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Review

Targeting Cellular Signaling Pathways in Breast Cancer Stem Cells and its Implication for Cancer Treatment

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Abstract. *Breast cancer is a public health problem both in developing and developed countries. The breast cancer stem cell (BCSC) hypothesis has grown in the cancer research community. These BCSCs comprise of a small subpopulation of cells within the tumor mass which exhibit stem cell-like characteristics and have emerged as being responsible for tumor development, recurrence and metastasis in BC. The complexity of control of gene expression in BCSC is commonly driven by a myriad of signaling pathways triggered by extracellular signals, mutations and epigenetic control. Thus, some signaling pathways have been highlighted in BC, especially those linked to stem cell phenotype, such as nuclear factor-kappa B, signal transducer and activator of transcription 3, wingless-type MMTV integration site family (Wnt)/β-catenin, Hedgehog and NOTCH. Moreover, these BCSCs can also be influenced by the tumor microenvironment, for instance, hypoxic areas. Given the importance of signaling pathways and tumor microenvironment for breast cancer, this review focuses on the relationship between cellular signaling and BCSCs and its therapeutic implications.*

Breast cancer is a public health problem in developing countries, as well as in developed countries. Breast cancer is the second most common type of cancer in women worldwide, representing 22% of new cancer cases each year (1) and is responsible for more than 522,000 deaths of women annually (2). In developing countries, the 5-year survival rate of patients with breast cancer is around 50-60% (1).

At the end of the 1970s, the idea of tumor heterogeneity emerged, inferring that tumor masses were composed of differentiated and undifferentiated malignant cells. It was believed that undifferentiated tumor cells were the target of carcinogenesis and kept their basal phenotype growth as malignant stem cells (3). Thereafter, Pierce *et al*. discussed similarities with normal stem cell phenotypes and the less-differentiated tumor cells, postulating that similar phenotypes occurred because of mutations in the tumor stem cell and not because of a dedifferentiation process (4). Some data have demonstrated that this depends on the cancer type: some cancer stem cells arise from the transformation of normal stem cells, whereas other differentiated cells acquire stem cell properties through tumorigenesis (5, 6). In fact, several articles have reported that tumors are more complex than expected (6).

The origin of cancer stem cell terminology is still much discussed and some researchers have preferred the term 'tumor-initiating cells' because of the ability of self-renewal and initiating a new whole tumor, like normal stem cells. The first terminology, 'cancer stem cell', was probably given due to the discovery of hematopoietic stem cells, the main source of all differentiated functional blood cells and responsible for leukemia development. These hematopoietic stem cells are represented by a small population characterized by hematopoietic progenitor cell antigen cluster of differentiation 34^+ ($CD34^+$) and $CD38^-$ cell surface markers and, in leukemia, these are highly enriched and very much able to transfer disease (7). Independently of the nomenclature, how important are these stem cells for cancer? What are the main characteristics of these cells? What are their special cellular signaling systems which maintain or transform their treatment-resistant and aggressive phenotype?

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It is consolidated that cancer is a multistep process that usually starts with DNA reprogramming by mutations, epigenetics alterations or changes in cellular metabolism (8). These alterations result in a loss of the control of the expression of essential genes responsible for correct cell functions. In this review, we discuss the discovery of BCSC, characterizing them, and explore the current main signaling pathways involved in the BCSC phenotype. We also discuss promising targets of these pathways with therapeutic potential.

The Discovery of BCSCs: The Past

In solid tumors, a small proportion of cancer cells are clonogenic *in vitro* and *in vivo*. To test the tumorigenic potential, uncultured specimens of breast cancer cells from patients were fractioned by flow cytometry, based on the expression of surface molecules, and injected into the mammary fat pads of non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. This experiment identified a small subpopulation of the cancer cells which expressed cluster of differentiation 44 (CD44, hyaluronic acid receptor) and had low expression of cluster of differentiation 24 (CD24, a ligand for P-selectin) (*i.e.* $CD44^{+}CD24^{low/-}$) but were more tumorigenic than $CD44^{-}CD24^{+}$ cells. These cells were termed BCSCs or breast tumor-initiating cells (9). Additionally, the $CD44^{+}CD24^{low/-}$ population regenerated grafted tumors with a similar cell heterogeneity as exhibited in the breast tumor. This suggests that tumor heterogeneity present within breast tumors arises *via* differentiation of BCSCs into non-tumorigenic breast cancer cells (9). As well as these surface markers, the expression of aldehyde dehydrogenase 1 (ALDH1) has also been included as a characteristic of BCSCs (6). These cells have extensively been associated with tumor initiation, progression, metastasis, and resistance to radio/chemotherapy. Consequently, they are also associated with cancer recurrence and therapeutic failure (10).

