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Mini-review

Determinants of resistance to chemotherapy and ionizing radiation in breast cancer stem cells

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ABSTRACT

Breast cancer cells are suggested to be organized in a hierarchical manner with a subpopulation of stem cells, termed as breast cancer stem cells (BCSCs), which contribute significantly to tumorigenesis, cancer recurrence and metastasis. BCSCs have been demonstrated to exhibit significant resistance to conventional chemo- and radiotherapy. Recent evidence suggests that treatment of breast cancers with radiation or chemotherapy agents induces stem cell-like properties in non-stem cells. Herein, we provide an overview of the key determinants of resistance to chemotherapy and radiation in BCSCs. To this end and by the use of bioinformatics, the molecular pathways, the defining markers, as well as the microenvironmental and genetic factors, which are implicated in the maintenance of stemness, chemo- and radioresistance in BCSCs, are identified and presented. Our findings could provide the foundation for the design of targeted chemo- or radiotherapeutic regimens in order to eliminate or sensitize BCSCs to cytotoxic therapies and prevent tumor relapse and metastasis.

Introduction

According to the hierarchical model, there is a rare cellular subpopulation in solid tumors that exhibits stem-like properties, capable of extensive self-renewal, clonal expansion and differentiation, the so called tumor-initiating (TI) cells or cancer stem cells (CSCs) [1,2]. CSCs display increased tumorigenesis, aggressiveness and resistance to various types of anticancer therapeutic strategies, including radiation therapy and chemotherapy [3,4]. CSCs were identified first in breast cancer in a seminal work by Al-Hajj and coworkers [5]. Several solid tumors including breast, colon, head and neck, hepatic, lung, ovarian, pancreatic, prostate, renal, stomach, are generated from, and are sustained by CSCs [6,7].

In this review, we focus on breast CSCs (BCSCs). A combination of well-established biomarkers can be utilized to identify or isolate BCSCs [8]. One of the defining characteristics of BCSCs is their ability to form mammospheres, that is, a clump of mammary cells generated from a single cell through clonal expansion when grown under non-adherent, non-differentiating conditions [9]. Elevated expression of certain aldehyde dehydrogenase (ALDH) isoforms, detoxification enzymes that catalyze the oxidation of intracellular aldehydes, is another biomarker often used to isolate malignant breast stem/progenitor cells [10,11]. Moreover, Al-Hajj and coauthors [5] demonstrated that in mammary cancer cells grown in severe combined immunodeficient mice, a fraction of cells with the phenotype of $CD44^{+}/CD24^{-/low}$ were able to generate new tumors [5]. In breast cancers, the *HER2/ERBB2* gene encoding the receptor tyrosine kinase is preferentially over-expressed in the BCSC population [12]. In addition, the expression of the ATP-binding cassette (ABC) transporter ABCG2/BCRP1 (ATP-binding cassette subfamily G member 2/breast cancer resistance protein 1) is markedly elevated in tumor progenitor/stem cells [13].

An important mechanism underlying both chemotherapy and radiotherapy is the induction of DNA damage, thereby activating DNA damage response (DDR) pathways. The complex DNA damage induced by ionizing radiation (IR) or some chemotherapeutic agents initiates diverse DNA damage response and repair pathways, collectively called DDR/R [14]. Therefore, the ability of cancer cells, including BCSCs, to withstand high doses of IR or chemotherapeutic drugs

Abbreviations: CSCs, cancer stem cells; BCSCs, breast cancer stem cells; DDR, DNA damage response; DSBs, double strand breaks; EMT, epithelial–mesenchymal transition; HR, homologous recombination; IR, ionizing radiation; NHEJ, non-homologous end-joining; ROS, reactive oxygen species; SSBs, single strand breaks; TI, tumor-initiating; TNBC, triple negative breast cancer.

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relates primarily to enhanced DDR/R pathways that respond to and repair DNA damage like double strand breaks (DSBs) much more efficiently than non-stem cancer cells [15–18]. IR and radiomimetic drugs, such as bleomycin, are well known to induce complex DNA damages, including a combination of DSBs, single strand breaks (SSBs) and non-DSB lesions, like oxidized bases, in a small volume DNA [19,20].

The presence of CSCs in breast tumors is known to increase greatly a breast cancer patient's probability of post-therapy recurrence or relapse. The underlying mechanisms of therapy resistance remain elusive though. Herein, the BCSC-specific molecular mechanisms and the associated factors implicated in the intrinsic and acquired resistance of BCSCs to radiation treatment and chemotherapy, as well as the mechanisms that govern the maintenance of "stemness" of breast cancer cells are discussed. Elucidation of these mechanisms is of profound importance in improving the clinical efficacy of chemo- and radiotherapy in breast tumors.

Bibliographic search and use of bioinformatics

The search engine GLAD4U [21] was employed to mine the biomedical bibliographic database PubMed [22] in order to retrieve genes/gene products related to the maintenance, radioresistance and chemoresistance of BCSCs using a combination of key terms such as 'breast cancer stem cells', 'chemoresistance', 'radioresistance', 'chemotherapy resistance', and 'radiation therapy resistance'. The search results were curated manually, and only the experimentally verified genes were selected. For these gene lists, the official HGNC [23] gene symbols were used (Table S1).

