REVIEW

Targeting stem cells in the realm of drug-resistant breast cancer

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Abstract: Since its first documentation, breast cancer (BC) has been a conundrum that ails millions of women every year. This cancer has been well studied by researchers all over the world, which has improved the patient outcome significantly. There are many diagnostic markers to identify the disease, but early detection and then subclassification of this cancer remain dubious. Even after the correct diagnosis, more than half the patients come back with a more aggressive and metastatic tumor. The underpinning mechanism that governs the resistance includes over-amplification of receptors, mutations in key gene targets, and activation of different signaling. A plethora of drugs have been devised that have shown promising results in clinical settings. However, in recent times, the role played by cancer stem cells in disease progression and their interaction in mediating the resistance to cellular insults have come into the limelight. As breast cancer stem cells (BCSCs) are dormant in nature, it is highly likely that they fail to directly respond to the cytotoxic drugs which are meant for ablating rapidly proliferating cells. Furthermore, the absence of well-characterized, drug-able surface markers to date, has limited the application of targeted therapies in complete eradication of the disease. In this review, our intent is to discuss versatile therapeutics in practice followed by discussing the upcoming therapy strategies in the pipeline for BC. Furthermore, we focus on the roles played by BCSCs in mediating the resistance, and therefore, the aspects of new therapeutics against BCSCs under development that may ease the burden in future has also been discussed.

Keywords: chemoresistance, cancer stem cell, BCSC, tumor microenvironment, breast cancer, conventional therapies, TRAIL therapy

Introduction

The first reported case of any kind of cancer was that of a breast cancer (BC) in around 1600 BC in Egypt, which is not surprising as the organ allowed easier identification. BC, since its first documentation, has been well-studied but is still a leading cause of deaths in women worldwide.[1] Extensive research has shown that this is due to the heterogeneous nature of the disease itself, which predicts the therapeutic response. Based on the presence or absence of the well-established biomarkers on the surface of BC cells, they are classified into luminal A, luminal B, HER2, and triple negative breast cancer (TNBC).[2] Each subclass has diverse risk factors for incidence, disease progression, therapeutic response, and favored organ sites of metastases. Out of these subtypes, TNBC is the most aggressive subtype and shows high metastatic potential. This can be attributed to the high number of cancer stem cell (CSC) population present within the tumor. There is a plethora of therapeutic modalities administered to BC patients on the basis of their initial diagnosis. Landmark discoveries like radical

mastectomy, lumpectomy, radiation implants, tamoxifen, trastuzumab, and so on have remarkably helped in improving disease outcome, for a wide number of patients (Figure 1). However, >50% of treated patients come back with a more aggressive disease. This aggressive behavior and resistance to the predicted therapies have marked a major challenge for BC clinics. Reports have shed a light onto the various mechanisms that might be responsible for these resistance mechanisms. Knowledge of the signaling cascades has led to the development of numerous molecules with potential to reduce the burden of the resistance encountered in BC. Even though chemotherapy and radiotherapy have undergone substantial improvements and refinements in efficacy and administration in the past decade, orthodox cancer treatments remain futile for many patients, predominantly whose cancer has been diagnosed at a later stage.[3] Delay in cancer diagnosis reduces the overall treatment efficacy mainly due to the increased likelihood of the manifestation of metastatic disease, but also partly because more advanced disease requires more intensive treatment, which may, itself, cause treatment intolerance. In this review, first we discuss the con-ventional chemotherapeutics in practice followed by the novel therapeutics that are being developed against drug-resistant BC. Further, we focus on the role of BCSCs in mediating the resistance. Lastly, aspects of currently developing treatment strategies against BCSCs are discussed.

BC therapy: conventional approaches

In the genome era, rapid advances in molecular understanding have subdivided BC into ten interclasts.[2] However, based on the current clinical practices, BC is known to have four primary subtypes. Luminal BCs are positive for steroid hormone receptors (estrogen receptor [ER] and progesterone receptor [PR]), which is further classified into two groups (A and B). The luminal A (ER+/PR+/HER2−) type tumors are less aggressive than other subtypes and take much longer to grow as well. These cancer cells also respond better to hormonal interventions and have a better prognosis.[4] Luminal B subgroup (ER+/PR+/HER2+) typically shows high Ki67 proliferative index marker as well as *HER2* expression. BC cells belonging to luminal B subgroup usually show poorer
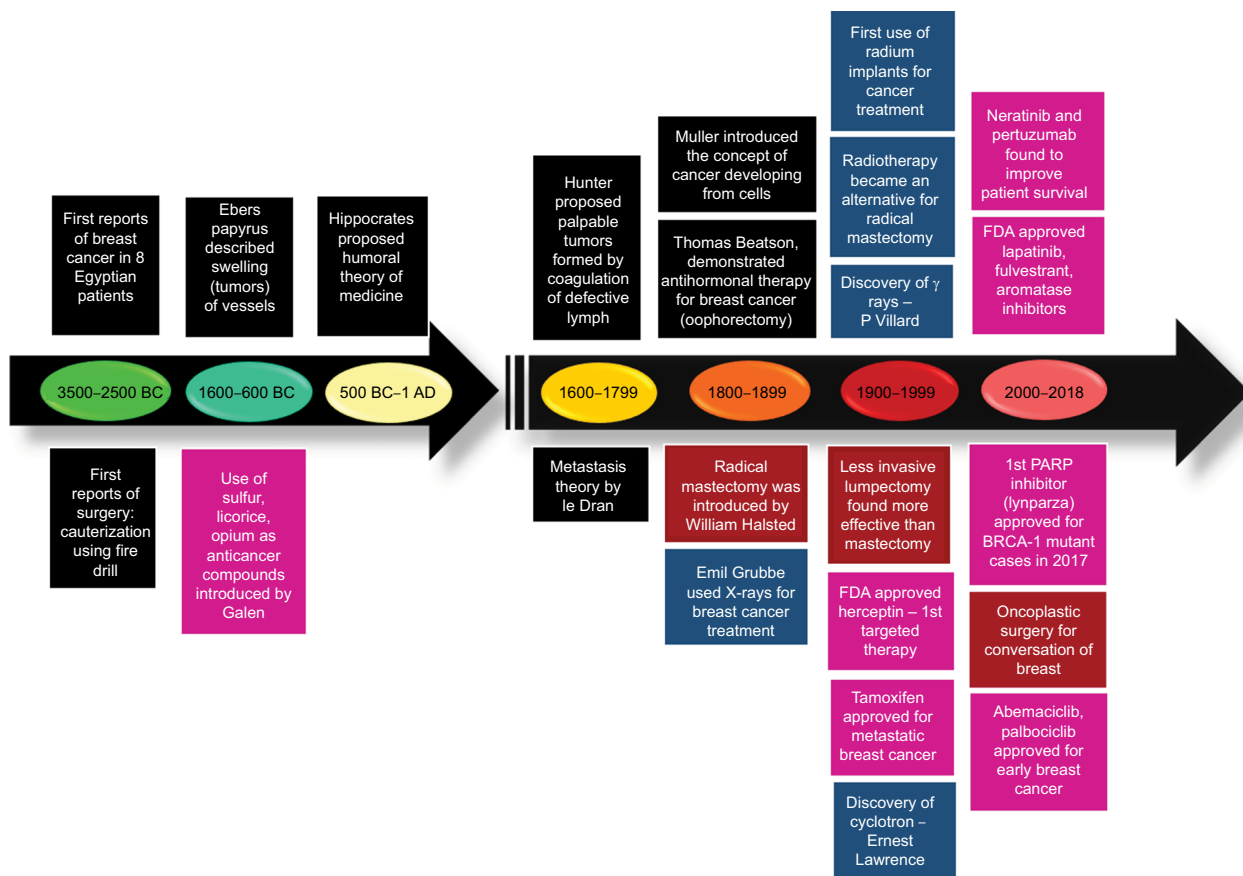
Figure 1 Image depicting the development of therapeutic regimens against breast cancer.
Abbreviation: FDA, US Food and Drug Administration; PARP, poly(ADP-ribose) polymerase.

prognosis than luminal A, but respond better to standard chemotherapy. Since patients of this subgroup also show high *HER2* expression, targeted therapy for *HER2* might also be employed in some cases.[4] In HER2+, BCs, which have amplification or overexpression of the HER2/ERBB2 oncogene, are generally treated with anti-HER2 therapies including the antibody drug trastuzumab and small molecule inhibitor lapatinib. Basal-like BC lacks the hormonal receptors as well as HER2 receptor and therefore is often known as triple negative breast cancer (TNBC). Standard chemotherapeutic regimens involving platinum-based drugs are majorly administered for treating TNBCs.