Understanding BCSC Biology: The Present

Cellular signaling pathways are responsible for coordinating cellular and molecular characteristics. In BCSCs, this signaling represents the main route for self-renewal, treatment-resistance and aggressiveness. Analogously to the normal stem cells, BCSCs have an intrinsic relationship with embryonic-related pathways, such as wingless-type MMTV integration site family (Wnt)/β-catenin, Hedgehog and NOTCH. In addition, inflammatory-related signaling responses, such as nuclear factor-κB (NF-κB) and signal transducer and activator of transcription (STAT) 3 have contributed to this phenotype. Moreover, together with these signaling pathways, hypoxic tumor areas have been described to shelter and stimulate stem cell properties in breast cancer cells.

Wnt/β-catenin signaling and BCSC. Wnt/β-catenin signaling plays important roles in developmental processes, including patterning and cell fate determination (11). Secreted Wnt, which is palmitoylated by Porcupin, binds to both co-receptors Frizzled and low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6). Wnt–receptor interaction leads to recruitment of Axin and Dishelved proteins to the cell membrane and induces inhibition of glycogen synthase kinase (GSK)-3β protein. GSK-3β is a negative regulator of the Wnt pathway driven by β-catenin to proteasomal degradation induced by phosphorylation. Once GSK-3β is inhibited, β-catenin accumulates in the cytoplasm, moves to the nucleus and acts as co-transcriptional activator together with cAMP response element binding protein (CREB) binding protein (CBP) and T-cell factor/lymphoid enhancing factor (TCF/LEF) transcription factors. The β-catenin–CPB–TCF/LEF complex up-regulates oncogenes, such as *c-myc, Cyclin D1* and *Wnt* genes (11, 12).

In breast cancer, Wnt signaling is constitutively activated by an autocrine mechanism. In colon cancer and melanoma, genetic alterations of β-catenin and adenomatous polyposis coli (APC) have been identified; however, it has not had a significant impact on breast cancer cells (13). In BCSC, the role of Wnt signaling has been associated with the maintenance of stem cell properties: inhibition of WNT1 alters the phenotype to $CD44^{+}CD24^{-}ALDH1^{-}$, reducing *in vitro* and *in vivo* tumor formation and cellular migration (14).

Zhao *et al*. suggested that pyruvate kinase isozyme M2 (PKM2) is able to induce mammosphere formation, which could be mediated by promoting β-catenin transcriptional activity (15). Furthermore, Yeon Kim *et al*. demonstrated that suppression of GSK3/β-catenin signaling *via* the inhibitory activity of protein kinase D1 (PRKD1) was sufficient to reduce the stemness features of breast cancer cells (16).

The Hedgehog pathway and BCSCs. The hedgehog (Hh) family comprises of Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh). This family encodes signaling molecules that regulate several processes during embryogenesis, including cell proliferation, cell fate determination and patterning. This signaling also bears an important role in maintenance of the stem cell population in adult organisms (17).

The three Hh transcription factors zinc finger protein 1/2/3 (GLI-1/2/3) are held in the cytoplasm in an inactive state by interaction with constitutive cytoplasmic proteins kinesin family member 7 (KIF7) and suppressor of fused [SU(FU)]. In the inactive Hh pathway, patched1 (PTCH1) blocks migration of smoothened (SMO), a G protein-coupled receptor, to the cell membrane. SMO is responsible for transduction of Hh signaling inside the cell by coordinating activation of GLI transcription factors. The Hh protein released by cancer cells contributes to enhancing the activity

of stromal cells from the tumor microenvironment (18). Once the ligand Hh is secreted, it binds to PTCH1 receptor on responding cells, allowing SMO activation by its migration to the cell membrane where it starts Hh signal transduction. Activated SMO is phosphorylated by casein kinase-1 (CK1) and protein kinase A (PKA) and becomes able to remove GLI from KIF7-SUFU, Hh repressor proteins. Transcription factor GLI is then released to translocate into the nucleus, resulting in the expression of target genes (17, 19).

