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REVIEW

Cancer stem cells: therapeutic implications and perspectives in cancer therapy

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Abstract The cancer stem cell (CSC) theory is gaining increasing attention from researchers and has become an important focus of cancer research. According to the theory, a minority population of cancer cells is capable of self-renewal and generation of differentiated progeny, termed cancer stem cells (CSCs). Understanding the properties and characteristics of CSCs is key to future study

Abbreviations: 5-FU, 5-fluorouracil; ABC, ATP-binding cassette; ABCB1, ATP binding cassette superfamily subfamily B member 1; ABCG2, ATP binding cassette superfamily subfamily G member 2; ALDH1, aldehyde dehydrogenase1; AML, acute myeloid leukemia; AMO, anti-miRNA oligonucleotide; APC, adenomatous polyposis coli; As_2O_3, arsenic trioxide; ASO, antisense oligonucleotide; ATRA, all-trans retinoic acid; bFGF, basic fibroblast growth factor; BMP4, bone morphogenetic protein 4; CBP, CREB(cAMP response element binding protein) binding protein; Chk, checkpoint kinase; CICs, cancer initiating cells; CRC-SCs, colorectal cancer stem cells; CSC(s), cancer stem cell(s); DDR, DNA damage response; DEAB, diethylaminobenzaldehyde; ECSCs, esophageal cancer stem cells; EGCG, (-)-epigallocatechin-3-gallate; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ESA, epithelial-specific antigen; FACS, fluorescence-activated cell sorting; FGF2, fibroblast growth factor 2; Gli, glioma-associated oncogene homolog; G-CSF, Granulocyte colony-stimulating factor; GSIs, γ-secretase inhibitors; HA, hyaluronic acid; HCS, high-content screening; HDAC, Histone deacetylase; Hh, Hedgehog; HIF-1α, hypoxia-inducible factor1α; HMGA2, high mobility group AT-hook 2; HSCs, haematopoietic stem cells; IFNα, interferon-α; Lgr5, leucine-rich repeat–containing heterotrimeric guanine nucleotide–binding protein–coupled receptor 5; LSCs, leukemia stem cells; MDR, multidrug resistance; MDR1, multidrug resistance transporter1; MMAF, monomethyl auristatin F; MSCs, mesenchymal stem cells; NICD, Notch intracellular domain; NOD/SCID, nonobese diabetic/severe combined immunodeficiency; NSAID, nonsteroidal anti-inflammatory drugs; OCT-4, octamer-binding transcription factor 4; PARP, poly(ADP-ribose) polymerase; PCR, polymerase chain reaction; PROM1, prominin-1; Ptch, patched; RA, retinoic acid; SAHA, suberoylanilide hydroxamic acid; SCLC, small cell lung cancer; SFRPs, secreted frizzled-related proteins; SHh, sonic hedgehog; siRNA(s), small interfering RNA(s); SLGC, stem-like glioma cells; SLNs, solid lipid nanoparticles; SMO, smoothened; SOX-2, sex-determing region Y-related HMG-box protein 2; SP, side-population; TCF, lymphoid enhancer factor; LEF, lymphoid enhancer-binding factor; TRAIL, tumor necrosis factor-related apoptosis induced ligand; TSCs, tumor stem cells; WIF1, Wnt inhibitory factor 1

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CSC marker;
CSC-targeted mechanism;
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Quiescent state;
miRNA expression;
Apoptosis;
Differentiation

on cancer research, such as the isolation and identification of CSCs, the cancer diagnosis, and the cancer therapy. Standard oncology treatments, such as chemotherapy, radiotherapy and surgical resection, can only shrink the bulk tumor and the tumor tends to relapse. Thus, therapeutic strategies that focus on targeting CSCs and their microenvironmental niche address the ineffectiveness of traditional cancer therapies to eradicate the CSCs that otherwise result in therapy resistance. The combined use of traditional therapies with targeted CSC-specific agents may target the whole cancer and offer a promising strategy for lasting treatment and even cure.