Majority of BC patients (~77%) have hormonal receptor-positive diseases, which comprise 23.7% from ER+/PR+/HER2− (luminal A) and ~53% from ER+/PR+/HER2+ (luminal B). Approximately, 23%–30% of BC patients show HER2 amplification. TNBC represents about 10%–12% of the total BC population.[4] Endocrine therapy is currently the gold standard treatment regimen to treat the hormone receptor+ BCs. This therapy works either by making the hormone effect ineffective or by lowering the hormone level itself. Therapeutic drugs prescribed to the patients include 1) tamoxifen, which acts by blocking the estrogen uptake by ER; 2) exemestane, anastrozole, and letrozole that belong to aromatase inhibitor class of drugs, which inhibits the conversion of androgens to estrogens thereby depleting estrogen in the body; 3) leuprolide and goserelin (luteinizing hormone-releasing hormone analogs), these drugs suppress the synthesis of hormone from the ovary; and 4) fulvestrant (a specific ER inhibitor), which makes it suitable for refractory BC patients. Administration of the above drugs for treating hormone receptor+ BC is recommended until there is clinical resistance or metastasis, where chemotherapy is employed.[5] As different endocrine drugs work by distinct mechanism, a combinatorial approach can show improved efficacy. However, the effectiveness of this combination treatment has not been proved well in the patient scenario.[5] Therefore, the current consensus is that both endocrine therapy-naïve advanced BC and high endocrine-sensitive patients can benefit from the combination endocrine therapy.[6]

The patient group having HER2 gene amplification or protein overexpression is generally administered molecular targeted therapy; a range of targeted drugs have been approved as single agent or in combination with standard chemo regimen. The receptor-targeted therapeutic agents include 1) trastuzumab (specific anti-HER2 monoclonal antibody [mAb]); 2) ado-trastuzumab emtansine, which is trastuzumab conjugated with emtansine (microtubule inhibi-

tor); 3) pertuzumab (specific anti-HER2 mAb with distinct binding site on HER2 extracellular region compared to trastuzumab); 4) lapatinib, a small molecule inhibitor (TKI) capable of inhibiting both HER2 and epidermal growth factor receptor (EGFR) signaling. The standard regimen for early stage HER2+ cases includes neoadjuvant therapy with a combination of HER2 targeted therapy and chemotherapy.[7] Subsequently, this treatment is followed by surgery, radiotherapy, and 1 year of HER2-targeted therapy. Endocrine adjuvant can be added based on the specific receptor status in patient. The successful advent of molecular targeted therapy against HER2+ BC can be seen by the substantial increase in overall survival (OS) of patients from ~1.5–5 years.[7]

TNBC is aggressive by nature and defiant to treat as well when compared to hormone-positive and HER2+ BC. TNBC can be further subdivided into six subtypes based on transcriptomic heterogeneity and response to chemotherapy. These subtypes are mesenchymal (M), a mesenchymal stem-like (MSL), basal-like (BL1 and BL2), a luminal androgen receptor (LAR), and an immunomodulatory (IM) type.[8] Both M and MSL subtypes have enhanced expression of factors regulating epithelial–mesenchymal transition (EMT), but intriguingly only the MSL subtype has diminished expression of genes involved in proliferation. The BL1 subtype is categorized by augmented expression of cell cycle and DNA damage repair genes, while the BL2 subtype shows higher expression of growth factor receptors and myoepithelial markers. The LAR subtype is regulated by the androgen receptor (AR) and characterized by luminal gene expression. The IM subtype comprises of BC cells encoding immune checkpoint regulatory genes such as programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1), antigens, and immunomodulatory cytokines. Detailed analysis shows activation of immune signal transduction pathways in this subtype, which is likely from both the tumor cells and infiltrating lymphocytes.[8]

Until now, standard chemotherapy remains the mainstay of treatment in TNBCs. The absence of the receptors precludes the application of targeted therapies against advanced stage disease. The only US Food and Drug Administration (FDA)-approved therapy is chemotherapy drugs such as anthracycline, taxane, and platinum drugs with or without bevacizumab.[9] The median OS of patients with metastatic disease ranges between 9 months and 1 year.[9] Given the suboptimal treatment outcome with standard therapeutic agents, identification of novel targets and therapy is the need of the hour.

Even with the development of so many different agents, the BC patient scenario is still very disappointing. This can

be attributed to the innate biology of the cancer cells to outsmart the current therapies. Over the past decade, it has been identified that cancer cells employ various strategies to overcome the cytotoxic effects such as activation of other signaling pathways, altered metabolism, change in the cell cycle machinery, and epigenetic changes to name a few. This knowledge has led to the development of agents with potential to overcome the resistance of BC cells (Figure 2). In the following sections, we discuss some of the promising therapeutic strategies that are being investigated to treat drug-resistant BC.

BC therapy: developments in the challenging dogma
Hormonal therapy-resistant BC

Development of resistance in hormone receptor-positive BC against their targeted treatment agents is now a well-established phenomenon. Resistant cancer is often metastatic in nature and the underpinning genomic alterations occur majorly in ER cascade. However, other signaling pathways might also get activated and are involved. Mammalian target of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/rapamycin (mTOR) (PI3K–Akt–mTOR) signaling circuit is considered as one of the prime contributing factors to the resistance in a variety of cancers including BC with hormonal drug resistance.[10] This signaling cascade is reported to be overactivated in almost 70% of BC, with PIK3CA (PI3K catalytic subunit p110α) being the frequently mutated and/or amplified genes.[11] In addition, activation of escape pathways like HER2 signaling as well as altered cell cycle kinetics has been observed to mediate resistance against ER therapies Thus, to tackle ER+ metastatic BC, there is a need to develop novel therapeutic approach with a potential to either minimize or reverse drug resistance. To address the conundrum of resistance, spectrum of different chemotherapeutic agents has been developed.

PI3K inhibitors

Combinatorial therapies targeting both hormonal receptors and PI3K/AKT/mTOR pathways have been appraised to reverse the resistance to hormonal therapies. Combinatorial treatment with PI3K inhibitors and aromatase inhibitors has been employed as a second-line of treatment for advanced luminal A cases. Buparlisib (a pan-class I PI3K inhibitor) was reported to considerably improve progression-free survival (PFS) in patients, specifically in those having PIK3CA mutation. However, PI3K inhibitors such as pilaralisib,[12] voxtalisib,[12] and buparlisib[13] cannot be employed in treating

patients due to their high toxicity. Recently, taselisib and alpelisib are also under Phase III trials (NCT02340221 and NCT02437318, respectively) and are reported to be efficacious primarily due to their high selectivity and lesser toxicity. These α-specific PI3K inhibitors showed promising results in patients harboring PIK3CA mutations.[14] Irrespective of PIK3CA status, both taselisib[15] and pictillisib[16] in combination with letrozole or anastrozole were found to augment antitumor effects in early luminal A patients when employed as neoadjuvant treatment. Buparlisib and alpelisib are currently under Phase II efficacy investigation (NCT01923168).
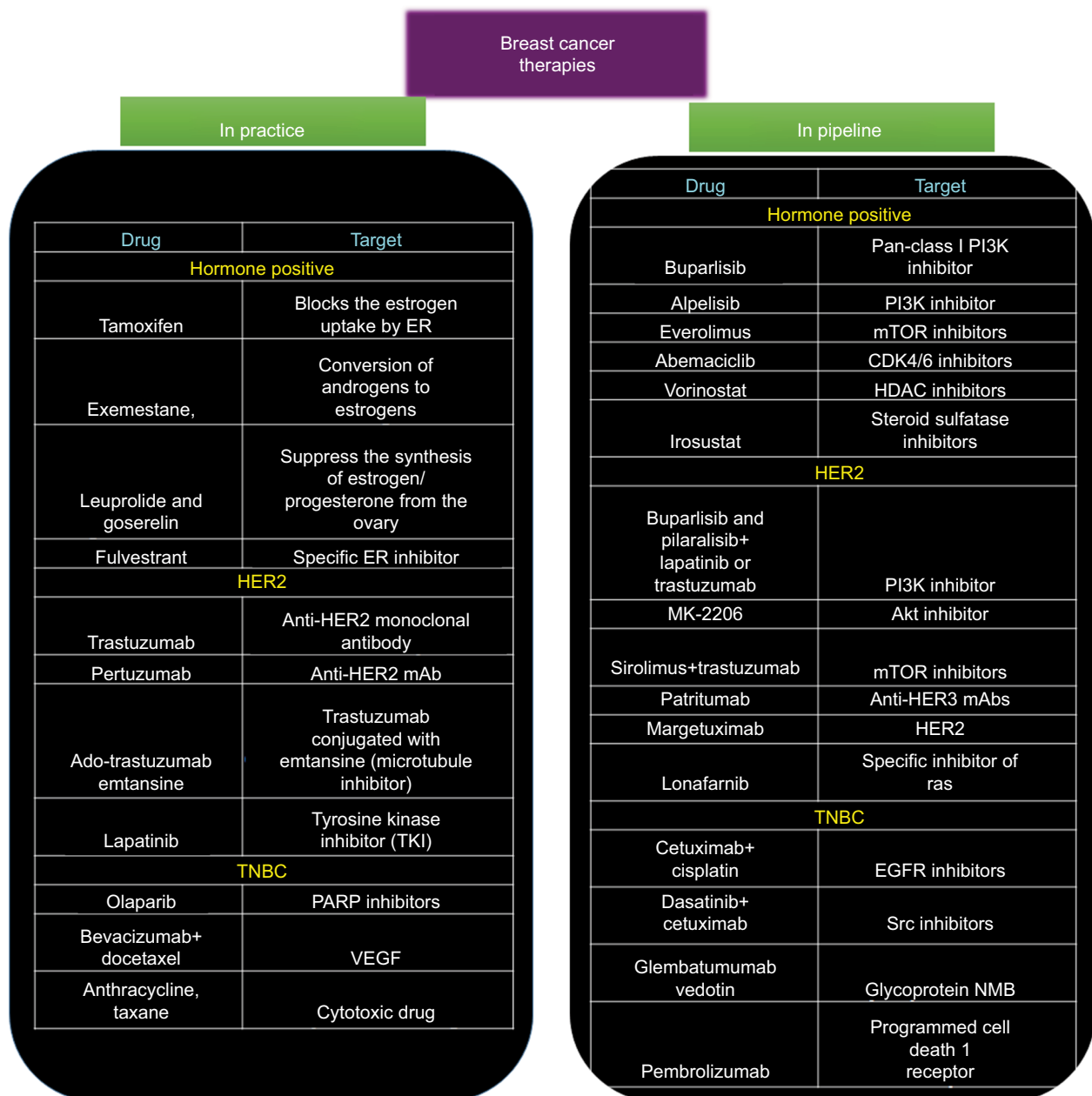
mTOR inhibitors

