 integrin, whose expression is increased in breast CSCs; 3) upregulation of downstream signalling pathways, due to loss of PTEN and PI3K/Akt pathway that is critical in breast CSCs survival; 4) activation of pro-survival signalling pathways, such as XIAP, surviving, telomerase, PP2A, CD40, with resistance to apoptosis; 5) activation of Notch signalling, that promotes self-renewal and proliferation; 6) induction of epithelial to mesenchymal transition (EMT) (57).

4.2.1.3. DC-based vaccination

Recently, the examination of adoptive transfer of HER-2-specific T-cell clones clinically suggests the potential to use an antigen-specific therapy to eliminate specific single tumour cells but that additional treatments are needed to reduce the solid tumour (96). DC-based vaccination strategies encompass a variety of different approaches that can be divided into two groups: antigen-defined vaccines and polyvalent vaccines.

Targeting of a single tumour antigen may allow for regression of the tumour by gene deletion or downregulation (97-98). However, immunotherapy that targets single antigens may also fail to target the underlying cells responsible for cancer initiation or tumour metastasis, thus limiting the long-term success of this treatment. To avoid these problems, it appears that several epitopes need to be targeted simultaneously for an effective therapy through the use of a polyvalent vaccine. One way to achieve this is use whole tumours in the vaccine. Fusions of DCs and breast tumours have been shown to elicit CTLs against autologous tumour cells (99). However, to induce long-lasting clinical responses using immunotherapy, targeting cancer stem cells may be required. To achieve this, specific antigens expressed on the cancer stem cells but ideally not by normal stem cells must be found and targeted. While there are not yet antigens fulfilling this description, they are likely to be present considering the various pathways identified as different between these two cell populations. Hence, it is important to identify as many antigens as possible on BCSCs in order to develop a polyvalent vaccine approach targeting several antigens.

4.2.1.4. Blockers of the extrinsic signals at CSC niche

In addition to intrinsic pathways regulating stem cell functions, such as Hh, Notch and Wnt, normal and malignant stem cells are regulated by extrinsic signals generated in the microenvironment or CSC niche. In the breast, niche is composed of immune cells, mesenchymal elements that include fibroblasts, endothelial cells, adipocytes and extracellular matrix components (100). If the cellular microenvironment plays an important role in the regulation of the CSCs growth and survival, the strategies aimed at interfering with these interactions

represent a rational approach to target breast CSCs (101). In breast cancer cell lines, gene expression profiling of ALDH1 positive cells revealed overexpression of CXCR1, a receptor for the cytokine IL-8. CXCR1 expression was limited to a subpopulation of Aldefluor+ cells, and the addition of recombinant IL-8 increased the CSC population as well as its propensity to invasion (17, 102). IL-8 has been implicated in tumor metastasis in preclinical models of prostate cancer; in addition, tissue damage induced by chemotherapeutic agents may induce IL-8 as part of injury response. This suggests that strategies able at interfering with the IL-8-CXCR1 axis may be able to target CSCs. In a recent study, Ginestier et al examined the effects of CXCR1 blockade on breast CSC population, using both *in vitro* assays and mouse models. They showed that using CXCR1-blocking antibodies or repertaxin, a small molecule CXCR1 inhibitor, there was a selective decrease in CSc population *in vitro* as well as in NOD7SCID xenograft models. CXCR1 blockade induced massive apoptosis in bulk tumor cells via a bystander effect mediated by FASL/FAS mediated signalling. Administration of repertaxin retarded tumor growth and reduced the development of breast cancer metastasis in mice (101). CSCs niches are likely to be well endowed with a blood supply as a result of enhanced angiogenesis (103). Therefore, blockage of action of vascular endothelial growth factor (VEGF) signalling with bevacizumab could be an alternative approach. Although the CSC niche is conceptually attractive and might offer possibilities to disrupt the cellular communication required for CSC maintenance, the components involved are still poorly defined. The CSC niches themselves might have aberrant properties due to their adaptation to the requirements of CSCs. The simultaneous interference with the autonomous tumour cell pathways driving their growth and survival and the interference with the supportive microenvironment seems like a promising strategy. Chemotherapeutic and anti-angiogenic agents might cooperate effectively (104).

5. PERSPECTIVES

Recent research recognizes that CSCs play a central role in initiating and spread of tumor growth. Undoubtably, this is a relevant advance in the field and stimulated novel therapeutical approaches. However, only in the next years controlled clinical trials will answer the question whether or not these new therapeutical approaches alone or combined with the ongoing treatments significantly improve the outcome of these patients.

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