Maintenance of breast cancer cell stemness

The stemness properties of breast cancer cells present potential targets for anti-BCSC-directed therapeutic strategies [24]. Toward this end, the molecular mechanisms, as well as the related genes/gene products, underlying the regulation of stemness, growth, survival, self-renewal, and expansion in breast cancer cells [25] were explored (Table S1 and Fig. 1).

Microenvironmental niche

The tumor microenvironmental conditions contribute to the maintenance and regeneration of BCSCs [26,27]. There are three niches that potentially affect the stemness properties of breast cancer cells: (a) a perivascular niche consisting of microvascular network structures that nurture and promote BCSC outgrowth [28]; (b) an invasive niche, where certain BCSC subpopulations can undergo epithelial–mesenchymal transition (EMT), a complex process that is implicated in cell invasion and cancer metastasis, and in the generation of cancer cells with stem cell-like properties. BCSCs acquire an invasive behavior that enables them to metastasize to distant organs [29–31]. The pluripotency associated genes *POU5F1* and *NANOG*, which are implicated in the self-renewal of undifferentiated embryonic stem cells, promote EMT in $CD44^{+}/CD24^{-}$ BCSCs [32]. Moreover, over-expression of another pluripotency gene, *SOX2*, which facilitates metastasis of breast cancer cells by inducing EMT, was shown to enhance mammosphere formation in breast tumors [33,34]. In addition, Gupta and colleagues [35], showed that inhibition of the EMT marker CDH1, increased the stem cell population in breast tumors [35]; (c) a hypoxic niche which promotes the acquisition of progenitor/stem-like properties by non-stem cells. CSCs depend largely on hypoxia inducible factors (HIFs) for survival, self-renewal, and tumor propagation [36].

Signal transduction pathways

An increasing amount of evidence suggests that proper functioning of specific developmental signal transduction pathways is required for the regulation and maintenance of BCSCs [37]. These pathways can induce reprogramming of cancer cells, thereby resulting to the generation of induced BCSCs (iBCSCs) from non-BCSCs [2,38].

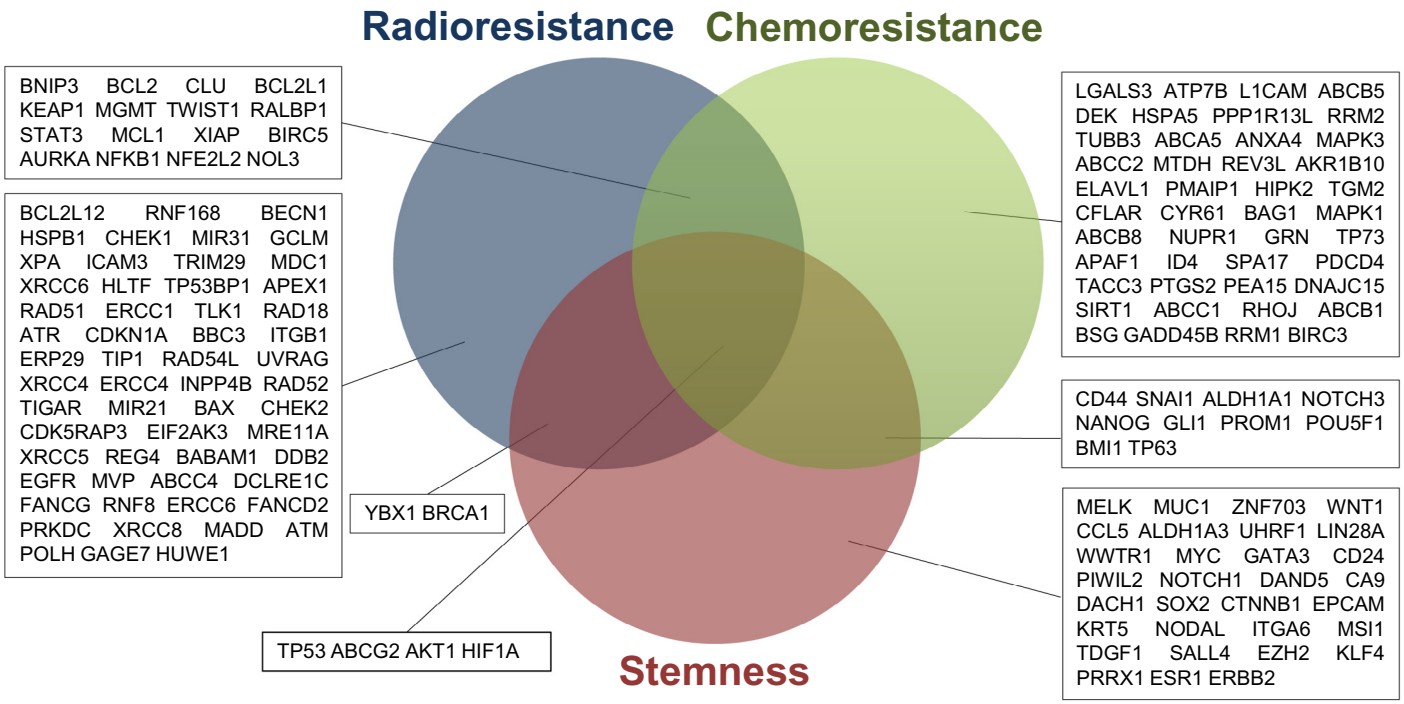

Fig. 1. A Venn diagram displaying the intersections of the retrieved gene/gene products relating to cell stemness and resistance to either ionizing radiation or chemotherapy or both.