Everolimus (derived from sirolimus) has been approved by the US FDA for treating ER/PR + advanced BC in combination with exemestane. Everolimus has also been employed in combination with letrozole, but its clinical efficacy was a failure as it could not reverse the resistant BC.[17] Another derivative of sirolimus, that is, temsirolimus, was a complete defeat as it could hardly show any clinical benefits either as first-line therapy in combination with letrozole or as a single agent in second-line therapy in advanced ER/PR + BCs.[18]

Cyclin-dependent kinases 4 and 6 (CDK4/6) inhibitors

It is known that cancer cells have aberrant cell cycle machinery that aids them to divide and proliferate infinitely. Thus somehow, inhibiting the cell cycle in these cancer cells might provide a way to overcome the resistance in cancer cells. The proteins involved in regulating cell cycle belong to cyclin-dependent kinase (CDK) family. In addition, CDK4/6 has been shown to regulate the cell cycle progression by its reversible interaction with cyclin D1. Thus, among the emerging therapies against CDKs, CDK4/6 inhibitors such as abemaciclib, ribociclib, and palbociclib are the most promising candidates. These CDK4/6 inhibitors block the phosphorylation of retinoblastoma protein, resulting in the downregulation of E2F-response genes to mediate cell cycle arrest at the G1-S stage. These small molecule inhibitors have also been reported to dephosphorylate the forkhead box protein M1 (transcription factor), causing inhibition in cellular proliferation.[19] Interestingly, it was observed that hormone-resistant tumors are still dependent on CDK4/6-cyclin D1 for their growth and proliferation.[20]

Promising results have led to the FDA approval of combination treatment using ribociclib and palbociclib along with aromatase inhibitor as the first-line treatment for ER+/PR+/HER2− advanced BC. They have been reported to

Breast cancer therapies

In practice

Drug	Target
Hormone positive	
Tamoxifen	Blocks the estrogen uptake by ER
Exemestane,	Conversion of androgens to estrogens
Leuprolide and goserelin	Suppress the synthesis of estrogen/progesterone from the ovary
Fulvestrant	Specific ER inhibitor
HER2	
Trastuzumab	Anti-HER2 monoclonal antibody
Pertuzumab	Anti-HER2 mAb
Ado-trastuzumab emtansine	Trastuzumab conjugated with emtansine (microtubule inhibitor)
Lapatinib	Tyrosine kinase inhibitor (TKI)
TNBC	
Olaparib	PARP inhibitors
Bevacizumab+ docetaxel	VEGF
Anthracycline, taxane	Cytotoxic drug

In pipeline

Drug	Target
Hormone positive	
Buparlisib	Pan-class I PI3K inhibitor
Alpelisib	PI3K inhibitor
Everolimus	mTOR inhibitors
Abemaciclib	CDK4/6 inhibitors
Vorinostat	HDAC inhibitors
Irosustat	Steroid sulfatase inhibitors
HER2	
Buparlisib and pilaralisib+ lapatinib or trastuzumab	PI3K inhibitor
MK-2206	Akt inhibitor
Sirolimus+trastuzumab	mTOR inhibitors
Patritumab	Anti-HER3 mAbs
Margetuximab	HER2
Lonafarnib	Specific inhibitor of ras
TNBC	
Cetuximab+ cisplatin	EGFR inhibitors
Dasatinib+ cetuximab	Src inhibitors
Glembatumumab vedotin	Glycoprotein NMB
Pembrolizumab	Programmed cell death 1 receptor

Figure 2 Snapshot of various chemotherapeutic modalities that are being prescribed in clinics and the novel therapeutic drugs that are being developed.
Abbreviations: EGFR, epidermal growth factor receptor; ER, estrogen receptor; HDAC, histone deacetylase; mAb, monoclonal antibody; mTOR, rapamycin; PARP, poly(ADP-ribose) polymerase; PI3K, phosphatidylinositol 3-kinase; TNBC, triple negative breast cancer; VEGF, vascular endothelial growth factor.

significantly improve the PFS in advanced BC patients by 10 months and the PFS rate by 20% after 18 months, respectively, compared to letrozole alone.[21] Abemaciclib has been shown to prolong the median PFS by 7 months,[22] when employed as second-line treatment in combination with fulvestrant in ER+/PR+/HER2− advanced BC. To assess the efficacy of ribociclib and abemaciclib alone, they are currently in Phase III trials (NCT02422615 and NCT02246621). Although the mechanism of action of these CDK4/6 inhibitors is alike, abemaciclib showed greater monotherapy response and

induced lesser neutropenia as compared to other inhibitors, primarily due to its more specific CDK4 inhibition.[21] In addition to the conventional/direct approach of targeting the signaling cascade involved in mediating resistance, there has been development of inhibitors which have the potential to reverse the resistance.

Histone deacetylase (HDAC) inhibitors

Resistance to conventional hormonal therapy has also been attributed to the histone deacetylation-mediated loss of ER

expression in ER+ patients.[1] In line with this observation, application of HDAC inhibitors has been shown to upregulate the expression of ERα and aromatase, thereby aiding in suppression of the signaling governed by ER.[23] Entinostat when combined with exemestane and vorinostat in combination with tamoxifen has shown promising antitumor activity when employed as second-line treatment for ER+/PR+ advanced BC in combination with, compared to, their monotherapy counterparts.[23]

Steroid sulfatase inhibitors

Steroid sulfatase also known as arylsulfatase C is a sulfatase enzyme involved in the metabolism of steroids. Interestingly, the enzymatic activity of steroid sulfatase was reported to be substantially increased in ERα-positive BC cells.[24] Thus, inhibiting the enzymatic activity reduces the estrogenic steroids and suppresses tumor growth. Encouraging results were reported from the Phase II trial of the combinatorial treatment using irosustat along with conventional aromatase inhibitor.[25] SR16157 (dual-acting steroid sulfatase inhibitor) which is a direct inhibitor of steroid sulfatase and releases ERα modulator has also been assessed for its effects in hormone-dependent BC.[26]

Resistant BC against HER2 targeted therapy

The growing reports of primary and acquired resistance to lapatinib or trastuzumab are alarming and severely hampering its clinical significance as HER2+ BC therapeutics. Thus, identifying the resistance mechanisms and discovery of potential therapeutic agents to tackle the resistance is the need of the hour. Various groups have identified and verified numerous therapeutic agents that might play an important role in the fight against metastatic HER2+ BC.

PI3K/Akt/mTOR inhibitors

The aberrant activation of PI3K/Akt/mTOR pathway is also considered to be mediator of resistance in HER2+ BC, thus combining the inhibitors of this pathway with HER2 targeted candidates and studying the efficacy of the drugs is an area of active research. Buparlisib and pilaralisib (pan-class I PI3K inhibitors), when administered with lapatinib,[27] trastuzumab,[28] or trastuzumab and paclitaxel,[29] was proven to be efficacious and safe in patients having HER2+ advanced disease. In addition, MK-2206 (an Akt inhibitor) also showed promising antitumor activity when combined with trastuzumab and paclitaxel[28] or trastuzumab[30] alone in patients with HER2+ advanced BC. To directly target the mTOR,

everolimus was combined with trastuzumab and vinorelbine; however, the clinical outcome of advanced HER2+ BC patients did not improve.[31] Surprisingly, this combination demonstrated better anticancer activity than trastuzumab alone in HER2+ patients who are hormone receptor negative.[31] Recent drugs such as sirolimus[32] and ridaforolimus[33] when administered in combination with trastuzumab have demonstrated promising results in refractory HER2+ BC.