Several pieces of evidence suggest an important role of the Hh pathway in breast cancer, for example, Hh overexpression or *PTCH1* mutation/polymorphism results in constitutive activation of SMO and up-regulation of this signaling pathway (20). Moreover, these alterations have been linked to a more aggressive phenotype, such as a triple-negative breast cancer (20). Hh signaling is essential for maintaining the BCSC phenotype (21) and regulating self-renewal (22). Current data support the importance of Hh signaling breast cancer and pancreatic cancer stem cells, and to chronic myeloid leukemia, in which the Hh proteins are often up-regulated, promoting growth and self-renewal (23, 24).

He *et al*. reported that MCF-7 breast cancer mammospheres expressed increased levels of Hh pathway members in comparison to MCF-7 cells cultured as monolayers (25). They also observed positive correlation between the expression of SMO and GLI1 with BCSC markers $CD44^{+}CD24^{-}$.

Memmi *et al*. demonstrated that p63 (other important factor for promoting self-renewal in normal epithelial stem cells) contributes to the maintenance of BCSCs, using *in vivo* models (26). This transcription factor is responsible for up-regulates the levels of some Sh signaling members in BCSC context.

NF-κB signal transduction pathway and BCSCs. The NF-κB family consists of five conserved proteins, RELA (p65), RELB, c-REL, p50 and p52. All of them share the conserved REL homology domain (RHD) which is responsible for DNA binding, dimerization and association with the repressor protein inhibitor of kappa B (IκB). The NF-κB pathway comprises the canonical and the non-canonical pathways. The canonical pathway is downstream of the pro-inflammatory cytokine receptors, such as tumor necrosis factor alpha (TNF-α), Interleukin 1 beta (IL-1β); toll-like receptor (TLR) family; T-cell receptor (TCR) and B-cell receptor (BCR) that ultimately leads to ubiquitin-dependent degradation of the repressor IκBα though phosphorylation by the IκB kinase (IKK) complex. IκBα degradation releases the p65–p50 dimer to translocate into the nucleus and activate the transcription target genes (27, 28). The non-canonical pathway is downstream of CD40L, receptor activator for nuclear factor κB (RANK) and B cell-activating factor receptor (BAFF-R) pathways that leads to NF-κB-inducing kinase (NIK) activation, which phosphorylates the homodimer IKKα that in turn phosphorylates the p100 subunit to be processed into p52. This ultimately leads to nuclear translocation of p52–RELB dimer (27, 29).

NF-κB transcription factors play essential roles in biological processes, including differentiation during embryogenesis, inflammation, immunological responses, cell proliferation and survival (30). NF-κB is a regulator of innate immunity and it is responsible for inducing the expression of cyclooxygenase 2 (COX2) and nitric oxide synthase (NOS), inflammatory cytokines such as IL-1, IL-6, IL-8 and TNF-α, chemokines such as C-C motif chemokines ligand 2 (CCL2) and C-X-C motif chemokines ligand 2 (CXCL8) (31). It is also described as inhibiting the intrinsic apoptotic pathway by inducing the expression of several anti-apoptotic genes, including the B-cell lymphoma 2 (BCL2) family [*BCL2, BCL-xl* and Survivin (*BIRC5*)]; for inducing the proliferation through up-regulation of cyclin D1, the key cyclin of G_1/S phase, and by increasing angiogenesis through vascular endothelial growth factor (*VEGF*) and *IL6* up-regulation (27). In addition, NF-κB is a key factor in both radio- and chemoresistance (27).

The constitutive activation of NF-κB has been reported in several types of cancer, including breast cancer (32). In breast tumors, constitutive activation of NF-κB contributes to cellular proliferation, angiogenesis and evasion of apoptosis, and is mostly described in human epidermal growth factor receptor 2 (HER2/neu) and triple-negative tumors. Liu *et al*. reported that NF-κB is required for initiation of HER2-positive murine mammary tumor growth (33). This transcription factor governs the initiation of HER2 tumors and its inhibition was sufficient to reduce the CD44+ (marker of human cancer stem cells) cell population and reduced the tumor microvessel density in models. When HER2 murine cells expressed IκBα mutant, which constitutively repressed the NF-κB pathway, a dramatic reduction in mammosphere numbers and down-regulation of embryonic stem cell factors sex determining region Y-box 2 (SOX2) and homeobox transcription factor Nanog (NANOG) (33) was observed. These findings together provide the evidence that the NF-κB pathway contributes to BCSC phenotype. Pratt *et al*. reported that NF-κB is activated during differentiation of luminal progenitor cells and is required for the early steps of tumorigenesis in breast cancer (34). The high level of NF-κB found in triple-negative tumors is due to epidermal growth factor receptor (EGFR) overexpression, which is positive in part of this group (35) and ultimately activates NF-κB (36). In a recent report, Kendellen *et al*. showed consistently that the canonical and non-canonical NF-κB signaling is required for self-renewal and to form xenograft tumors for triple-negative breast cancer cells (37).

