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Chemoresistance in breast cancer stem cells.

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Abstract

Breast cancer is the most prevalent cancer in women worldwide, contributing to 14% of all new cancer cases and 6.8% of all cancer deaths in 2014. A new area of cancer research has arisen from the discovery of cancer cells with stem cell-like properties in several tumor types including the colon, head and breast. Cancer stem cells, which are undifferentiated cells, have the ability of self-renewal, self-replication and differentiating into malignant daughter cells. Breast tumors containing breast cancer stem cells have increased resistance to chemo- and radiotherapy, a higher relapse rate and increased susceptibility to metastasis. Potential targets for the treatment of chemoresistance include signaling pathways of breast cancer stem cells such as the β-catenin-, Notch and Hedgehog pathways. Chemoresistance of these breast cancer stem cells potentially elucidates failure to achieve complete remission post-therapy, and, thus, relapses of breast cancer. By unraveling the mechanism behind the chemotherapeutic resistance of breast cancer stem cells, researchers could develop more ef¿cient treatment strategies towards breast cancer.

Keywords: Breast cancer, Cancer stem cells, Chemoresistance, Biomarkers, Signaling pathways.

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Introduction

Breast cancer is the most prevalent cancer in women worldwide, contributing to 14% of all new cancer cases and 6.8% of all cancer deaths in 2014 [1]. Invasive breast cancers can be divided into groups according to their hormone receptor status and human epidermal growth factor receptor 2 (HER2) levels [2, 3]. Breast cancer cells that have hormone receptors for either progesterone or estrogen are grouped as hormone receptor positive (ER) breast cancers [2, 4]. Breast cancer cells that lack hormone receptors are classi¿ed as hormone receptor negative and tend to be more resistant to hormone therapy drugs. HER2 positive and HER2 negative breast cancers depend on the presence of the HER2 protein [2, 3]. The presence or lack of estrogen, progesterone and HER2 determine if the breast cancer is classi¿ed as triple negative or triple positive [2, 4]. Classi¿cation of breast cancer provides essential information about tumor behavior and possible treatment options.

In 1997 Dick et al. proposed that there is a certain subpopulation of cells in tumors that possess stem cell-like properties [5]. These subpopulations where termed cancer stem cells (CSC) or tumor-initiating cells [6]. CSC possesses self-renewal properties and can differentiate into daughter cells. However, they differ from stem cells since they are tumorigenic and thus have the ability to form tumors when transplanted into animals whereas stem cells do not have this ability [7]. CSC also has increased chemoresistance, can promote metastasis and can survive treatment resulting in new tumors causing relapses [5, 6].

The origin of these cancer stem cells are still under investigation, but three possible hypotheses have been formulated (Figure 1) [8, 9]. The ¿rst hypothesis states that CSC is formed because of mutations in stem cells. Stem cells are pluripotent and have self-renewal abilities. If CSC originates from stem cells, no differentiation is required and CSC simply makes use of the self-renewal pathways of stem cells [9,10]. The second hypothesis states that progenitor cells undergo mutations leading to CSC. Progenitor or precursor cells are partially differentiated cells that divide into mature cells. Progenitor cells are more abundant in adult tissue and have a partial capacity for self-renewal [9, 11]. The third hypothesis states that differentiated cells also undergo dedifferentiation to possess stem-like phenotypes and self-renewal properties [8, 12]. In all of the hypotheses the self-renewal genes are turned on [9].

CSC promotes the multi-step process of metastasis. From the CSC pool a subset of CSC known as metastatic cancer stem cells (mCSC) arise [9, 11]. These mCSC are responsible for the tumors ability to migrate from the primary tumor via the circulatory system to a