Inhibitors targeting HER-family receptors

Receptor ligands switching between HER-family members (HER1 [EGFR], HER3, or HER4) can activate the signaling cascade, this phenomenon is reported to make trastuzumab redundant.[34] In addition, HER2/HER3 heterodimers have also been associated with trastuzumab resistance.[35] Thus suppression of the HER-family members may be promising to deal with the conundrum of resistance in HER2+ advanced BC. In view of this, an irreversible TKI, neratinib was developed that has the ability to inhibit HER1/HER2/HER4. It has been reported that administration of neratinib after trastuzumab adjuvant therapy has significantly improved the 2-year invasive disease-free survival in HER2+ patients.[36] Preclinical study has also shown encouraging antitumor activity of an anti-HER3 mAb (patritumab) by inhibiting HER2/HER3 heterodimers. Further, it was shown that it is efficacious and has lower toxicity in patients with advanced HER2+ disease.[37]

A novel mAb margetuximab (HER2 targeting) was also assessed in a first Phase I trial for its antitumor activity and was found to be well tolerated and had promising activity even as a single therapeutic agent.[38] Further, this antibody inhibitor is currently undergoing trials to test its efficacy as a single agent (NCT02492711) and/or in combination with pembrolizumab (targets PD-1 receptor of lymphocytes) (NCT02689284).[38] Among other ongoing efforts, trastuzumab is conjugated with emtansine (microtubule inhibitor) which utilizes the specificity of trastuzumab for targeting HER2+ BC cells and microtubule cytotoxicity for killing the cells.[39] It has been approved as a second-line treatment for lapatinib/trastuzumab-relapsed/refractory HER2+ BC patients.[40]

Immunotherapy

The use of immune system against tumor cells serves as an area of extensive research with the aim to develop a vaccine against cancer. One of the first devised immunotherapeutic agents was nelipepimut-S, derived from the extracellular region of HER2. It has been extensively analyzed as a potential vaccine to prevent relapse in high-risk BC patients.[41] The

combinatorial application of nelipepimut-S and trastuzumab in HER2+ early BC is studied in Phase IIb clinical trial (NCT02297698). Recombinant HER2 protein (dHER2) was also studied for the potential vaccine and exhibited immunogenicity to augment T-cell-mediated response against HER2+ BC.[42] Follow-up studies are being carried out to elucidate its role as monotherapy in HER2+ advanced BC as well as advanced BC refractory to trastuzumab or lapatinib.[43]

TNBC

Among the BC subtypes, TNBC has fewer choice of therapeutic drugs, primarily due to the lack of well-characterized molecular targets. Therefore, the need of the hour is to identify novel targets and develop effective agents against these targets to achieve improved clinical benefits. Till now the agents developed against TNBCs are primarily based on drug repurposing.

Anti-angiogenic agents

Vascular endothelial growth factor (VEGF), which is a key angiogenic factor implicated in various cancers, has been reported to be higher in TNBC as compared to non-TNBC BC.[44] A well-known anti-VEGF mAb, bevacizumab, demonstrates suppression of tumor neovasculature growth and inhibits metastasis. It was also reported in a Phase III trial that supplementation of bevacizumab to docetaxel (first-line chemotherapy) resulted in improved response rate.[45] It was also observed that the combination of bevacizumab and docetaxel had minimal or no side effects when compared to docetaxel alone.

Poly(ADP-ribose) polymerase (PARP) inhibitors

A major breakthrough toward the understanding of the heterogeneity of the TNBCs came in the form of detection of a subclass of sporadic TNBC that has deficiency in the homologous-repair pathway, which is a characteristic of BRCA1/2-mutated BC. In line with this observation, the therapeutic drugs administered to these patients incorporate PARP inhibitors or platinum drugs (DNA targeting) like carboplatin[46] along with conventional chemotherapy.[47] BRCA1/2 genes are responsible for encoding tumor-suppressor genes that are involved in repairing DNA double-stranded breaks via homologous recombination. Whereas, PARP enzymes repair the single-stranded breaks. Patients harboring germline BRCA1/BRCA2 mutation (gBRCA+) benefit the most after administration of PARP inhibitors, probably due to synthetic lethality.[48]

When considering PARP inhibitors, olaparib seems to be a success story. In addition to olaparib, other inhibitors targeting PARP such as talazoparib is currently in Phase III trial (NCT01945775). Talazoparib has shown promising preclinical results, which can be attributed to its strong affinity to DNA by trapping PARP–DNA complexes.[49] It has also demonstrated strong anticancer activity as a monotherapeutic drug in advanced gBRCA+ BC.[50] Rucaparib (Phase II, NCT02505048) and niraparib (Phase III, NCT01905592) are being explored in case of gBRCA+ advanced BC patients as a single agent as well as in combination with standard chemotherapy (niraparib: Phase I/II, NCT02657889; rucaparib: Phase II, NCT01074970).

The decision of using either PARP inhibitors or carboplatin in TNBC is generally determined by three DNA-based homologous recombination deficiency scores, which highly correlates with the germline genetic defects in BRCA1/2.[51] However, none of the abovementioned therapeutic agents is beneficial against all TNBC because of the heterogeneous nature of TNBC. Thereby, an urgent need for the identification and characterization of novel clinically important molecular biomarkers for further refinement of the in-practice treatment approaches.

EGFR inhibitors

The EGFR has been reported to be overexpressed in TNBC. Therefore, numerous clinical trials are underway to evaluate the antitumor activity of cetuximab in combination with platinum-based drugs like cisplatin in metastatic TNBC patients.[52,53] Identification of a subpopulation of TNBC patients that might respond well to EGFR inhibitors is an area of active research efforts.[54] Low expression of α-crystalline B chain, lack of KRAS expression, and higher expression of PTEN in tumors might be correlated with favorable response.[54]

SRC inhibitors

SRC is a non-receptor signaling kinase which is a downstream molecule of several growth factor receptors such as PDGFR, EGFR, HGFR, and IGF-1R which have been reported to be deregulated in TNBC. Dasatinib, when tested as monotherapy for TNBC in Phase II trial (CA180059), showed substandard result.[55] However, when tested in cell lines, dasatinib in combination with anti-EGFR mAb: cetuximab and cisplatin showed synergistic antitumor activity in different TNBC cell lines.[56] The combination of three drugs resulted in more prominent induction of apoptosis and inhibition of MAPK and EGFR phosphorylation than all the other combinations.[56] In addition,

cancer cell migration and invasiveness were also substantially suppressed by dasatinib as well as combination treatment with dasatinib, cetuximab, and cisplatin in TNBC cell lines.[56] Thus, clinical investigations are required to further access the use of dasatinib-containing amalgamations in TNBC patients that have tumors expressing both EGFR and c-Src.

Monoclonal antibodies

Glembatumumab vedotin is a mAb conjugated with a cytotoxic drug aimed at targeting glycoprotein NMB-overexpressing (gpNMB+) TNBC.[57] gpNMB is a transmembrane protein that has been linked with tumor invasion and promote metastasis and is overexpressed in about 40% of TNBC.[58] Phase II trial done on gpNMB+ advanced TNBC patients showed significant improvement in PFS and OS in glembatumumab vedotin-treated patients as compared to conventional therapy.[59]

Breast cancer stem cells (BCSCs): the troublemakers

BC is widely understood as a heterogeneous disease which in turn contributes to therapy failure and disease progression.[60] There is not only intratumoral heterogeneity, that is, diversity within a tumor in context to phenotypic, functional, and genetic variations, but also intertumoral diversity, that is, the diversity between primary and metastasized tumor. To explain the intratumoral heterogeneity, two theories have been put forward. The first one was clonal evolution theory/stochastic theory, introduced by Peter Nowell, according to which cancer is an evolutionary process in which most neoplasms arise from single cell and progression of tumor results from stepwise accumulation of mutations within original clones following selection of more aggressive subclones. Accordingly, each dominant subclone possess similar tumorigenic potential.[61]

The second theory proposed is CSC theory. According to this hypothesis, only a small population of cells, called CSCs, are capable of self-renewal and have the potential to initiate tumor. In CSC model, cancers originates from the malignant transformation of a stem or progenitor cells through the deregulation of self-renewal program or from transformation of committed cells through dedifferentiation of mature cells that gain a self-renewal potential.[62]

The first CSCs from solid tumors were identified in breast tumors,[63] subsequently CSCs were isolated from other organs. Al-Hajj et al were the first to identify a subpopulation of BC which had the potential to form tumors in immune-deficient Nonobese Diabetic (NOD)/Severe Combined Immuno-deficiency (SCID) mice.[63] They used a set of cell surface markers to isolate cells with increased tumorigenic capacity. In particular, cells that were CD44+CD24lowEpCAM+ and lineage negative (cells lacking markers CD2, CD3, CD10, CD16, CD18, CD31, CD64, and CD140b), isolated from one primary breast tumor and eight metastases, were able to form heterogeneous tumors eight out of nine times and were termed as BCSCs. Surprisingly, as few as 200 CD44+CD24lowEpCAM+lin- cells transplanted into NOD/SCID mice could form tumors with 100% efficiency, while CD44−CD24+EpCAM− cells could not form tumors. Different subtypes of BC constitute different proportion of BCSCs contributing to different disease outcome. Among the BC subtypes, the highest amount of CSCs was observed in patients with TNBC (basal) subtype and has been correlated with its aggressiveness.[64]

With momentous discovery of CSCs, their pivotal role in driving key processes during cancer development such as tumor growth, metastasis, recurrence, as well as treatment resistance was established. However, the signaling pathway that regulates CSCs and that might be involved in promoting the resistance toward the conventional therapies remains largely elusive. Hedgehog, Notch, and Wnt pathways have been shown to play crucial role in promoting resistance to therapy. These pathways are generally involved in the development of embryo and adult tissue homeostasis. Deregulation of the Notch and Hedgehog pathways, which normally regulates stem cell self-renewal and differentiation, results in BCSC phenotype.[65] The Wnt pathway plays an important role in maintaining and preserving undifferentiated state of stem cells.[66] Hedgehog pathway, which is an embryonic development organizer pathway, is also deregulated in BC, thereby activating *Gli1* and *Ptch1* genes (positive modulators of the hedgehog pathway) and thus leading to BCSC proliferation.[67] The Notch pathway is involved in cell differentiation during both embryogenesis and adulthood. Notch pathway deregulation activates genes important for regulating proliferation and apoptosis inhibition in cancer cells.[68] The transcription factors targeted by Notch signaling include CDKN1A, cyclinD1, c-myc, and HES-related repressor protein. These pathways have been reported to be activated in BCSCs.[69] In addition, other transcriptional factors involved in maintaining the potency of BCSCs have also been identified. The transcriptional factors such as Sox2, Oct4, and Nanog act as master regulators of pluripotency and maintain the undifferentiated state of BC cells.[70] Of the basal-like breast carcinomas, 43% exhibit higher Sox2 expression, indicating a less differentiated phenotype.[71] Another member of the Sox family, Sox4, induces changes associated with the