The Notch signal transduction pathway is activated by the binding of Notch receptors (NOTCH1-4) to ligands [39]. It has been shown that the Notch signaling cascade and its complex interactions with other signaling pathways play a pivotal role in BCSC self-renewal [40]. For instance, Notch-mediated activation of ALDH1A1 through deacetylation increased the stem cell population in breast cancer, whereas acetylation of ALDH1A1 decreased the BCSC population [41]. Furthermore, breast cancer cells with elevated Notch4 activity exhibited increased mammosphere formation and expression of BCSC markers such as ALDH1 [42].

The serine/threonine kinase Akt, or protein kinase B (PKB), mediated signaling pathway is activated upstream by phosphatidylinositol 3-kinase (PI3K), as well as by PI3K's antagonist, the tumor suppressor PTEN [43]; Akt transactivates β-catenin via phosphorylation [44]. It has been demonstrated by Korkaya et al. [45] that in a mouse breast tumor model, the continuous growth of the stem/progenitor cells depended on the cross-talk between the PTEN/PI3K/Akt and the Wnt/β-catenin signaling pathways. In the same study, pharmacological inhibition of Akt resulted to decreased stem and progenitor cell population [45]. Moreover, oncogenic activation of the Wnt/β-catenin signaling network led to the amplification of mammary stem/progenitor cells [46,47]. Conversely, abrogation of Wnt/β-catenin signaling suppressed self-renewal and migration of BCSCs [48]. Furthermore, the PCNA-associated factor (PAF), which promotes breast tumorigenesis, was transactivated by the stemness-associated factors POU5F1 and NANOG. PAF was shown to upregulate BCSC markers, such as ALDH, and maintain stemness in BCSCs through PAF/Wnt signaling [49].

The Hedgehog (Hh) signaling pathway also plays a critical role in the regulation of mammary CSCs. In particular, it has been demonstrated by Liu and coworkers [50] that Hh pathway components, such as PTCH1, GLI1 and GLI2, are up-regulated in human breast stem/progenitor cells when cultured as mammospheres; these genes were down-regulated substantially when cells underwent differentiation [50]. In the same study, activation of the BMI1-dependent Hh signaling pathway increased mammosphere formation and mammosphere size, whereas inhibition of Hh signaling reversed these effects [50]. The expression of the Hh pathway components and BMI1 was elevated in $CD44^+/CD24^-$ BCSCs compared to normal tumor cells [50].

Cytokine signaling is also involved in the regulation of BCSCs. In particular, the interleukin IL-6 was shown to activate the Notch3-mediated up-regulation of the Notch ligand Jagged-1, resulting to mammosphere formation in metastatic breast cancer cell lines [51]. Another interleukin, IL-8, is implicated in the regulation of BCSCs through its cognate receptors, CXCR1 and CXCR2, by transactivating ERBB2-dependent signaling cascades [52]. Moreover, pharmacological inhibition of CXCR1 reduced the viability of BCSCs [53].

In addition, activation of the oncogenic transcription factor Y-box binding protein-1 (YBX1) via a p90 ribosomal S6 kinase (RSK)-dependent pathway, induced the BCSC surface marker CD44 in breast cancer cells resulting to self-renewal and mammosphere growth [54]. Suppression of RSK signaling led to eradication of BCSCs in triple-negative breast cancers (TNBCs) [55], signifying its contribution to the maintenance of BCSCs.

The tumor suppressor TP53

Several lines of evidence have highlighted a prominent role of the tumor suppressor TP53 in regulating stemness properties beyond its established role in cancer [56]. Restoration of TP53 activity in ERBB2-over-expressing mammary cancer stem cells resulted to restoration of asymmetric cell division and reduction of mammosphere formation and tumor growth [57]. Moreover, the TP53 isoform Delta133p53beta was shown to promote stemness properties in metastatic breast cancer cells, by upregulating the expression of key factors in the regulation of pluripotency SOX2, POU5F1, and NANOG [58]. In a study by Chang and coworkers [59], TP53 was shown to suppress EMT-associated properties in mammary epithelial cells by modulating miR-200c. Re-activation of miR-200c, also, enhanced stemness by inhibiting the EMT-associated genes *BMI1*, *KLF4*, and *ZEB1* [59]. Importantly, TP63, the sister homolog of TP53, is also involved in the self-renewal and clonal expansion of BCSCs through modulation of the Sonic Hedgehog signaling pathway [60].