Interestingly, Nakshatri *et al*. reported that progression of rat mammary carcinoma from an estrogen receptor (ER)-positive, non-malignant phenotype to an ER-negative, malignant phenotype was accompanied by a constitutive activation of NF-κB signaling (38). These findings were in concordance with Wang *et al*., who described the repression of RELB in ER-positive breast cancer cells (39). Both studies reinforce the significance of NF-κB signaling to hormone-independent breast cancer.

NF-κB signaling is considered pivotal in the cancer stem cell context for generating secondary mammospheres in $CD44^+$ murine cells and seems to be a key player in resistance to therapies (32). Hence, the NF-κB pathway is a promising target for cancer therapy.

The notch pathway and BCSCs. The translocation-associated Notch protein (NOTCH) signaling controls self-renewal and asymmetric divisions of normal stem cells and appears to be reactivated in epithelial cells to contribute to tumorigenesis in the earliest cancer phases. Notch signaling is mainly promoted by cell-to-cell interaction: it is triggered by contact of Notch receptor (represented by NOTCH1, 2, 3 and 4) present in the surface of the receiving cell, with Delta or Jagged proteins, located on the surface of sending cells. The Delta/Jagged–Notch interaction promoting release of Notch intracellular domain (NICD), cleaved by A-disintegrin and metalloproteinase (ADAM) proteases and by enzymatic complex γ-secretase. Once cleaved, NICD diffuses into nucleus, interacts with the co-activators Mastermind (MAM) and p300 to regulate Notch target genes (40, 41).

Clarck *et al*. reported the role of NOTCH1 expression in breast epithelial stem cells in regulating asymmetrical cell division and supposed that it was the BCSC source (42). Furthermore, NOTCH1 expression was up-regulated in $CD44^+CD24^{low/-}$ breast cancer cells and has been associated with radioresistance (43). Sansone *et al*. also demonstrated that mammosphere self-renewal induced by IL-6 was NOTCH3 signaling-dependent (44). The findings of D'Angelo *et al*. confirmed the role of the Notch pathway in the self-renewal ability of BCSCs when they demonstrated that breast tumor cells with high activity of Notch overexpressed cancer stem cell markers and significantly increased both mammosphere formation (*in vitro*) and *in vivo* tumor-forming capabilities (45).

Hypoxia signaling and BCSCs. The hypoxia-inducible factor (HIF) is a heterodimer composed of one alpha subunit, which is regulated by oxygen, and a beta subunit referred to as constitutive, known as aryl hydrocarbon receptor nuclear translocator (ARNT) (46). There are three alpha subunits isoforms: HIF-1α, HIF-2α and HIF-3α. While the HIF-2α subunit is restricted to certain tissues, HIF-1α is widely expressed, being the first to respond to hypoxia (47, 48). The function of HIF-3α is not fully known, however, a splice variant is known to be able to inhibit HIF-1α activity (49). HIF-1α activation occurs under condition of low oxygen, known as hypoxia. At normal oxygen concentrations, known as normoxia, prolyl hydroxylase enzymes (PHDs) drive hydroxylation of HIF-1/2α proline amino acid residues, which are then recognized by von Hippel Lindau (VHL) protein. This hydroxylation results in proteolytic degradation of HIF-1α (50).

In hypoxia, HIF-1α is not hydroxylated by PHDs, which makes it stable in the cytoplasm. HIF-1α moves to the nucleus, where it dimerizes with HIF-1β to form an active heterocomplex able to regulate the expression of several genes (51). These target genes allow the cell to survive in the hostile environment represented by a low level of oxygen, acidic and with nutrient scarcity (52, 53).

Hypoxia is a hallmark of solid tumors, in which the blood supply is not sufficient to meet the demand of the growing tumor mass (54). It is known that a hypoxic environment has a negative clinical impact, since hypoxic cells are more radiotherapy- and chemotherapy-resistant, contributing to poor prognosis (55-58). The relationship between hypoxia and poor prognosis is commonly associated with post-treatment relapse and metastasis. In part, it is probably justified by the observation that cancer stem cells are located in hypoxic microenvironment areas, which confers a high metastatic potential and increases resistant to conventional treatments in these cells. Hypoxic cancer stem cells are mainly responsible for repopulating *in situ* and distant tumors after treatment (59, 60).