EMT process that is responsible for increased invasiveness and mobility of cancer cells in vivo.[72] Recently, the ability of BCSCs to undergo EMT has been scrutinized, leading to the identification of partial EMT. Reports suggest that the circulating tumor cells (CTCs) survive in blood by exhibiting both epithelial and mesenchymal (E/M) phenotypes. The CTCs employ the collective cell migration properties of the epithelial cells and enhance their attachment to the extracellular matrix by achieving mesenchymal properties.[73] This significantly enhances the chance of survival and promote distant metastasis. Several evidences suggest that the expressions of Oct3/4, Nanog, and Sox2 are strongly associated with different CSCs, including BCSCs.[74]

Apart from the genes that maintain the potency of stem cells, the BCSCs can be distinguished based on the following unique features:

1. Presence of classical cell surface marker such as CD44$^+$CD24$^-$, in addition CD133, CD44$^+$ CD49 f^{hi} CD133/2^{hi}. CD49f and CD61 have also been introduced as BCSC marker.[75] These markers can be detected by flow cytometer, via employing specific mAbs.
2. High expression of BC resistance protein 1, also known as ATP-binding cassette (ABC) transporter G family ABCG2 or CD338.[75] This can be tested by using orthodox side population assay.
3. Ability to form mammospheres in suspension culture and the overexpression of aldehyde dehydrogenase-1 (ALDH1).[75]

Chemoresistance to the conventional therapies can be divided into two main groups, namely intrinsic resistance due to genetic alterations and extrinsic resistance including microenvironment influences (Figure 3).[76] Intrinsic resistance includes overexpression of ABC transporter, overexpression of ALDH1, enhanced DNA repair mechanism, an altered cell cycle, and resistance to apoptosis. The extrinsic cause of resistance includes all microenvironment influences such as hypoxia or EMT.

Intrinsic factors of resistance

The small pool of cells, that is, BCSCs that evade chemotherapy is possibly because of the presence of ABC transporters. Increased level of ABCG2 in BCSCs was shown to enable rapid expulsion of cytotoxic drugs, conferring cellular resistance to antitumor drugs.[77] Increased levels of P-glycoprotein which belongs to ABC transport family also confer resistance to antineoplastic drugs by manipulating several cellular processes like the p53 network which plays a role in mediating chemoresistance. New tumors

arising from BCSCs show a chemoresistant phenotype and are often accompanied by activating mutations.[75] Hu et al[78] observed that Akt signaling altered the subcellular localization of BCRP, thereby regulating drug efflux activity in CSCs. Inhibitors of PI3K, blocked Akt signaling, resulted in the suppression of cancer cell proliferation, but also enhanced the sensitivity of chemoresistant cells.[78]

Aldefluor assays indicated that highly tumorigenic BC cells were ALDH positive. These BCSCs had the similar differentiation and self-renewal properties when compared to CSC.[79] ALDH1A1 and ALDH3A1 are important in the protection and the differentiation of CSCs via the conversion of retinol to retinoic acid.[80] ALDH1 has the ability of metabolizing toxic chemotherapeutic agents into nontoxic molecules, particularly cyclophosphamide class, by converting aldophosphamide to carboxyphosphamide and thus eliminating the lethal effects of the acrolein and phosphoramide mustard (metabolite of cyclophosphamide).[80,81] It has been observed that metastatic breast tumors overexpress ALDH.[82]

Alteration of cell cycle kinetics is another alternative intrinsic mechanism of resistance reported in BCSC.[83,84] This feature aids the BCSC to evade death due to chemotherapeutic agents targeting rapidly dividing cells.[76,81] This quiescent state of BCSC is also responsible for relapsed disease after a long-periods of time. A dexterous DNA repair mechanism in the BCSCs is another example of intrinsic resistance mechanism.[84] BCSC uses the augmented activity of ChK1 and ChK2 allowing them to escape from mitotic catastrophe and to repair their damaged DNA proficiently.[84,85] This state of dormancy and robust DNA repair mechanism contributes to the resistance of BCSC against standard chemotherapeutic regimes.

In recent times, miRNAs are also shown to govern and regulate the BC resistance against the standard therapies. miRNAs are short, non-coding RNAs that regulate crucial biological processes and are frequently deregulated in cancer. Suppression of miR200c has been shown to promote tumorigenicity of BCSCs and normal mammary stem cells. In addition, it was shown that suppression of miR-200c triggers migration and invasion of cancer cells in the neighboring tissues.[86] Loss of miR-205 in BCSC populations has been shown to result in drug resistance properties.[87] Another report demonstrated that miR-141 is inhibited in BCSCs, contributing to the dedifferentiation of BC cells into stem-like cells which in turn enhances the stem population.[88] Alternatively, reduced expression of miR-34a in human BC resulted in inhibition of stem cell properties. A report has shown that miR-34a regulates Notch-1 pathway in sustaining stem cell properties of BCSC populations, thereby suggesting that the
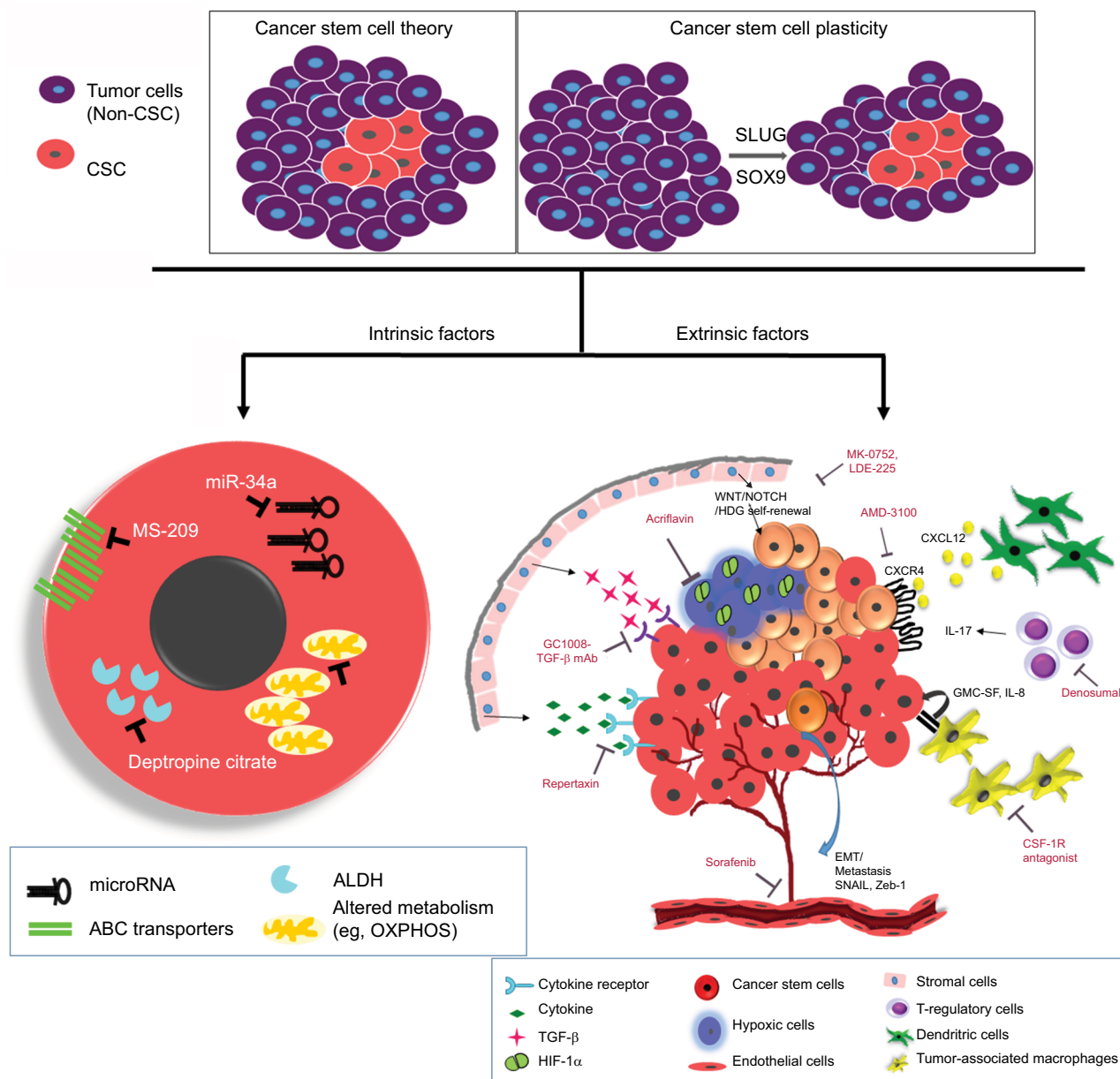
Figure 3 Schematic diagram showing intrinsic and extrinsic factors that aid the breast cancer stem cells to evade and survive against therapeutic insults.
Notes: Two widely accepted theories regarding the origins of CSCs are highlighted. According to "Cancer stem cell theory," an inherent subpopulation of dormant cancer cells that are pluripotent in nature with the ability to repopulate the depleted pool of cancer cells following therapy. The "cancer stem cell plasticity theory" describes that therapeutic insults and EMT triggers a few breast cancer cells to undergo a switch, converting epithelial cells to pluripotent CSCs. The interplay of microenvironment and intrinsic cascades of CSCs aids them to elude the conventional therapy. Intrinsic factors depict microRNAs, drug transporters, ALDH, and altered cellular metabolism as pivotal processes that can be targeted. Further, extrinsic factors include a plethora of various components from the tumor microenvironment. Some of the important players being hypoxia, angiogenesis, EMT, immune cells, and stromal cells promoting proliferative signaling like cytokines, TGF-β, and self-renewal signals like Wnt/Notch/Hedgehog. Some of the inhibitors/mAbs against these resistance promoting factors have also been depicted.
Abbreviations: ABC, ATP-binding cassette; ALDH, aldehyde dehydrogenase; CSC, cancer stem cell; EMT, epithelial–mesenchymal transition; GMC-SF, granulocyte-macrophage colony-stimulating factor; HIF-1α, hypoxia inducible factor-1alpha; OXPHOS, oxidative phosphorylation; TGF-β, transforming growth factor beta.