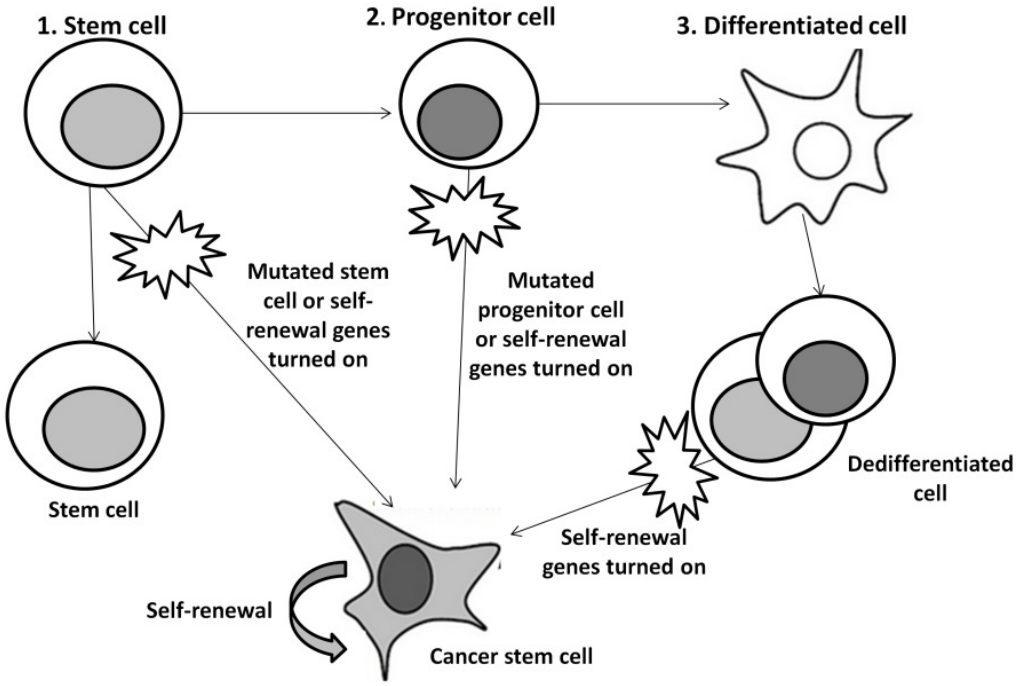

Figure 1: Three hypotheses of how cancer stem cells arise. Cancer stem cells arise due to mutations caused to either stem cells, progenitor cells or differentiated cells due to the activation of the self-renewal genes. Image created with Microsoft PowerPoint® 2010 (Microsoft Corporation, California, United States of America)

secondary location for secondary tumor formation [9, 11]. The pre-metastatic niche of the secondary location secretes anchorage and homing factors such as osteopontin (Opn) and oxygen gradients which assist the circulating mCSC. These secreted factors play an important role in determining the tissue tropism of the future metastatic lesions [11]. Once the mCSC reach the secondary location, the niche (microenvironment) helps to determine its fate. The mCSC can either form metastatic lesions resulting in secondary tumor formation, or the mCSC's can enter a dormant period [11]. Herman et al. (2007) showed that a subpopulation of these mCSC strongly expressed C-X-C chemokine receptor type 4 (CXCR4) in pancreatic cancer in vivo through histological analysis [13]. CXCR4 is a receptor for stromal cell-derived factor 1 (SDF1) which plays a critical role in cell migration and has been associated with tumor metastasis due to its metastatic potential. CXCR4 could thus be a future target for cancer therapy through use of CXCR4 inhibitors [13].

Al-Hajj et al. (2003) ¿rst reported in 2003 that breast cancer potentially originates from a subpopulation of cells known as breast cancer stem cells (bCSC) [14]. These bCSC are aggressive and contribute to breast cancer relapses. Since 2003 there has been an increase in breast cancer stem cell research regarding resistance to chemo- and radiation therapy [15].

Signaling Pathways

There are several signaling pathways identi¿ed to play a role in the self-renewal abilities of breast cancer stem cells. The Hedgehog (Hh)- , Notch- , Wingless-type (Wnt)/β-catenin- and the inÀammatory pathways are examples of these signaling pathways (Figure 2) [8, 16-19].

Notch Signaling Pathway

The Notch signaling pathway plays a critical role in stem cell fate determination, cell cycle progression and normal embryonic development [17, 19]. Notch pathway action has been indicated in bCSC models where mammary tumor formation is promoted due to the change in morphogenetic properties caused by Notch-4 overexpression [8,20]. The Notch pathway also contributes to the maintenance of breast cancer stem cells and other tumor stem cells by interacting with erythropoietin and the Erb2 (HER2) promoter binding sequence [20, 21].