MicroRNAs

MicroRNAs (miRNAs) are small (approximately 20–22 nucleotides) non-coding RNAs that regulate gene expression by targeting the 3′-untranslated region (UTR) of mRNAs, either for translational repression or mRNA degradation [61]. Emerging evidence suggests that deregulated miRNAs play an important role in regulating sets of stemness-associated genes through different pathways, acting either as oncomirs or tumor suppressive miRNAs [62,63]. For example, according to Yu et al. [64], forced expression of let-7 in breast TI cells reduced mammosphere formation, whereas suppression of let-7 expression promoted self-renewal of BCSCs [64]. Shimono and coauthors [65] identified 37 miRNAs that are differentially expressed between BCSCs and non-tumorigenic cells. In the same study, three clusters of miRNAs, miR-183-96-182, miR-200b-200a-429 and miR-200c-141, were found down-regulated in human BCSCs. Importantly, miR-200c inhibited clonogenicity of BCSCs in murine breast tumors and tumorigenicity of human BCSCs [65]. Furthermore, miR-34a suppressed BCSC-like properties by inhibiting Notch1 signaling [66], while miR-27b is involved in the inhibition of BCSC generation [67]. Another onco-suppressive miRNA, miR-1, was found to suppress proliferation and migration of BCSCs via inhibition of the Wnt/β-catenin signaling cascade [68]. Moreover, ectopic expression of the tumor-suppressive miR-33b in aggressive metastatic breast cancer cell lines was shown to inhibit stemness, as well as metastasis and invasion of breast cancer cells [69]. This was achieved by targeting *HMGA2*, *SALL4* and *TWIST1*, which are involved in CSC self-renewal [69]. On the other hand, the oncomir miR-221 was found to be up-regulated in BCSCs and enhance stemness of mammary cancer cells [70].

Resistance of BCSCs to chemotherapy and radiation

Chemoresistance

Chemotherapeutic agents are administered to breast cancer patients to decrease the size of the breast tumors and induce a remission. However, these agents do not eradicate breast tumor stem cells. As a result, the patients acquire pan-resistance to chemotherapy leading to post-treatment relapse [71,72]. There is increasing evidence to suggest that BCSCs contribute to chemoresistance through various molecular mechanisms/pathways and associated factors (Table S1 and Fig. 1).

Increased ALDH expression

Cyclophosphamide, an alkylating agent that forms crosslinking of DNA, is one of the most commonly used chemotherapeutic agent in the treatment of breast cancers [73]. Increased levels of two ALDH isoforms, ALDH1A1 and ALDH3A1, were observed in breast cancer patients that either were given cyclophosphamide therapy or did not respond to this therapy [73]. Since ALDH1A1 and ALDH3A1 are overexpressed in BCSCs [74], it is most likely to be implicated in BCSC resistance to cyclophosphamide. Another antineoplastic drug, disulfiram, was shown to strongly inhibit ALDH1A1 and target BCSCs [75]. Moreover, Tanei et al. [76] showed that there is a link between ALDH1-positive, but not $CD44^+/CD24^-$, BCSCs and resistance to the sequential chemotherapy with the drugs paclitaxel and epirubicin

[76]. In a more recent study by Kida et al. [77], ALDH1 expression was found to be a powerful predictor of chemoresistance by breast cancer subtype [77].

Upregulation of ABC transporters

The main function of ABC transporters, which are generally located in the cytoplasmic membrane, is to excrete harmful toxins and xenobiotic compounds out of the cells [15]. The ABC transporters play a pivotal role in chemoresistance by effluxing a wide variety of anticancer drugs out of cancer cells. Regarding BCSCs, Wright et al. [78] showed that BCSCs resistant to the chemotherapeutic drug doxorubicin have increased expression of ABCB1 (ATP binding cassette subfamily B member 1) [78]. Moreover, in metastatic breast cancer patients, upregulation of the multidrug resistance transporter ABCG2 and increased resistance to the anticancer agent mitoxantrone was found in stem cells compared to non-stem cells [79]. In a study by Chun and colleagues [80], ERBB2^{+} BCSC-enriched MCF-7 tumorspheres were shown to be resistant to the drugs doxorubicin, fluorouracil and paclitaxel. Pharmacological inhibition of ERBB2 with lapatinib sensitized BCSCs to doxorubicin by inhibiting the ABC transporters ABCB1 and ABCG2 [80].

Deregulation of apoptosis

Inactivation of apoptosis may render BCSCs more resistant to chemotherapeutic drugs [81]. The efficacy of chemotherapeutic agents can be increased by suppressing cellular antiapoptotic factors [82]. The therapeutic agent evodiamine was demonstrated to selectively target breast cancer stem-like cells by activating the pro-apoptotic TP53 and its transcriptional target p21 [83]. Moreover, reduced expression of the tumor suppressor miR-34a, due to TP53 mutation, in murine chemoresistant metastatic breast cancer cells decreased the stem cell population and sensitized these cells to doxorubicin by directly targeting the gene *NOTCH1* [84]. In addition, increased levels of the anti-apoptotic protein B-cell lymphoma 2 (BCL2) were found in CD44^{+}/CD24$^{-/low}$ BCSCs [85]. BCSCs were shown to be sensitized to doxorubicin by down-regulating the anti-apoptotic *BCL2* gene [71].

Increased DNA damage repair

The DNA-damaging chemotherapeutic agents administered to breast cancer patients induce DNA damage and tumor cell death [72,86]. These agents preferentially target the S-phase of breast cancer cells, where DNA replication takes place. The cancer cells, due to defective DNA repair mechanisms and enhanced proliferation, are not able to adequately repair the damage, leading to cell death [72,86]. BCSCs, as opposed to the non-stem breast cancer cells, have demonstrated enhanced DNA repair capacity [5]. Transcriptional profiling of BCSCs from the mammary gland of *p53*-null mice revealed the up-regulation of DNA damage response and repair genes, such as *Bmi1*, *Brca1*, *Chek1*, *Ezh2*, *Hus1*, *Nek1*, *Sfpq*, *Ung*, *Uhrf1*, *Xrcc5* [87]. Increased DNA repair in CSCs contributes to chemoresistance in breast cancer [72]. In particular, it has been shown that CSCs isolated from the mammary tumors developed in *Brca1/p53* knockout mice are implicated in resistance to cisplatin [88], a member of platinum-containing antineoplastic compounds that form various DNA interstrand or intrastrand crosslinks [89,90].