miR-34a/Notch-1 pathway might be a potential therapeutic target for treating BC.[89] Further, enhanced expression of let-7 miRNA has been shown to be involved in tumorigenesis of BCSCs. Other report has revealed that isoform let-7c along with Wnt signaling cascade regulates BCSC renewal in vivo.[90] In addition, miR-1 has been shown to be associated with Wnt signaling pathway which is critical for the aggressiveness

of BC.[91] Most of the knowledge gained in recent times has provided a foundation for the future development of miRNA-based therapy against BCSCs.

Extrinsic cause of resistance

The interaction between the microenvironment and CSC is a dynamic process resulting in continuous remodeling of

both.[84] EMT plays a crucial role in chemoresistance and aids in cancer metastasis.[92] Augmented drug efflux, suppressed apoptotic signaling pathways, and slow cellular proliferation are associated with EMT, and this contributes to the resistance of BC cells against anticancer drugs.[93] Gefitinib or erlotinib, prescribed to BC patients with high EGFR, often relapses. EMT-associated transcription factor Snail is reported to enhance the expression of AXL receptor tyrosine kinase. Signals transduced by AXL allow the BC cells to override the cytostatic effects of EGFR inhibitors. EMT also triggers other processes that enable the BC cells to elude the lethal effect of cytotoxic T cells. Elevated expression of PD-L1 is one such major evasion mechanism employed by BC cells.[75] PD-1, an inhibitory immune-checkpoint receptor, expressed by cytotoxic T cells recognizes the PD-L1 on the cancer cells and diminishes their function. Further, enhanced secretion of thrombospondin-1 by mesenchymal cells induces the development of regulatory T cells within the tumor microenvironment that suppresses the cytotoxic T cells.

In addition to chemoresistance, EMT process also equips the BC cells to evade cytotoxic effects of radiation. Radioresistant BC cells that acquire mesenchymal properties have been reported to be more invasive, attributed to enhanced traction forces and membrane ruffling.[94] The intricate program of EMT is not only responsible for the development of BCSC, but deeper understanding in the process would help us to develop novel approaches to target cells that evade conventional therapeutic regimens. Paracrine signals from the Notch/Wnt/Hedgehog pathway influence EMT by cytoskeleton rearrangements which results in mesenchymal-like phenotype.[95]

BCs have the tendency to recruit mesenchymal cells from the normal breast stroma[96] or from the bone marrow.[97] For instance, mesenchymal stem cells (MSCs) expressing ALDH1 are selectively recruited to areas of actively dividing tumor, where they interact with BCSCs via cytokine loops of CXCL7 and IL-6.[97] These cytokine signaling augments the self-renewal of BCSCs.[97] In addition, MSCs have also been shown to protect the BCSCs via recruitment of regulatory T cells.[98] Immunohistochemical analysis has established the presence of such interacting MSC/BCSC in tumor biopsies of BC patients.[97] High ALDH1 expression in BC cells has been shown to be an independent predictor of poor outcome in patients with BC.[79] Further, MSCs have the capability to differentiate into adipocytes and tumor-associated fibroblasts, which might also interact with tumor cells and can influence disease progression.[99]

Gabbiani and Majno were the first who reported the morphological alterations in the stimulated myofibroblasts of dormant tumor- and wound-associated fibroblasts.[100] Confirming the observation, in an experimental mouse model, it was demonstrated that acute wounding of the mammary gland by dermal incision augmented BC growth and metastasis.[101] While the exact underpinning mechanisms remain elusive, it is believed that paracrine signals from the developing tumors induce epigenetic changes in the neighboring stromal fibroblasts.[102] Certainly, the expression profile of cancer-associated fibroblasts (CAFs) is similar to that of wound-associated fibroblasts; this profile has been linked with poor outcome of patients.[103,104] A report suggests that transforming growth factor beta (TGF-β; growth factor) may be involved in regulating the epigenetic changes, leading to fibroblast activation.[105] In addition, cytokines like CXCL12 (also known as SDF-1) released by BC-associated fibroblasts might help in the proliferation of cancer cells, which expresses CXCR4 (SDF-1 receptor).[106] The high levels of free SDF-1 in serum has been correlated with poor outcome in BC patients.[107,108]

Interleukin-6 (IL-6) and IL-8 have been associated with both chronic inflammation and tumor growth.[109,110] Many cell types present in the tumor microenvironment including macrophages, immune cells, and mesenchymal cells have been reported to secrete both IL-6 and IL-8.[110] In addition, the high levels of both of these cytokines in serum have been related to poor BC patient outcome.[111,112] IL-6 has been shown to promote angiogenesis, tumorigenicity, and metastasis.[113] In clinics, correlation of high IL-6 serum levels and poor outcome in BC patients justifies the studies aimed to elucidate the role that these cytokines play in tumorigenesis. A report demonstrated that IL-6 is directly involved in BCSC self-renewal, that was mediated by the IL-6 receptor/GP130 complex via STAT3 activation.[114] IL-6 has been shown to be a vital element of positive feedback loop that regulates these MSCs and BCSCs.[97] Utilizing relative gene expression profiling, it was identified that CXCR1 (IL-8 receptor) was overexpressed on BCSCs and also IL-8 was able to induce the self-renewal of the BCSCs.[115] Further, blocking the receptor activity in mouse xenografts significantly reduced the population of BCSCs, resulting in decreased tumorigenicity and metastasis. The production of inflammatory cytokines such as IL-6 and IL-8 is controlled by the NF-κB signaling pathway.[116]

Hepatocyte growth factor (HGF), released by mammary stromal cells, might also play an important role in developing mammary tumors.[117] HGF serves as a co-stimulatory signal

to activate the Wnt pathway during colon carcinogenesis;[118] however, involvement of similar pathways in breast carcinogenesis is still unknown. Another vital growth factor released by activated fibroblasts includes fibroblast growth factors (FGFs). It was recently reported that estrogen regulates the BCSC population via paracrine signaling cascade involving FGF9.[119] Additional factors such as PDGF, IGF, Wnt, Hedgehog ligands, Notch ligands, and matrix metalloproteinases (MMPs) are released in the tumor microenvironment that controls tumor proliferation, invasion, and metastasis.[120–125]

Endothelial cells are involved in blood vessel formation and might play an important role in developing the tumor microenvironment via direct interaction with tumor cells. Endothelial cells have been reported to be a significant constituent of normal neuronal and hematopoietic stem cell niches.[126,127] It has been observed that cytokines produced by endothelial cells regulate CSCs.[128,129] Remarkably, the tumor vasculature is substantially different from the normal vasculature, as exemplified by the differential expression of almost 1,000 genes between them, including JAK3, MMPs, and FGF receptors.[128] Even though several pro-angiogenic factors have been recognized, VEGF is the principal facilitator of this process,[130] and because of this, it has become the primary target of many anti-angiogenic therapeutics. Bevacizumab and two small molecule multi-kinase VEGF inhibitors, sunitinib and sorafenib, are currently approved for clinical application. Bevacizumab was approved against metastatic BC as it can prolong the time taken to tumor progression.[131] However, more recent studies have suggested discouraging the result that the effect is severely limited and that the combination of bevacizumab and cytotoxic chemotherapy failed to increase the OS of patient.[132] These results are corroborated with reports in mouse models that application of anti-angiogenic agents might accelerate BC invasion and metastasis.[133,134] By studying mouse model of human BC, a report also suggests that these anti-angiogenic agents increase the CSC pool through tissue hypoxia.[135] Anti-angiogenesis drugs might also augment tumor growth by stimulating HGF production from tumor-associated stromal cells.[136]

New approaches to develop therapeutics against BCSCs

As stated above resistance to conventional therapeutic regime such as radiation and cytotoxic chemotherapy has been the motivation behind the development of specific agents capable of targeting the CSC population. BCSCs show enhanced expression of CD44 and ABC transporters promoting survival of these stem cells. These surviving cells again give rise to tumors that have enhanced chemo-tolerance and metastatic ability resulting in relapse. Here we would discuss some of the current approaches used for targeting BCSC. The promising therapies employed against BCSCs have been summarized in Table 1.