There are four transmembrane Notch receptors (Notch1-Notch4) and 5 ligands consisting of Delta-like proteins (DLL1, DLL3, DLL4) and Jagged-proteins (JAG1, JAG2) [17-19]. The ligands bind to the outer membrane receptor proteins resulting in the intramembrane cleavage of the receptor. The latter is due to a disintegrin and metalloproteinase (ADAM)/γ-secratase proteolytic cleavages [17, 21]. This allows translocation of the intracellular domain (NCID) toward the nucleus [17-19]. In the nucleus, NCID interacts with mastermind-like proteins 1/2 and 3 (MAML1/2/3), as well as CSL (CBF1/Lag1/RBP-Jκ) factors. This leads to the transcriptional activation of Notch genes, including cyclin D or nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Figure 2A) [17]. Cyclin D regulates cell cycle progression and overexpression due to increased Notch pathway activation demonstrating uncontrolled growth [19].

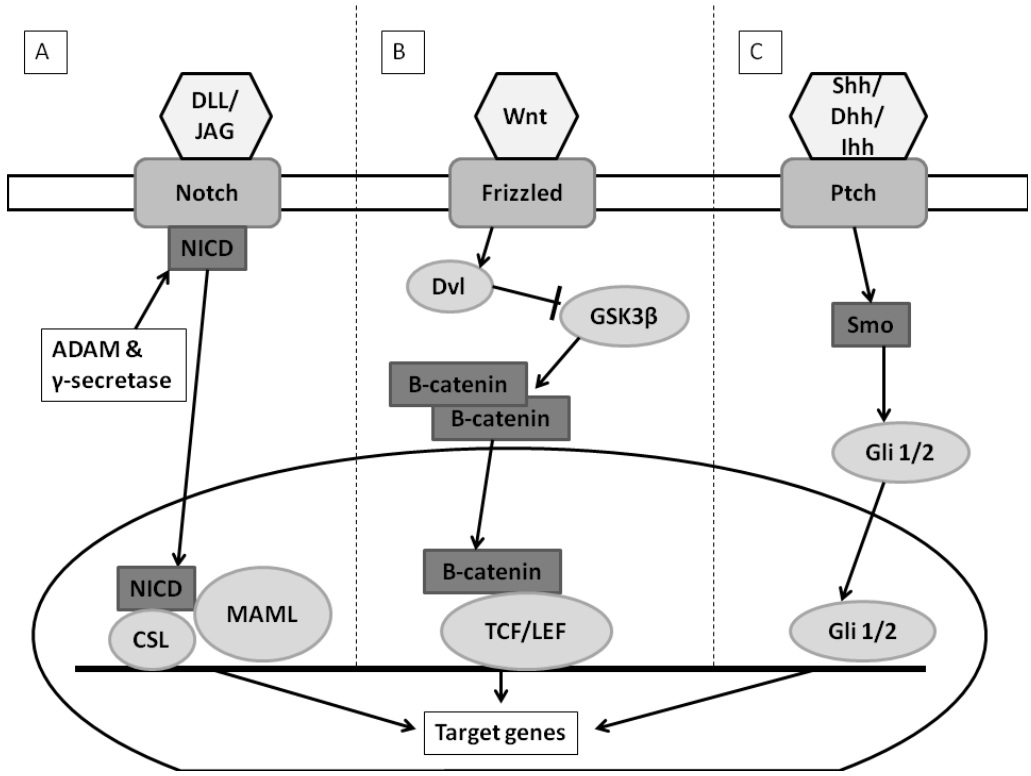

Figure 2: Notch, Wnt and Hedgehog signaling pathways. A) Notch signaling pathway: Notch ligands (DLL/JAG) bind to Notch receptors allowing for NCID to be released into the cytoplasm from membrane. NCID is translocated to nucleus where it interacts with MAML/CSL factors. Transcription of Notch genes is activated. B) Wnt signaling pathway (in presence of Wnt ligand): Wnt ligand binds to and activates the Axin/Frizzled/LRP complex which allows for Dvl to be released. Dvl inhibits phosphorylation of β-catenin. β-catenin accumulates in cytoplasm and is translocated to the nucleus where it binds to TCF/LEF. Transcriptions of Wnt genes are activated. C) Hedgehog signaling pathway (In presence of Hh ligand): Hh ligand relieves inhibited Smo through binding to Ptch which allows Gli 1/2 transcription factor to be translocated to the nucleus and activates Hh gene transcription. Image created with Microsoft PowerPoint® 2010 (Microsoft Corporation, California, United States of America)

Wnt/β-catenin Signaling Pathway

Alexander et al. (2004) demonstrated that up regulation of the Wnt/β-catenin pathway contributes to breast cancer by playing a role in breast cancer stem cell self-renewal in transgenic mice [22]. They observed an increase in the number of mammary stem cells due to activated β-catenin in mammary epithelium or overexpression of Wnt ligands in mammary stroma [22].