Interestingly, breast cancer cells may acquire cancer stem cell phenotype after exposure to hypoxia (61, 62). This phenomenon is promoted through transcription factors responsive to hypoxia, HIF-1α and HIF-2α, able to induce cell tumor dedifferentiation to BCSC characteristics (63). This process comprises HIF-1α and NOTCH1 interaction, which leads to maintenance of the undifferentiated state of the stem cell, such as occurs in embryonic stem cells (64). Schwab *et al*. showed that HIF-1α interferes in BCSC viability regulation; supporting the formation of mammospheres and the increase of the $CD44^+CD24^{low/-}$ population *in vivo* by up-regulating CD133 expression and cellular markers of basal phenotype in breast tumors, which are Notch pathway targets (65). These results suggest that the underlying mechanism might involve regulation of HIF-1α-dependent components present in the Notch signaling pathway. Moreover, *in vitro* experiments carried out under hypoxic conditions using triple-negative breast cancer cell lines showed an increase of CD44 expression mediated by HIF-1α, described as a BCSC marker (66). In turn, HIF-2α also contributes to BCSC phenotype by directly promoting expression of octamer-binding transcription factor 4 (OCT4), that is involved in maintaining stem cell properties (67).

Additionally, hypoxia increases the expression of other factors involved in stem cell maintenance, such as NANOG, SOX2 and Kruppel-like factor 4 (KLF4) in several tumor cell lines (68).

Xing *et al*. reported that hypoxia significantly increased the expression of NOTCH1 ligand, JAGGED2, in breast cancer cells present in the invasive tumor front of migratory process (69). Furthermore, JAGGED2 expression was also increased in hypoxic regions of bone cells and was mainly associated with self-renewal. Since bone is a major site of metastasis of breast cancer, bone hypoxic areas are suitable niches for BCSC metastasis, promoting BCSC self-renewal.

STAT3 pathway and BCSCs. STAT proteins comprise a family of cytoplasm transcription factors that mediate intracellular signaling triggered by many molecules and thereby transmit it to nucleus. Among STAT proteins, STAT3 intermediates extracellular signals stimulated by cytokines, mainly IL-6, growth factors and other molecules (70). Briefly, the receptor tyrosine kinase Janus kinases (JAK) recruit STAT3, which dimerizes and is translocated to the nucleus where it recognizes specific DNA sequences and activates the transcription of target genes. This transcription factor regulates many cellular processes, including proliferation, apoptosis, invasion, angiogenesis and metastasis (71).

In breast cancer, STAT3 protein is constitutively activated and much evidence suggests that aberrant STAT3 signaling promotes cancer initiation and progression (70). Interestingly, STAT3 is highly involved in BCSC development and progression and its persistent activity is crucial to stem cell-like and mesenchymal properties during metastasis (72-74). Recently, STAT3 was also described as pivotal in inflammatory signaling and maintenance of BCSCs by controlling self-renewal and differentiation, which confers a poor prognosis and highlights its therapeutic potential (70, 74).

A great amount of evidence supports the role of STAT3 in BCSCs, although its relevance in breast cancer initiation and progression is not completely known (75). The BCSC phenotype is regulated by many signal transduction pathways, which include IL-6–JAK2–STAT3 pathway, which is preferentially activated in $CD44^+CD24^-$ BCSCs when compared to other breast tumor cells (72, 76). However, the expression of other cytokines, such as IL-1α and IL-8, is also positively correlated with the expression of $CD44^+CD24^{low/-}$ markers in breast cancer cell lines (76).

Moreover, the classical co-receptor for growth and angiogenic factors in inflammatory response stimulus CD138 has been described as responsible for linking STAT3, Wnt and NF-κB signaling to modulate the BCSC phenotype (77). The self-renewal observed in BCSCs was referred to as being epigenetically regulated in the IL-6–STAT3–phosphatidylinositol 3-kinase (PI3K) pathway (78).