Hypoxia and cell cycle status

Solid tumors contain hypoxic areas, and the cells located in these areas are more likely to develop resistance to chemotherapy [91]. In a hypoxic environment, BCSCs enter a quiescent and slower cell cycle progression state which enables them to become more resistant to cell cycle targeting chemotherapeutic agents [92,93]. Of importance, critical hypoxia-associated factors, such as the hypoxia-inducible factor-1 (HIF1A), are required for resistance of BCSCs to chemotherapy [94,95]. In particular, Samanta et al. [95] demonstrated that treatment of human TNBC and MCF-7 cell lines with the chemotherapeutic agent paclitaxel or gemcitabine induced HIF1A expression and transactivation of its downstream targets, CA9 and ENO1. Upon HIF1A induction, the BCSC population was enriched via IL6 and IL8 signaling and the transporter ABCB1 was activated. Inhibition of HIF1A rendered BCSCs susceptible to chemotherapy and prevented tumor relapse [95].

Chemoresistance-associated signal transduction pathways

BCSCs could be eradicated by applying chemotherapeutic agents that selectively target various BCSC-related signaling pathways [15]. For instance, the anti-inflammatory drug aspirin sensitized BCSCs to chemotherapy drugs (cyclophosphamide, fluorouracil and doxorubicin) by disrupting the inflammatory NFKB1/IL6 pathway which promotes stemness in non-stem cells [96]. Moreover, BCSCs treated with disulfiram and copper inhibited NFKB1-mediated pathways and rendered these cells vulnerable to the drug paclitaxel [97]. Zhang and colleagues [98] showed that PYGO2, a component of the Wnt/β-catenin signaling axis, increased the acquisition of chemoresistance in breast cancer cells by inducing ABCB1 expression through Wnt/β-catenin signaling. Silencing of PYGO2 sensitized cancer cells to chemotherapy and reduced the BCSC population [98]. In addition, abrogation of RSK signaling was found to sensitize CD44^{+} TNBC cells to chemotherapy by eradicating the BCSC population [99]. In a study by Wang et al. [100], up-regulation of caveolin-1, a membrane transporter protein, was shown to be correlated with the acquisition of resistance in BCSCs to genotoxic drug combination (epirubicin, paclitaxel and fluorouracil). Inhibition of caveolin-1 sensitized BCSCs to chemotherapy by downregulating the β-catenin/ABCG2 signaling pathway via GSK3B induction and Akt suppression [100]. Furthermore, the expression of components of the Hh signaling pathway (SMO, PTCH1, GLI1 and GLI2) was increased in MCF-7 mammospheres enriched in BCSCs that were sensitive to the potent anticancer drug salinomycin [101].

Radioresistance

Ionizing radiation presents a widely used modality of breast cancer therapy [102]. BCSCs have evolved resistance to radiation due to intrinsic and extrinsic mechanisms, genetic mutations and epigenetic modifications [103]. In a study by Phillips et al. [104], it was demonstrated that after radiation treatment of established breast cancer cell lines, a side cell population with stem-like properties was enriched; conversely, these cells displayed considerable resistance following radiation [104]. The BCSC biomarker ERBB2 also plays an important role in the response of these cells to radiation. For example, ERBB2-positive BCSCs were shown to be more radioresistant compared to ERBB2-negative BCSCs. NFKB1 was required for radiation-mediated ERBB2 transactivation [105]. Moreover, BCSCs with the phenotype ERBB2^{+}/CD44^{+}/CD24$^{-/low}$ displayed increased resistance to radiation compared to their ERBB2-negative counterparts [106]. The molecular pathways and the related factors that modulate the radiation response of BCSCs are discussed below (Table S1 and Fig. 1).

Hypoxic environment and quiescence

Oxygen is considered a powerful radiosensitizer that can increase the efficacy of radiation by forming DNA-damaging reactive oxygen species (ROS) [107]. Therefore, in a hypoxic environment tumor cells become less vulnerable to radiation [16,108]. Furthermore, similar to chemoresistance, HIF1A has a profound impact on the net response of breast tumors to radiation [109,110]. In particular, following radiation, increased expression of HIF1A-regulated cytokines led to enhanced tumor radioresistance as a result of cytokines' capacity to abate vasculature destruction [110]. Con-

versely, suppression of HIF1A significantly enhanced tumor radiosensitivity due to pronounced vasculature damage that resulted to massive tumor killing [110]. Of importance, CSCs, including BCSCs, tend to be more resistant to radiation treatment by becoming quiescent under hypoxic conditions [92]. In general, rapidly proliferating cells are more sensitive to radiation, whereas quiescent cells are more radioresistant [111,112].

Enhanced DNA damage repair

Radiation treatment, as in the case of chemotherapy, exerts its therapeutic effect by inducing complex DNA damage in tumor cells and eventually cell death. There are a great number of proteins that respond to radiation by participating in the molecular pathway of aberrant DNA detection, cell cycle arrest, DNA damage repair and apoptosis. Deregulation of these pathways and their associated factors can lead to genomic instability and carcinogenesis [102].