In bCSC ampli¿ed amounts of β-catenin are present along with increased Wnt ligand expression [22]. In the Wnt/β-catenin pathway (canonical pathway), Wnt ligand binds to a receptor complex of Frizzled receptors and low-density lipoprotein receptor-related proteins 5 and -6 (LRP5/6) [17, 19, 21]. In the absence of the Wnt ligand a destruction complex forms resulting in β-catenin accumulating in the cytoplasm that cannot be translocated to the nucleus [17,19]. β-Catenin then binds to glycogen synthase kinase (GSK-3β), axin and adenomatous polyposis coli (APC) which form the β-catenin destruction complex [19].

β-Catenin is phosphorylated through GSK-3β which leads to degradation of the complex [21]. Presence of the Wnt ligand activates dishevelled (Dvl) inhibits the destruction complex resulting in β-Catenin translocation to the nucleus (Figure 2B). In the nucleus β-catenin binds and activates the transcription factor T cell factor/lymphoid enhancing factor (TCF/LEF) resulting in the transcription of the Wnt genes such as cyclin D and ¿bronectin [18, 21].

Hedgehog Signaling Pathway

The Hedgehog pathway (Hh) was ¿rst discovered in Drosophila as being essential in segmental embryo patterning [19, 21]. Since then it has been discovered that the Hh pathway also plays a role in cell proliferation, migration and differentiation [17]. In several solid tumor CSC models including breast, increased activation of the Hh pathway has been identi¿ed [8]. The 3 ligands of the Hh pathway are the Sonic (SHH), the Indian (IHH) and Desert (DHH). Other components are the Patched membrane receptor (PTCH1/2) and the Smoothened signal transducer (Smo) which form a complex with each other in the absence of the Hh ligands [17, 19]. PTCH will inhibit Smo which prevents the modulation of the glioma-associated oncogene family zinc ¿nger 1/2/3 (GLI 1/2/3) transcriptions factors [17, 19]. GLI subsequently form a multi-protein complex with Fused (FU), Suppressor of Fused (SUFU) and Drosophila costal 2 (Cos 2). When the Hh ligands are present, Smo will not be inhibited [17, 19]. This allows GLI to translocate to the nucleus (Figure 2C) where it can activate many genes for transcription including cyclin D and cyclin E [19].

Inflammatory Signaling Pathway

Chemokine–and/or cytokine-mediated inflammatory signaling pathways play a role in breast cancer stem cell maintenance [16]. Some inflammatory genes that are involved in this regulation include NF-κB, Interleukin 6 and 8 (IL6/8) and tumor-necrosis factor α (TNF-α) [16]. IL6 plays a role in the self-renewal of breast cancer stem cells through a paracrine/autocrine Notch-3/JAG-1 loop [16]. IL6 forms a positive feedback loop with NF-κB, maintaining mammosphere formation as demonstrated by Iliopoulos et al. (2009) [23]. High IL6 levels are maintained by means of IL6 transcription activation by NF-κB. Reciprocally, high levels of IL6 activate NF-κB [23]. TNF-α and interferon-γ (INF-γ) pathways are up regulated in bCSC and the two pathways activate NF-κB and vice versa [24]. A pro-inflammatory chemokine, IL8, binds to the CXCR1 receptor activating protein kinase B (Akt) resulting in β-catenin translocation to the nucleus forming a complex with T-cell factor (TCF) [16].