Regarding breast cancer treatment, current studies have been elucidating the relevance of STAT3 signaling to BCSC maintenance. Wang *et al*. proposed that STAT3 signaling might mediate tamoxifen resistance in BCSCs by up-regulation of STAT3 in $CD44^+CD24^{low/-}$ subpopulation of tamoxifen-resistant MCF-7 cells (79). In addition, MDA-MB-231 stem-like cells treated with the antibiotic salinomycin resulted in blockage of cell migration and invasion by inhibition of STAT3 activation which consequently led to a down-regulation of its regulatory targets cyclin-D1, matrix metallopeptidase 9 (MMP9) and 2 (MMP2) (80). A recent study reported that a natural product obtained from fermented blueberry preparation (polyphenol-enriched blueberry preparation) reduced the formation of cancer stem cells in MDA-MB-231 and MCF-7 cells when they were cultured both *in vitro* and *in vivo* through modulation of IL-6–STAT3 signaling (74).

Zhang *et al*. showed that the ectopic expression of microRNA-7 significantly down-regulated the expression and activation of STAT3 through inhibition of SET domain bifurcated 1 (SETDB1), which result in inhibition of epithelial–mesenchymal transition properties in $CD44^+CD24^-$ epithelial-specific antigen-positive MDA-MB-231 cells and of metastasis *in vivo* (75). Together, the evidence corroborates the relevance of the STAT3 pathway to BCSC development and maintenance and its potential as a therapeutic target.

Perspectives for BCSCs: The Future

Based in BCSC characteristics, there is a growing interest in understanding BCSC biology, which could provide more information about potential therapy targets. Were these targets identified, the elimination of resistant cells responsible for maintaining the tumor would be possible. Ultimately, it might improve treatment efficacy and have positive outcomes for patients with breast cancer. Thereby, the search for relevant potential molecular targets should be encouraged.

Recent research related to the embryonic pathways Wnt, Hh and Notch have evaluated potential inhibitors. Regarding the Wnt pathway, LGK974, a Porcupine inhibitor, blocks WNT palmitoylation (81), and PRI-724, CREB binding protein/catenin antagonist (82), have shown interesting results and are in phase I trial for breast cancer and other solid tumors.

Jang *et al*. developed the WNT/β-catenin inhibitor CWP232228. This small molecule showed satisfactory results through preferential inhibition of growth of BCSCs and other breast tumor cells (83).

The inhibition of Hh signaling pathway has shown promising results for BCSC therapy. Fu *et al*. reported that salinomycin, a carboxylic polyether ionophore, was

selectively cytotoxic to BCSCs in MCF-7 mammospheres instead of monocultured MCF-7 cells. The observed cytotoxicity was mediated by inhibition of Hh signaling since its components expression decreased after treatment (84).

Moreover, one promising inhibitor of SMO, called LDE225, is in a phase II trial for breast cancer and other solid tumors (85). The most promising inhibitors for Notch signaling, the compounds MK-0752 and PF-03084014, aiming for the inhibition of γ-secretase activity, are in Phase I trial (86, 87).

Furthermore, Simões *et al*. demonstrated that anti-estrogen therapy using Tamoxifen or Fulvestrant, increased the activity of the BCSCs though activation of NOTCH4–JAG1 axis (88). The same group also showed that a hormone therapy combined with NOTCH4 inhibition, using γ-secretase inhibitor RO4929097, might abrogate anti-estrogen therapy resistance.

A recent study has suggested that capsaicin, a compound derived from chilli peppers is able to induce cell death in BCSCs *via* apoptosis, in a dose-dependent manner. The mechanism could be *via* inhibition of NICD translocation into the nucleus, reducing its regulatory activity (89).

In order to avoid intestinal toxicity provoked by inhibition of all Notch receptors promoted by γ-secretase inhibitors, new specific antibodies against certain receptors have been developed (90). Yen *et al*. demonstrated the effectiveness of anti-NOTCH2/3 (OMP-59R5, tarextumab) in reducing the proportion of BCSCs using *in vivo* models (91).

Anticancer research has highlighted a number of NF-κB inhibitors. The protease inhibitor bortezomib has shown interesting results in treatment of refractory myeloma to inhibit degradation of the IκB complex, keeping NF-κB transcription factor in the cytoplasm (92). Additionally, a more promising inhibitor is dehydroxymethylepoxyquinomicin, reported to inhibit the development of tumors, including breast cancer, in murine models (93). Recently, this compound was described as disturbing the epithelial–mesenchymal transition found in aggressive phenotypes of breast cancer (Pires *et al*., unpublished data), being able to inhibit mammosphere formation in HER2 cells (94). Overall, NF-κB inhibition is an attractive field in cancer therapy.