It was shown that both normal and cancer breast stem cells produced fewer ROS upon radiation compared to the non-stem cells [104]. Moreover, Diehn et al. [113] showed increased expression of free-radical scavengers and the formation of fewer DNA strand breaks in BCSCs after irradiation, indicating that stem cells have developed mechanisms to protect their genome from oxidative damage [113]. In the same study, depletion of ROS scavengers in BCSCs decreased markedly their clonogenic potential and resulted to radiosensitization [113].

BCSCs are capable of enhanced repair of damaged DNA. In particular, Phillips et al. [104] showed that BCSCs have lower expression of phosphorylated H2AX (γH2AX) in response to radiation, signifying efficient repair of DNA double-strand breaks [104]. Using extensive bibliographic search, a great number of DNA damage repair-associated factors were found to be implicated in the response of BCSCs to radiation treatment (e.g., ATM, ATR, BCL2, BCL2L1, BRCA1, CDKN1A, CHEK1, CHEK2, ERCC1/4/6, MRE11A, RAD18/51/52/54L, TIGAR, TP53, XIAP, XPA, XRCC4/5/6/8) (Table S1). For example, increased expression of the phosphorylated checkpoint kinases CHEK1/2 (pCHEK1/2) in MCF-7 breast cancer cells was shown to enhance the resistance of these cells to radiotherapy [17].

Of importance, CSCs, including BCSCs, prefer to repair major radiation-induced DNA lesions, like DSBs, primarily via homologous recombination (HR) [16,114] instead of non-homologous end-joining (NHEJ), the latter of which is less accurate and prone to errors compared to the practically error-free HR [115]. Recent studies suggest that breast cancer stem-like cells show dominant homologous recombination due to a larger S-G2 fraction [116].

Radioresistance-related signaling pathways

Radiation leads to the activation of either pro-apoptotic or pro-survival signal transduction pathways in the irradiated BCSCs [16]. For example, radiation exposure activated the PIK3-mediated Notch signaling pathway in BCSCs and induced the expression of the Notch ligand Jagged-1, indicating that Notch signaling may contribute to radioresistance [104]. Moreover, in the breast cancer MCF-7 cell line, the stem cell population was enriched upon radiation treatment, β-catenin was elevated, and the γH2AX nuclear foci were resolved very fast [117]. This signifies the potential role of the Wnt/β-catenin signal transduction cascade in radioresistance and enhanced DNA repair in BCSCs. Furthermore, in a p53-deficient mouse mammary tumor model, abrogation of the Akt and Wnt signaling pathways was correlated with decreased efficiency of DNA damage repair in stem cells and sensitization of these cells to ionizing radiation [117]. Another signaling axis, RAF-MEK-ERK, was shown to be implicated in the radioresistance of ERBB2^{+}/CD44^{+}/CD24$^{-/low}$ BCSCs through transactivation of the MAPK phosphatase DUSP1. Inhibition of DUSP1 resulted to sensitization of BCSCs to radiation [118].

Repopulation

An additional mechanism by which breast tumors develop radioresistance is the accelerated repopulation of the recurrent tumors by BCSCs, as the fraction of BCSCs was shown to increase after radiation therapy [16]. During treatment, BCSCs accumulate genomic alterations. Consequently, only the fittest BCSCs with the beneficial genomic mutations can adapt and survive. Therefore, radiation selectively kills the most radiosensitive BCSCs, whereas a percentage of BCSCs remains viable and acquires further resistance to radiation [16]. Furthermore, iBCSCs are generated from irradiated non-stem breast cancer cells that may, along with the surviving BCSCs, repopulate the tumor bulk [119].

Chemo- and radioresistance of BCSCs

For the successful treatment of breast cancer patients, a combinatorial therapeutic approach of anti-oncogenic drugs and radiation is recommended [71,92]. Therefore, in order to enhance the efficacy of targeted therapy, it is of paramount importance to identify the determinants of both chemo- and radioresistance. To this end, in the present study, the common factors contributing to the maintenance of stemness, radioresistance and chemoresistance in BCSCs were detected and the potential functional associations among them were investigated.

A Venn diagram depicting the overlapping genes/gene products among the three BCSC-related biological processes (Table S1) was constructed with the usage of BioVenn [120]. TP53, AKT1, HIF1A, and ABCG2 appear to participate in all three events of BCSCs (Fig. 1), suggesting a critical role of these factors in the stem cell-dependent treatment resistance in breast cancer.

Moreover, the interactions among the chemo- and radioresistance associated genes were investigated and visualized by using the Ingenuity Pathway Analysis (IPA) (Ingenuity Systems, Inc., Redwood city, California, http://www.ingenuity.com/) software. IPA is based on a built-in library, the Ingenuity Pathways Knowledge Base (IPKB), a large curated pathway database which extracts knowledge from various sources. The input gene entries, collectively 134, used to construct the molecular interactome (Fig. 2) were retrieved from the biomedical literature and are listed in the second and third column of Table S1. Totally, 61 of these genes appear to form a highly interconnected network (Fig. 2), indicating a possible functional association among them or their products in radiation/chemotherapy response. As expected, the node that corresponds to the cardinal tumor suppressor TP53 is the most highly connected, representing a hub in the interactome network. Also, several DDR/R factors (e.g., ATM/ATR for DDR and BRCA1/RAD51 for HR) are implicated in the network, underlying the paramount role of DNA damage repair in chemo-and radioresistance (Fig. 2).