Targeting signaling cascades

There are significant reports of dysregulation of Notch pathway in a substantial fraction of human BCs.[137,138] Of the various approaches, one of the most clinically promising candidates is γ-secretase inhibitor. Activation of Notch signaling is regulated by this proteolytic enzyme (γ-secretase), which cleaves Notch receptors and releases the intracellular domain, which in turn acts as a transcription factor and regulates important oncogenic gene functions.[139] Therefore, γ-secretase inhibitors were designed for treating BC patients in an early-phase clinical trial. The most severe effect observed has been the gastrointestinal toxicity due to goblet cell hyperplasia, which is an on-target effect of Notch inhibition.[140] Although, a moderate dosage along with the administration of high dose of corticosteroids was able to lower the toxicity.[141] Combination of γ-secretase inhibitor with taxane chemotherapy has also been clinically investigated in a Phase I trial.[141] Other pathway regulating BCSC is the Hedgehog pathway. This pathway has been reported to be active in tumor cells as well as in the tumor stroma.[142] Oral Hedgehog inhibitors were clinically tested, and they appear to be fairly nontoxic.[143] Phase II clinical studies employing this drug compounds in combination with conventional cytotoxic agents are underway.

Despite the outstanding clinical efficiency of HER2-targeted therapy, almost one third of HER2-positive BC patients do not respond to these agents, and chemoresistance may develop in these patients with chronic exposure. Increasing evidence indicates that resistance may be associated with the activation of other receptor kinases, gain of function mutations of PI3K, loss of PTEN tumor suppressor gene, or truncation of the extracellular domain of HER2.[144] These mutations cause aberrant activation of the downstream PI3K/Akt/mTOR pathway and are generally correlated with poor prognosis after conventional trastuzumab therapy.[144] Confirmatory evidence has recently shown that the PI3K/Akt/mTOR pathway plays a significant role in regulating BCSC pool. This ensues via Akt activation of the Wnt pathway through phosphorylation of GSK/3β and direct phosphorylation of β-catenin on serine552 amino acid which results in its nuclear transport.[145] This observation suggests that suppressing Akt that is downstream of HER2 signaling might efficiently target BCSCs in

Table 1 Upcoming therapeutic modalities against breast cancer stem cells

Drug	Biological target	Clinical trial
γ-Secretase inhibitors		
MK-07S2	Notch activation	Phase I
RO-4929097	Notch activation	Phase II
MK-0752 + docetaxel	Notch activation	PhaseI/II
RO-4929097 + letrozole	Notch activation	Phase Ib
OMP52M5 1	Anti-Notch monoclonal antibody	Phase I
Hedgehog pathway inhibitors		
LDE-22	Smoothened (SMO)	Phase Ib
TAK-441	Smoothened (SMO)	Phase I
PI3K/AKT/mTOR inhibitors		
Perifosine	Akt inhibitor	Phase II
MK-2206	Akt inhibitor	Phase II
Everolimus + exemestane	mTOR inhibitor	Phase III
Everolimus + tamoxifen	mTOR inhibitor	Phase II
ALDH inhibitors		
Benztropine mesylate	ALDH	Preclinical
Deptropine citrate	ALDH	Preclinical
Microenvironment		
EZN-2968	Hif-Iα mRNA	Phase I
Acriflavin	Hif-In/2 a PAS-B domain	Preclinical
Echinomycin	Hypoxia response element	Preclinical
Repertaxin	CXCR1/CXCR2	Phase I
PD0332991	CAF	Phase II
CSF-IR antagonist + paclitnxel	TAM	Preclinical
Anti-CD-47 antibody	Macrophage inducer	Phase I
Denosumab	Tregs	Phase III
Rebimastat	MMP	Phase II
AMD3100 (CXCR4 antagonist)	SDF-I/CXCR4	Preclinical
GC1O08	Anti-TGF-Q monoclonal antibody	Phase II
Imiquimod	TLR7agonist	Phase II
PEGPH20	Hyaluronan	Phase II
TH-302	Hypoxia	Phase III
Sorafenib	VEGFR/PDGFR	Approved
AMG337	MET kinase	Preclinical
Tumor metabolism		
Etomoxir	Carnitine palmitoyltransferase-1 inhibitor	Preclinical
VLX600	Mitochondrial OXPHOS	Preclinical
Salinomycin	Sodium potassium gradient	Preclinical
NK4	HGF/MET	Preclinical
TVB-2G40	Fatty acid synthase (FASN)	Phase I
XCT790	ERRn-PGC1	Preclinical
Transporter inhibitors		
MS-209	P-glycoprotein	Phase III
9-Deazapurine	P-glycoprotein + MRP-I+BCRP	Preclinical

Abbreviations: ALDH, aldehyde dehydrogenase; CAF, cancer-associated fibroblast; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase; OXPHOS, oxidative phosphorylation; TAM, tumor-associated macrophage.

HER2-resistant tumors. Indeed, perifosine (Akt inhibitor) has demonstrated promising prospect by effectively targeting the BCSC pool in breast tumor xenografts.[145] Encouraged by the abovementioned observations, a spectrum of PI3K and Akt selective inhibitors are being clinically investigated, providing us the direct assessment of the effects of these agents in controlling the stem cell population.

Targeting tumor microenvironment

The role of cytokine signaling in maintaining and promoting CSCs is well-documented. Among the cytokines, IL-6 and IL-8 play an important role in the maintenance of BCSC population. These two cytokines promote the inflammatory cascade via NF-κB pathway leading to chronic inflammation and augmentation of tumorigenesis. Interestingly, anti-inflammatory

agents such as statins show a decrease in BC risk.[146] Statins lower the levels of pro-inflammatory cytokines, as revealed by lowered CRP levels.[147] A recent report has demonstrated that antibodies against the CXCR1 (IL-8 receptor) or repertaxin (small molecule CXCR1/CXCR2 inhibitor) has the potential to target BCSC in mouse xenograft models impeding tumor growth and metastasis.[145] Repertaxin was initially developed to avert graft rejection and has shown to have promising effect in Phase I trials. Repertaxin was reported to mediate stem cell death in bulk cellular population through bystander effect involving release of Fas cell surface death receptor (FAS) ligand. CXCR1 inhibits FOXO3A localization and FAS ligand expression through AKT signaling. Treatment with repertaxin suppressed AKT, resulting in nuclear FOXO3A and FAS ligand expression. Conventional chemotherapeutics are also known to cause cell death via a bystander effect through FAS ligand, but that in turn induces IL-8 which protects BCSC from FAS ligand. This suggested that repertaxin might cause blockade of this effect and efficiently kill the BCSC population. Repertaxin also suppressed the BCSC population in vitro as well as in tumor xenografts. As a monotherapy, repertaxin had a nonsignificant effect on tumor growth, but drastically reduced tumor volume when employed in combination with docetaxel. Further, repertaxin was able to reduce metastatic lesions and secondary tumor formation. These promising results show that repertaxin can sensitize BCSC to bystander effect via FAS ligand and that CXCR1 blocking might represent a novel approach to targeting and eliminating breast CSCs. Further, mAbs targeting IL-6 or its receptor are currently being assessed in clinical trials for multiple myeloma.[148]

The downregulation of caveolin-1 (CAV1) in CAFs is a well-studied biomarker which is associated with oncogenic transformation. It has been observed that inhibition of CAV1 in CAFs resulted in hyper-proliferative phenotype of BC cells. The replacement of CAV1 with CAV1 mimetic eliminated the proliferative behavior of the cells.[149] It was also reported that CAFs and MSCs sensitized MCF7 cells to the RAD001 (an mTOR inhibitor) and augmented the cytotoxic effect of RAF265 (an RAF inhibitor) on MDA-MB-231 cells through the inhibition of ERK1/2 phosphorylation.[150] However, both CAFs and MSCs had no significant effect on the response to TKI258 (a PDGFR/FGFR/VEGFR inhibitor) in BC cell lines.[150] This demonstrates that CAFs may not be involved in all the mechanisms of drug resistance, but heterogeneity of CAFs should be taken into account during drug response.

Tumor and adjoining stromal cells are known to secrete CCL2, which is an essential chemoattractant for macrophages. CCL2 and its receptor, CCR2, are involved in monocyte recruitment onto the tumor periphery. Lu et al observed that overexpression of CCL2 promotes both bone and lung metastases in BC. Targeting the tumor-derived CCL2 via a neutralizing mAb reduced metastasis to bone and lung.[151] Adverse side effects of anti-CCL2 therapy have raised serious concern as it has shown to aggravate metastasis via increasing macrophage recruitment within weeks of treatment termination.