Given the role of hypoxia in tumor progression and the formation of a tumor stem cell niche in breast cancer (59, 60), novel therapeutic approaches that target hypoxia factors, especially HIF-1α and HIF-2α, are of interest (58). Combination of conventional chemotherapies with hypoxia factor inhibitors appears to be promising for therapeutic alternatives, including BCSC, thereby preventing the onset of metastasis and recurrence after treatment (62, 65). Lock *et al*. reported that inhibition of the expression or activity of carbonic anhydrase IX (CA-IX), which is one of the genes regulated by hypoxia, resulted in inhibition of the growth of metastatic BCSCs under hypoxia. CA-IX is a critical mediator of BCSC growth in hypoxic niches (95). Therefore, CA-IX represent a valid therapeutic target for selectively reducing BCSCs.

In addition to direct inhibition of hypoxia factors, many treatments aim at molecular targets related to hypoxia, such as anti-VEGF therapies. Although antiangiogenic therapies that target VEGF (VEGF-neutralizing antibody bevacizumab) or its receptor (VEGF receptor tyrosine kinase inhibitors sorafenib and sunitinib) are used in the treatment of breast cancer (96), their results are usually transient and involve recurrence of a more aggressive disease (97, 98).

Furthermore, preclinical studies have demonstrated that antiangiogenic therapy is able to generate intratumoral hypoxia, which promotes an increase in the BCSC population, due to HIF-1α-dependent activation of WNT–β-catenin pathway involved in cancer stem cell self-renewal (99, 100). This reinforces the idea that in order to achieve an effective therapy, combinations of angiogenic inhibitors with drugs targeting BCSCs are necessary (99). However, *in vitro* studies showed that the combination of antiangiogenic agent with multi-kinase inhibitor sorafenib associated with ionizing radiation was able to inhibit BCSCs preferably in hypoxic conditions, and suggested that this inhibition may be a consequence of reduction of HIF-1α expression (101). Harrison *et al*. demonstrated through *in vitro* and *in vivo* experiments that hypoxia affects BCSC activities differently according to ER status (102). In ER+ tumors, hypoxia led to increased mammosphere formation and tumor numbers from BCSCs in an HIF-1α and NOTCH1-dependent fashion. These characteristics were reversed using a Notch inhibitor.

On the other hand, since bone is a major site of metastasis of breast cancer, bone hypoxic areas are suitable niches for BCSC metastasis, promoting BCSC self-renewal, associated with JAGGED2 expression. Therefore, new therapeutic approaches that target JAGGED2 may have a significant effect by targeting metastatic BCSCs. (69).

Regarding STAT3 signaling, in HER2+ breast cancer subtype, Chung *et al*. demonstrated that HER2 signaling leads to the activation of STAT3, with subsequent up-regulation of stem cell markers, such as OCT4, SOX2 and CD44. They showed that the expression of these markers was down-regulated and spheroid formation was abolished after STAT3 inhibition by stattic. Moreover, they showed that combined treatment of Herceptin with stattic led to a higher rate of apoptosis than the separate treatments and this may help overcome the resistance derived from the enriched of cancer stem cells in breast tumors (103).

Mathematical models also have been useful in studying the population of cancer stem cells under targeted therapy. Sehl *et al*. recently reported that slowing self-renewal and disrupting the positive feedback loop between IL-6, STAT3 activation and NF-κB signaling by simultaneous inhibition of IL-6 and HER2 is the most effective combination of