Chemoresistance

Conventional chemotherapies used to treat aggressive breast cancers may be effective initially, but, over time many patients will relapse [25]. Although most of the breast cancer cells are killed by cytotoxic agents, bCSC survive. These bCSC then have the ability of regrowth, forming new tumors and causing patient relapses [25]. Chemoresistance of bCSC can be divided into two main groups namely intrinsic resistance due to genetic alterations and extrinsic resistance including microenvironment influences [26]. Intrinsic resistance include overexpression of adenosine triphosphate (ATP) binding cassette transporter proteins (ABC transporter), the adapted deoxynucleotide acid (DNA) repair mechanism, an altered cell cycle, overexpression of aldehyde dehydrogenase 1 (ALDH1) and resistance to apoptosis [26-28]. The extrinsic group includes all microenvironment influences such as hypoxia or epithelial-mesenchymal transitions (EMT) that lead to chemoresistance [27].

Intrinsic Resistance

The small population of cells that survive chemotherapy treatment is potentially due to ABC transporters found on bCSC [26]._ENREF_30 ABC transporter proteins use energy gained from ATP binding and hydrolysis to transport substrates such as anticancer drugs [27]. ABC transporters rapidly cause the efflux of these chemotherapeutic drugs out of the bCSC leading to increased chemoresistance [29]. Hirschmann-Jax et al. (2004) reported high levels of ABCG2 in bCSC accompanied with an improved survival rate due to increased capacity to expel cytotoxic drugs and the ability to confer cellular resistance to antineoplastic drugs [30]. High levels of P-glycoprotein (Pgp) also confer resistance to chemotherapeutic drugs by influencing many cellular processes like the p53 network which mediates chemoresistance [26]. Thus, the bCSC that express high levels of ABC transporters (ABCG2, Pgp) survive chemotherapy [26]. New tumors with a chemoresistant phenotype regrow due to cells that survived and mutations caused by chemotherapy [31]. Through use of an efflux pump mechanism, ABC transporter molecules protect the bCSC against any damage caused by chemotherapeutic drugs [28,31]._ENREF_30

ALDH, as previously mentioned, is a biomarker for breast cancer stem cell identification [28]. Through use of aldefluor assays Ginestier et al. (2007) indicated that breast cancer cells that were highly tumorigenic were $ALDH^+$. These bCSC had the same properties of self-renewal and differentiation when compared to CSC [32]. This detoxifying enzyme oxidizes aldehydes to form carboxylic acids. ALDH1A1 and ALDH3A1 play a critical role in the self-protection and differentiation of stem cells through the conversion of retinol to retinoic acid [33]. ALDH1 has the ability of metabolizing chemotherapeutic agents, especially cyclophosphamide through the conversion of aldophosphamide to carboxyphosphamide and thereby eliminating the toxic effects of the metabolites acrolein and phosphoramide mustard [28, 33, 34]. Metastasis is associated with overexpression of ALDH accompanied by a poor prognosis [35]. ALDH over expression has been indicated as one of the causes of chemoresistance [28, 33].

Altered cell cycle kinetics is another intrinsic resistance mechanism found in bCSC [27, 36]. This allows bCSC to escape death from chemotherapeutic agents that target rapidly dividing cells such as normal breast cancer cells [27, 28, 36]. This dormant state of bCSC can also explain the relapses of breast cancer after long-periods of time [36]. A proficient DNA repair mechanism in CSC is another intrinsic resistance mechanism [37]. bCSC use increased checkpoint (ChK) activation of ChK1 and ChK2 allowing escape from mitotic catastrophe after chemotherapy treatment and to repair their DNA proficiently [27, 37, 38]. This state of dormancy and proficient DNA repair mechanism can contribute to the chemoresistance of bCSC [36].

Extrinsic Resistance

The indirect mechanism of chemoresistance (extrinsic) takes into account the microenvironment and its influence on bCSC [26, 27]. The interaction between the microenvironment and cancer stem cells is a dynamic process leading to continuous remodeling of both [27]. Epithelial-mesenchymal transitions (EMT) play a critical role in bCSC chemoresistance and development of cancer metastasis [27]. Paracrine-acting signals such as self-renewal pathways (Notch/Wnt/Hh) induce EMT by activation of a transcriptional complex resulting in a cytoskeleton rearrangement towards a mesenchymal-like phenotype [39-41]. Cells typically found in the tumor-stroma that undergo these morphological changes, will gain pro-metastatic characteristics increasing stem-cell like markers and clonogenicity [27, 42].