Adipocytes are one of the main components of the breast and have been shown to play a role in tumor development. In line with this observation, a few chemopreventive agents have been tested for their efficacy against BC cells. Sulforaphane, which is a compound present in broccoli, has been extensively studied and shown promising results.[152] However, the limited understanding about the role of adipocytes in promoting tumorigenesis is limiting the scope of available options to target these adipocytes. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) is a well-known death receptor that can mediate ligand (TRAIL-R1, TRAIL-R2)-induced apoptosis, in tumor cells. TRAIL-mediated therapies are in Phase I clinical trials for TNBC.[153] It has been reported that TRAIL can be a valuable tool for targeting patients who have limited treatment options.[153] However, there are some tumors that show resistance to TRAIL therapy, with CSC being major contributors in therapy resistance. The overexpression of anti-apoptosis proteins like c-FLIP can lead to the resistance of anti-TRAIL therapy. Thus combining the c-FLIP inhibition with anti-TRAIL antibodies can lead to an effective eradiation of CSC and thus overcoming therapy resistance.[154]

Also, the macrophages and monocytes express a large amount of TRAIL receptors, that is, TRAIL-1R and TRAIL-2R. Thus, recombinant TRAIL therapy can help in selectively inducing apoptosis of the tumor-associated macrophages (TAM), which form a primary signaling arm for tumor microenvironment. Therefore, TRAIL therapy can provide dual benefits all together, where it can selectively eliminate tumor cells and also control the pro-tumor signals coming from TAM present in the microenvironment (Figure 4).[155]

Targeting CSC metabolism

CSCs show a distinct dependency on glucose and mitochondrial metabolism. It is shown that the multipotent cells rely majorly on glycolysis. The stem cell pool of basal-like BC, which is CD44$^+$/EPCAM$^+$, is dependent on aerobic glycolysis. Overexpression of FBP1, which promotes gluconeogenesis and inhibits glycolysis, reduces the number of spheroids in basal-like BC.[156] A well-known regulator for mitochondrial
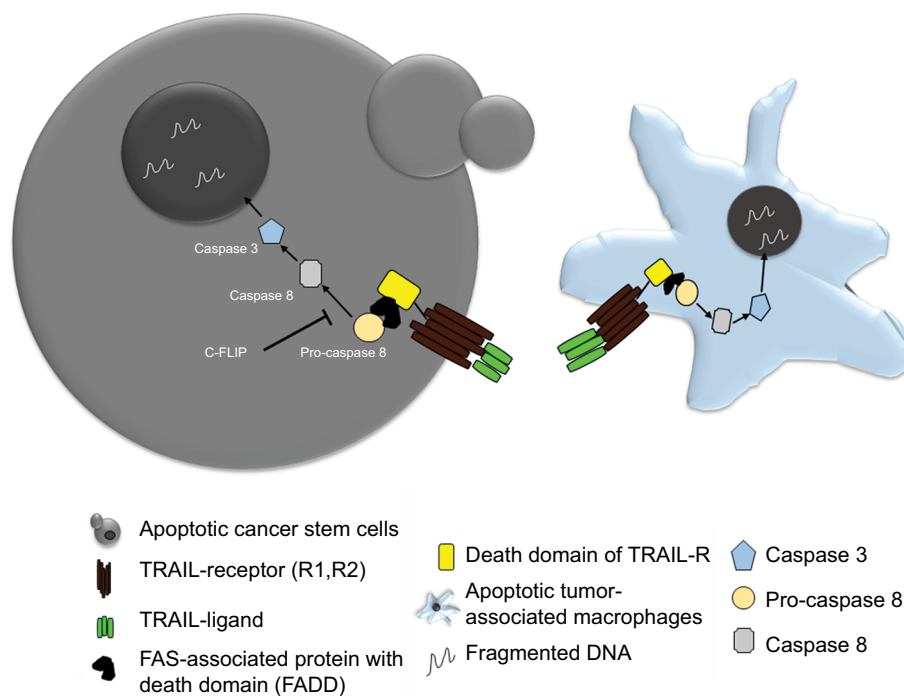
Figure 4 Schematic showing TRAIL therapy against CSC and TAM.
Notes: Recombinant TRAIL binding to its receptor on BCSCs as well as TAMs induces cell death via apoptosis.
Abbreviations: BCSC, breast cancer stem cell; CSC, cancer stem cell; FAS, Fas cell surface death receptor; TAM, tumor-associated macrophage; TRAIL, tumor necrosis factor-related apoptosis inducing ligand.

metabolism is BCL-2 protein, which forms a complex with Bcl-2-associated death promoter and glucokinase. Inhibition of BCL-2 activation can lead to the inhibition of oxidative phosphorylation (OXPHOS) leading to the reduction of CSC depending on OXPHOS.[157] Increase in mitochondrial activity can promote metastasis and confer resistance to DNA damage in BC.[158] A transcription factor peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PPARGC1A, also known as PGC-1α), is an important target for cancer cell metabolism as it couples with OXPHOS and supports migration and invasion of cells. This factor has been reported to be highly expressed in BCSCs and its inhibition lead to decreased stemness.[159] Fatty acid oxidation is another major arm of supporting tumor cell growth and proliferation. It has been reported that various stem cell pools rely on fatty acid oxidation.[160] NANOG is known to repress OXPHOS and activate fatty acid oxidation. Thus using etomoxir, a carnitine palmitoyltransferase-1 inhibitor, could reduce the spheroid formation ability of BC in vitro and also reduced in vivo tumorigenic potential.[161] A mitochondria inhibitor VLX600 has been reported to target the quiescent cell pool within the tumor, in vivo.[162] Salinomycin, which is an antibiotic extracted from *Streptomyces albus* has been shown to reduce the stemness by targeting the Wnt pathway, which is crucial for main-

taining stem cell proliferation.[163] Wnt signaling is a known regulator of cell metabolism, where a study has shown that using a therapeutic approach of administering Wnt antagonist frizzled-related protein 4 caused metabolic reprogramming, which led to apoptosis of CSC under variable glucose conditions.[164] Recent reports suggest that targeting iron metabolism can be fruitful in targeting CSC since altered iron metabolism can cause increase in ROS and oxidative stress.[165]

Nano-therapeutics against CSC

Nanoparticle (NP)-mediated therapy is an effective strategy of drug delivery for cancer therapeutics. NPs are also being employed for targeting stem cell subpopulations within tumor bulk, where CSC marker-targeted NPs offer an advantage of specificity and precision. Thus using biocompatible polymers like liposome, PLGA, and so on, which are coated with antibodies/aptamers against BCSC-specific markers, can help in specific delivery of chemotherapeutic drug, RNAi, or antibodies to the stem cell population. BCSCs generally show enhanced expression of CD44, and studies have shown that paclitaxel- and salinomycin-loaded liposomal NP coated with CD44 antibody can target the CD44+ CSC population of MDA-MB-231 cells.[166] Iron oxide magnetic NPs coated with CD44 antibody and loaded with gemcitabine have been used

for targeting the stem cell population in BC.[167] These particles have been shown to have an added advantage of hyperthermia. NPs containing a combination of chemotherapeutic agents along with autophagy inhibitor chloroquine (CQ) are an upcoming line of therapy which can target the tumor bulk as well as CSC pool within a tumor.[168] Doxorubicin and CQ NP have shown to reduce the ALDH high population of MDA-MB-231 cells.

Administration of decitabine, a DNA hypermethylation inhibitor encapsulated in NP made with polyethylene glycol, could sensitize the tumor bulk and CSC population to chemotherapy. Also, when these NPs were combined with doxorubicin, they could reduce the ALDH+ population in mammospheres of MDA-MB-231 cells.[169] Anticancer drugs are frequently being incorporated into liposomes, for efficient drug delivery. An anticancer compound ESC8 was used along with dexamethasone (Dex)-associated liposome (DX), to form ESC8-entrapped liposome named DXE, showed promising results in reducing the drug-resistant cell population.[170] Drug targets against Notch, TGF-β, and Wnt/β-catenin pathways are also being used in combination with NPs.

NPs are also used to deliver siRNA to tumor. A cationic lipid-based polymer was developed along with an siRNA and TGF-βR-I receptor inhibitor LY364947.[171] Similarly, a cationic liposomal delivery of miR-34a could reduce the expression of CSC markers ALDH and CD44, thereby delaying tumor growth.[172] An interesting observation made by a group showed that graphene oxide (carbon nanomaterial) itself has the potential to induce differentiation of stem cells and reducing their in vitro sphere forming ability, thereby it can be a good source to target tumor bulk as well as the CSC population.[173] Carbon nanotubes are capable of mediating a thermal effect, and they have been studied in BC cells where the BCSCs were found to be sensitive to these carbon nanotube thermal therapy.[174] Further, conjugation of these carbon-based nanomaterials with stem cell receptor targeting can help achieve specificity. Also, novel methods of gene delivery using NPs are being effectively used to reduce tumor burden. An interesting study showed that specific gene delivery targeting the glucocorticoid receptor using a cationic liposome has the potential to reduce tumor growth in vivo.[175]

Conclusion

BC is a complex and heterogeneous disease, a culmination of a variety of cells that exert influence on one another, thereby making the disease management complex. Cellular/clonal heterogeneity within tumors and disease relapse are the major threats from the clinical point of view. We have now started to understand the quiescent, self-renewable pool of cells within a tumor population, the so-called BCSCs, which can govern the therapy resistance, metastasis, and disease relapse. Also the stem cells have unique mechanisms to withstand drug/radiation insults, for example, presence of large number of drug efflux pumps, and enhanced DNA repair machinery and thus posing big in terms of future developments of cancer therapeutics.

Tumor microenvironment is another key domain helping in maintenance of CSCs. Various elements like cytokine flux, tumor-associated immune cells, stromal cells, and CAFs impact chemokine receptor signaling, cytoskeletal rearrangements, hypoxia, angiogenesis, as well as cell metabolism. Altered cancer cell metabolism is a consequence of cancer condition where the BCSCs depend particularly on glycolysis. Mitochondrial OXPHOS is an alternative backup for stem cell survival. A large number of preclinical and clinical studies are being conducted on today's date, targeting eradication of stem cell pool at the tumor site. Studies have also begun to explain the concept of CSC plasticity, where the non-CSCs can revert back to CSCs, and therefore, greater attention is needed as it will be indispensable for CSC management. The advancement in nanotherapeutics and nanomedicine is also greatly changing the face of treatment options by providing novel approaches of combining multimode treatment options. Together, all these dimensions added to BC research is surely going to give us an edge in reducing the impact of therapy resistance and improve disease outcomes in future days.

Disclosure

The authors report no conflicts of interest in this work.

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