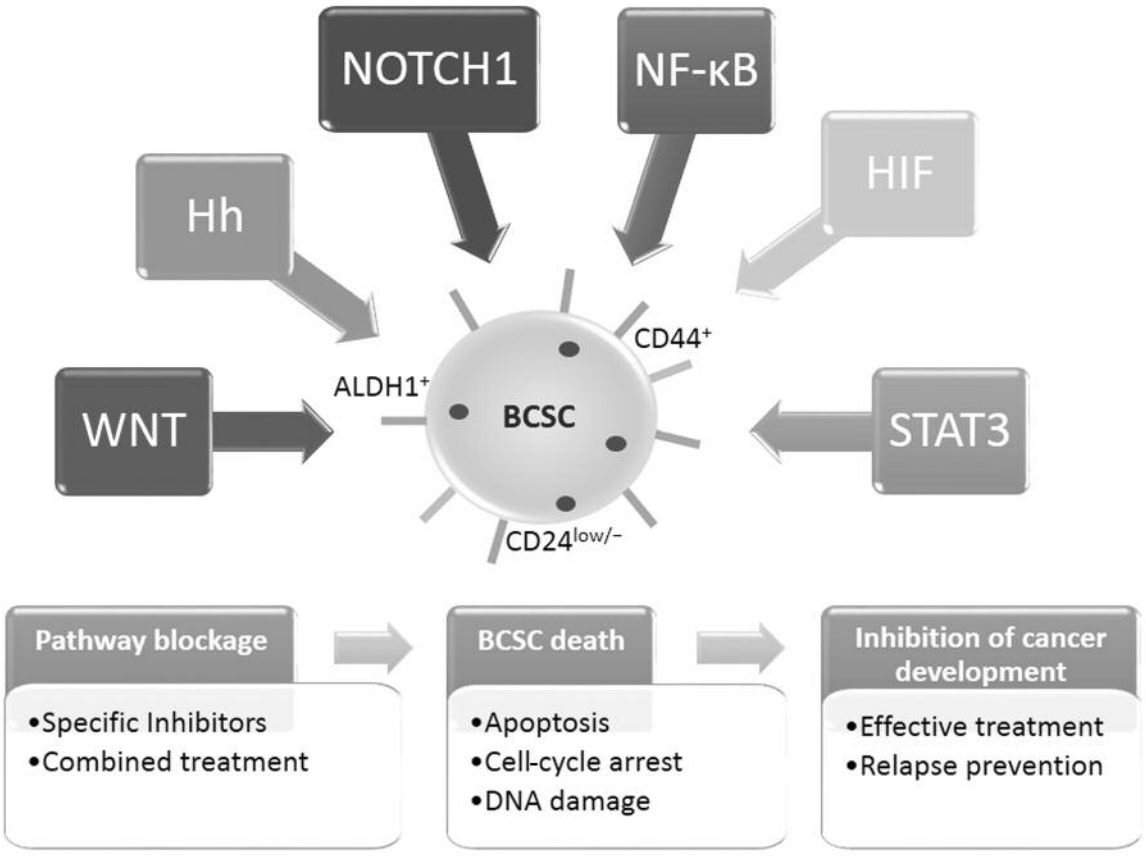

Figure 1. *The wingless-type MMTV integration site family (W)–β-catenin, Hedgehog (Hh), NOTCH1, nuclear factor-kappa B (NF-κB), hypoxia-inducible factor (HIF) and signal transducer and activator of transcription (STAT3) signaling cooperatively induce, in some cases, the breast cancer stem cell (BCSC) phenotype. This is characterized by stem cell features, such as self-renewal, and the following expression profile: cluster of differentiation 44 (CD44)$^+$, CD24$^{low/-}$ and aldehyde dehydrogenase 1 (ALDH1)$^+$. Treatments based on blockage of these signaling pathways is promising, since they might induce BCSC death in different ways. Ultimately, this could result in a more effective treatment against cancer development and prevention of relapse after conventional therapy.*

therapies to eliminate both mesenchymal and epithelial populations of BCSCs (104). The results show an excellent agreement with experimental data from cell line and mouse xenograft studies, highlighting the combined efficacy of HER2 and IL-6 blockade. Nevertheless, the authors clarify that the complexities of the microenvironment limit the ability of analytic models and emphasize the necessity for more detailed models of simulation (104).

A recent study linked tumor growth *in vivo* and metastasis to signaling driven by STAT3, PI3K/AKT and MAPK/ERK pathways. This study suggested that cancer stem cell growth and aggressiveness are associated with inflammatory cellular signaling (74).

These data support the effectiveness of some combined therapies and pathways as a new approach to combat BCSC development (Figure 1). Therefore, targeting these pathways could make treatments against breast cancer more effective, aiming at the source of disease.

Conclusion

The BCSC concept suggests the existence of subpopulations of neoplastic cells with increased tumor-initiating ability and resistance to therapies. Understanding the biology of BCSCs and the mechanisms that support them in breast cancer could help improve tumor treatment and prevent recurrence and metastasis. Therefore, therapeutic targeting in specific signaling pathway of these cells may remove residual disease and become an important component of a multimodality treatment.

Targeting the stem cell niche to eradicate BCSCs represents a new area of therapeutic development. The complex nature of many features of the stem cell niche, including both intracellular signals and microenvironment creates a challenge in choosing which elements to target, and whether alone or in combination. In this sense, mathematical modeling combined with experimental validation can be an alternative for optimizing the development of safe and

effective therapies that target the cancer stem cell and its niche. Many studies have focused on different targets concomitantly to eradicate breast cancer stem cells, but ultimately, is it possible to achieve this goal?

Conflicts of Interest

The Authors declare no conflict of interest.

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