The WebGestalt [121] toolkit was employed to identify statistically significant over-represented KEGG Pathway [122] terms (the threshold for the FDR adjusted p-value was set at 10^{-4}) across genes. The enriched KEGG Pathways in the 134 genes are shown in Table 1. A total of 52 out of 134 genes participate in 23 over-represented KEGG Pathways (Table 1). As expected, many of these pathways are related to cancer (Table 1). Notably, several enriched KEGG pathways participate in DNA damage repair such as cell cycle, NHEJ and nucleotide excision repair, highlighting the importance of these pathways in the resistance of BCSCs to chemo/radiotherapy. The p53 signaling pathway and apoptosis, key events in the repair of DNA damage, are also over-represented. The omnipotent transcription factor p53, as well as the kinase AKT1, appear to participate in most of the over-represented pathways (Table 1), indicating a nodal role in the regulation of many breast cancer stem cellular events primarily by eliciting signaling cascades.

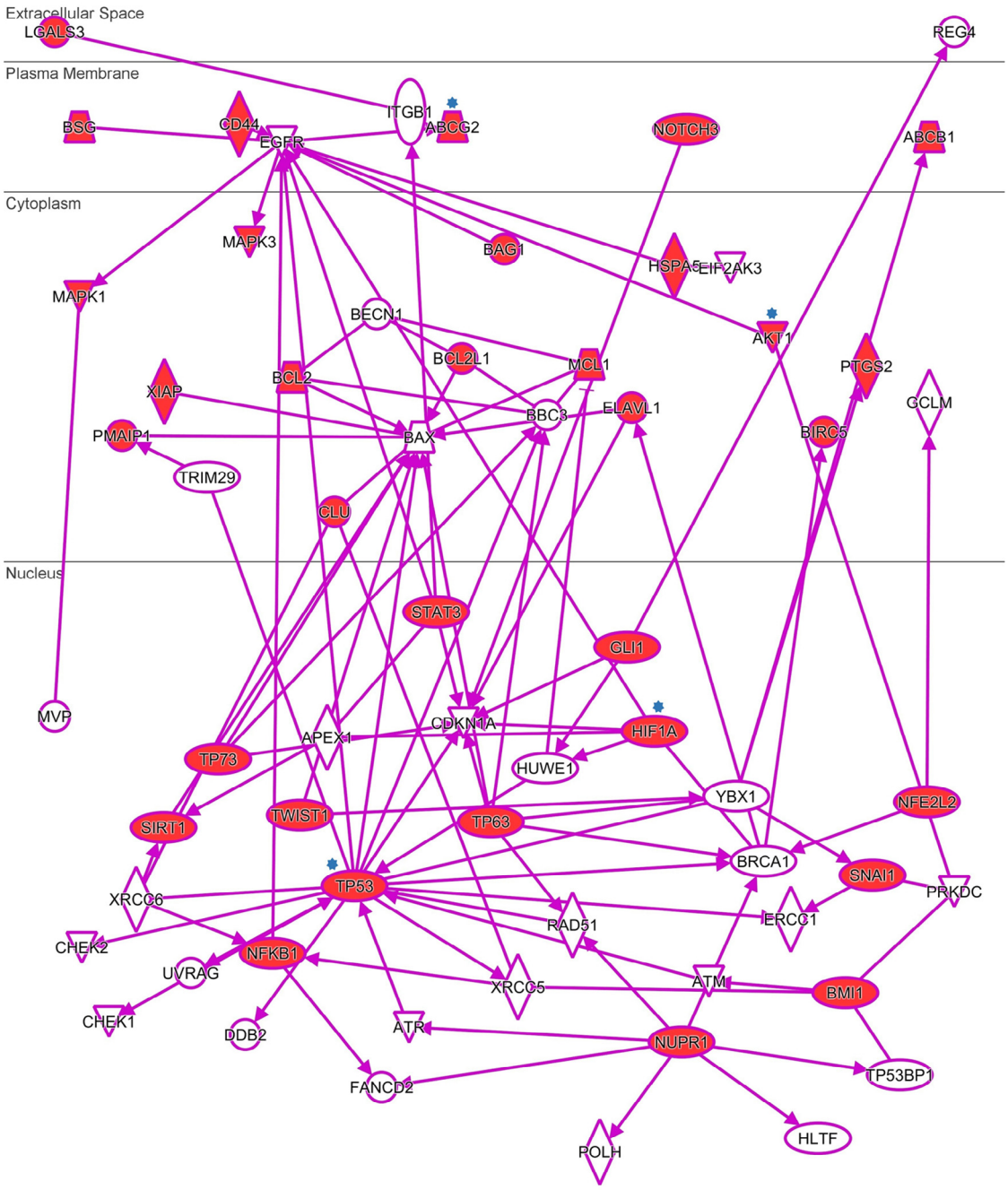

Fig. 2. The interactions among the protein factors implicated in chemo/radioresistance, as designed by Ingenuity Pathway Analysis (IPA; Qiagen). The IPA along in combination with Ingenuity Knowledge Base, which comprises ~5.1 million relationships, was used for the network analysis. The lines indicate interaction and the arrowed lines denote "acts on." The chemoresistance-associated factors are highlighted in red. The ones that are common in all three events of BCSCs are indicated by a blue asterisk. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Concluding remarks