Hypoxia has also been identified as a regulator of CSC since

the tumor growth is faster when compared to blood supply resulting in a hypoxic environment [27, 43]. Self-renewal properties of both stem cells and CSC are promoted by hypoxia. In the presence of low oxygen levels, hypoxia-inducible factor (HIF) is activated resulting in new blood vessel formation (angiogenesis) and promotes a pro-survival phenotype [15]. New blood vessels limit drug perfusion, due to their abnormal architecture resulting in lower concentrations of chemotherapeutics drugs in tumors [27, 43]. In addition, HIF-1 contributes to chemoresistance in bCSC through mechanisms of genomic instability and, abnormal cell cycles [15]. HIF is capable of reprogramming non-stem-like cells to have more stem cell-like traits such as self-renewal capabilities by inducing the expression of key stem cell genes like octamer-binding transcription factor 4 (Oct4) and myelocytomatosis cellular oncogene (c-Myc) [43]. In addition, hypoxia creates niches for CSC by means of increased lysyl oxidase (LOX) production [26, 27, 43]. Thus indirectly the microenvironment and hypoxia contribute to chemoresistance [27].

Applications

For optimal incapacitation of chemoresistance in cancer, the sub-population of CSC should be eliminated [21]. The identi¿cation of CSC is therefore required since both stem cells and CSC have similar signaling pathways and mechanism [37]. A variety of cell surface markers are speci¿c for bCSC and these are currently being used for identi¿cation (Table 1) including clusters of differentiation 44, 24 and 133 [8]. Highly tumorigenic breast cancer types such as those with a BRCA1 defect have been found to express high levels of CD44 and no or low amounts of CD24 (CD44+/CD24-) [8]. _ENREF_15 CD44 is a cell-surface glycoprotein involved in migration and cell adhesions and in addition binding of CD44 to hyaluronic acid (HA) is essential in tumor progression by inhibiting apoptosis [21, 44]. CD24, a glycoprotein, down regulates the CXCR4/SDF-1 pathway which contributes to breast metastasis due to its role in cell migration [45]. Processes that are essential to tumor metastasis such as their chemo-attraction, adhesion and locomotion of malignant cells are regulated by the pleiotropic effects exerted by SDF-1 [46]. ALDH1 is also highly expressed in bCSC, speci¿cally in estrogen receptor negative breast cancers and correlates with a lower overall survival rate [47]. This isoenzyme is not only used to identify CSC but also plays a role in and CSC self-protection, differentiation and expansion [21, 48]. Epithelial-speci¿c antigen (ESA), CD133 (prominin-1) and CXCR4 are other biomarkers used to identify breast cancer stem cells [8, 14]. ESA is used to differentiate between benign reactive epithelial cells and epithelial cancer cells. Populations containing ESA^{+}/$CD44^{high}$/$CD24^{low}$ biomarkers have an enhanced capacity for tumor and mammosphere formation [8, 14]. $CD133^{+}$ cells containing CSC characteristics have been identi¿ed in triple negative breast cancers and also show increase survival in vitro [8, 21].

By combining conventional and CSC targeted therapies, it will improve ef¿cacy of cancer therapy (Figure 3) [5]. These future combination cancer therapies may help improve cancer prognosis, speci¿cally for metastatic cancers [5, 49]. They may potentially reduce the chemoresistance of cancer and thereby also improve overall survival with a decrease in relapses [5, 37, 49].

Controversies

The existence of CSC has been debated since 1970 when scientists discovered two sub-populations in leukemia cells [50]. One school of thought endorses the existence of CSC and that they are a crucial target for cancer therapy. Opponents to this theory question the separate entirety of CSC and argue their importance in cancer pathology and treatment [51]. One drawback is that experiments for CSC have only been conducted on immunode¿cient mice raising concerns since results do not represent the reality of cancer initiation and progression in humans [49]. Other reports demonstrated that CSC does not necessarily lead to tumor formation, but rather that the clonal evolution model (stochastic) is a better explanation for this sub-population of cells origin [50, 52]. This model suggests that the heterogeneity found in cancer cells is due to variation in the levels of transcription factors that vary amongst the cells [50]. The CSC hypothesis still remains an attractive model despite all these controversies. Thus, continuing research is still required for distinguishing between stem cells and CSC for differential drug activity [8, 37]. Should this population of cells be fully de¿ned and characterized, they could provide a potential target for eradicating cancer.

Conclusion

Breast cancer recurrence occurs 40% of the time in part due to resistance to chemo- and radiation therapies [53].