Herein, the mechanisms and the relevant genes that potentially contribute to the process of stemness, chemo- and radioresistance of BCSCs were investigated using bioinformatics approaches. To overcome breast tumor relapse and recurrence following conventional chemo- or radiotherapy, it would be challenging to identify BCSC-specific gene networks implicated in chemo/radiotherapy resistance and selectively target them. In our study, chemo- and radioresistance associated genes/gene products were uncovered by a thorough literature search and, subsequently, interactome networks were constructed. The role of the factors found to participate in the three BCSC processes and to be highly interconnected should be further investigated. Genes such as *HIF1A* and *ABCG2*, which play a pivotal role in tumor-promotion and chemoresistance [123,124], respectively, should be taken into consideration in the development of effective therapeutic strategies.

Of importance, the tumor microenvironment influences the activity of signaling pathways, as well as the expression of particular factors, such as the angiogenesis regulator HIF1A, which contributes significantly to BCSCs' resistance to chemotherapeutic agents and radiation [103,125]. Therefore, therapeutic efforts should be also directed toward the breast tumor niche, in order to improve the effectiveness of the chemo- and radiotherapy. Promising results have

Table 1
The enriched KEGG Pathways and the genes that participate in them. The genes common in all three processes of BCSCs are underlined.

Pathway name	Genes	adjP
p53 signaling pathway	TP73 DDB2 PMAIP1 GADD45B BAX TP53 ATM RRM2 BBC3 ATR CHEK1 APAF1 CDKN1A CHEK2	7.62e-16
Pathways in cancer	BIRC3 AKT1 RALBP1 TP53 XIAP CDKN1A BCL2L1 EGFR MAPK3 PTGS2 RAD51 HIF1A BIRC5 NFKB1 ITGB1 BAX BCL2 MAPK1 STAT3 GLI1	1.12e-12
Apoptosis	BIRC3 AKT1 NFKB1 BAX TP53 XIAP ATM BCL2 APAF1 BCL2L1 CFLAR	2.53e-10
Pancreatic cancer	AKT1 RAD51 RALBP1 NFKB1 TP53 BCL2L1 MAPK1 STAT3 EGFR MAPK3	4.91e-10
Non-homologous end-joining	MRE11A DCLRE1C XRCC4 PRKDC XRCC6 XRCC5	1.65e-09
Small cell lung cancer	PTGS2 BIRC3 AKT1 NFKB1 ITGB1 TP53 XIAP BCL2 APAF1 BCL2L1	2.37e-09
ABC transporters	ABCC4 ABCB5 ABCA5 ABCB1 ABCG2 ABCC2 ABCB8 ABCC1	3.92e-09
Toxoplasmosis	BIRC3 AKT1 NFKB1 ITGB1 XIAP BCL2 BCL2L1 MAPK1 STAT3 MAPK3	1.38e-07
Prostate cancer	AKT1 NFKB1 TP53 BCL2 CDKN1A MAPK1 EGFR MAPK3	9.50e-07
Colorectal cancer	BCL2 AKT1 BIRC5 MAPK1 MAPK3 BAX TP53	1.12e-06
Hepatitis C	AKT1 NFKB1 EIF2AK3 TP53 CDKN1A MAPK1 STAT3 EGFR MAPK3	1.53e-06
Chronic myeloid leukemia	BCL2L1 CDKN1A AKT1 MAPK1 NFKB1 MAPK3 TP53	2.93e-06
Cell cycle	ATM ATR CHEK1 CHEK2 CDKN1A PRKDC GADD45B TP53	8.47e-06
Neurotrophin signaling pathway	TP73 AKT1 NFKB1 BAX TP53 BCL2 MAPK1 MAPK3	9.46e-06
Glioma	CDKN1A AKT1 MAPK1 EGFR MAPK3 TP53	2.01e-05
Melanoma	CDKN1A AKT1 MAPK1 EGFR MAPK3 TP53	3.16e-05
Bladder cancer	CDKN1A MAPK1 EGFR MAPK3 TP53	3.31e-05
Nucleotide excision repair	DDB2 ERCC4 ERCC1 ERCC6 XPA	3.95e-05
Dorso-ventral axis formation	NOTCH3 MAPK1 EGFR MAPK3	6.48e-05
Endometrial cancer	AKT1 MAPK1 EGFR MAPK3 TP53	8.16e-05
Amyotrophic lateral sclerosis (ALS)	BCL2 APAF1 BCL2L1 BAX TP53	8.54e-05
Non-small cell lung cancer	AKT1 MAPK1 EGFR MAPK3 TP53	8.93e-05

been obtained by targeting the hypoxic tumor environment in anti-oncogenic therapeutic regimens [108,125]. Our analysis and findings could further advance our understanding of the determinants responsible for BCSC-mediated treatment resistance in primary tumors, thereby leading to metastasis, relapse and recurrence. In addition, the identified genes offering resistance to radiation or chemotherapy in BCSCs could be used as a solid basis for developing diagnostic tools or biomarkers predicting the outcome of treatment and personalized therapy.

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Conflicts of interest

The authors declare no conflicts of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2016.07.018.

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