Table 1: Overview of cancer stem cell markers and their functions

Cell surface marker	Cancer types	Functions
$CD44^{+}$	Breast, Ovarian, Prostate, Colon, Pancreatic	Glycoprotein involved in migration, cell adhesions and chemoresistance
$CD24^{-}$	Breast	Down regulates the CXCR4/SDF-1 pathway
CD133	Ovarian, Glioblastoma, Lung, Prostate, Colon, Renal, Melanoma	Glycoprotein involved in cell growth, metastasis and chemoresistance
CXCR4	Pancreatic	Metastasis
ALDH1	Breast, Head and Neck	CSC self-protection, differentiation, expansion and chemoresistance

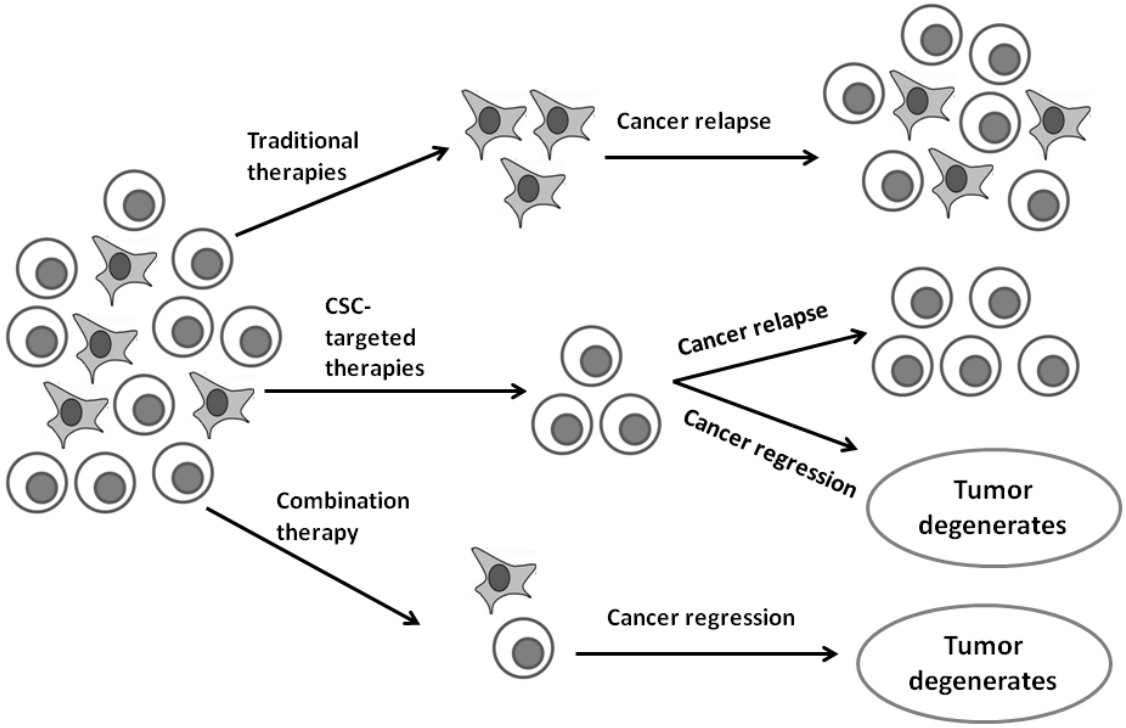

Figure 3: Therapeutic implications of combination therapies. Traditional therapies fail to target CSC and mostly only kill differentiated cancer cells. CSC can thus survive and could lead to relapses. CSC-targeted therapies either eliminate CSC or result in differentiation where after these differentiated cancer cells may also undergo cell death via apoptosis. However, combination therapy could be most effective for tumor elimination. Image created with Microsoft PowerPoint® 2010 (Microsoft Corporation, California, United States of America)

By better understanding the CSC phenotype causing chemoresistance, more improved cancer treatments can be developed to prevent relapses [9, 14]. One method to overcome chemoresistance in bCSC is for future cancer therapies to focus on drugs that inhibit signaling pathways (Wnt/Hh/Notch) responsible for the self-renewal of CSC [37, 49]. The role that the microenvironment and hypoxia play should also be taken into account due to their contribution to chemoresistance [37].

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