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1. Introduction

Solid tumors similar to aberrantly developed organs and tissues are composed of many types of cells including neoplastic cells, supporting vascular cells, inflammatory cells, and fibroblasts[1]. The majority of cells in bulk tumors have limited self-renewal ability and are non-tumorigenic. Only a small subpopulation of cancer cells are long-lived with the ability of extensive self-renew and tumor formation. This small population is called cancer stem cells (CSCs), or cancer initiating cells (CICs), or tumor stem cells (TSCs)[2,3].

The concept that cancer might evolve from a small population of cells with stem cells properties was proposed about 150 years ago[4,5]. Huntly and Gilliland[6] outlined the evolution of cancer stem cell (CSC) research from 1855. The leukemis stem cells (LSCs) were the first to be described as CSCs in human acute myeloid leukemia (AML)[7]. Bonnet and Dick[8] demonstrated that a subpopulation of $CD34^+$ $CD38^-$AML cells possessed LSCs with the capacities of differentiation, proliferation and self-renewal, and were able to reconstitute a heterogeneous cell population in NOD/SCID (nonobese diabetic/severe combined immunodeficiency) mice. With the advances in stem cells biology and rapid development of detecting technologies and rational animal models to measure CSC properties, the CSC hypothesis is gaining validation[5]. Schepers et al.[9] demonstrated that Lgr5 (leucine-rich repeat–containing heterotrimeric guanine nucleotide–binding protein–coupled receptor 5) marked a subpopulation of adenoma cells that fueled the growth of established intestinal adenomas, revealing $Lgr5^+$ stem cell activity in mouse intestinal adenomas. Another study revealed that a quiescent subset of endogenous glioma cells which related to CSCs propagates glioblastoma growth[10]. Driessens et al.[11] presented experimental evidence for the existence of CSCs during unperturbed solid tumor growth by clonal analysis. CSCs have also been defined in many other tumors including cancers of breast[12,13], pancreas[14], prostate[15], head and neck[16], colon[17,18], liver[19], bladder[20] and lung[21,22].

Most commonly used cancer therapies depend on chemotherapy, radiation, or surgical resection solely or in combination. Schiller et al.[23] found that neither the chemotherapy regimens of cisplatin and gemcitabine, cisplatin and docetaxel, or carboplatin and paclitaxel, nor the regimen of cisplatin and paclitaxel offered a significant advantage over other treatments (*e.g.*, mitomycin, ifosfamide and cisplatin) of advanced non-small-cell lung cancer. On the other hand, Bonner et al.[24] found that when treated with high-dose radiotherapy plus cetuximab, patents with local/regionally advanced head and neck cancer survived longer than patients treated with radiotherapy alone. Many other clinical trials have shown improvements in cancer survival of combined therapy compared to chemotherapy or radiotherapy[25–28]. However, the combined therapy is not effective against all types of cancer, and the severe toxicity cannot be ignored[29]. In addition, in later rounds of therapy, the cancer tends to relapse and metastasize, and often develops resistance to previous therapies[29].

The standard oncology treatments have incomplete and temporary effects that only shrink the tumor, and the tumor tends to relapse mainly due to the multiple resistant mechanisms existing in CSCs. Newer CSC-based therapies focus on eliminating the tumor initiating cells. In this review, we introduce the basic information about CSCs including the definition, origination, and the main characteristics; compare different techniques used to isolate and identify the minority of CSCs among the bulk tumors; analyze the reasons for the failure of traditional therapies (chemotherapy, radiotherapy) and the resistant mechanisms inherent in CSCs and the micronvironment; and discuss the multiple therapeutic implications of targeting CSCs.

2. CSCs: definition, origination and characteristics

The widely accepted definition of a CSC is a cell within the tumor that possesses the capacity to self-renew and to generate the heterogeneous lineages of cancer cells that comprise the tumor[7,30]. We also use the term “tumorigenic cell” or “tumor initiating cell” to describe the pluripotent CSCs. Although only a small subpopulation ($<1\%$) of the overall cancer cells have the ability to proliferate extensively and form new tumors[2,31], they are the crucial component leading to tumor recurrence, therapy resistance, and metastasis[5,32]. CSCs may undergo a symmetrical self-renewing cell division into two identical daughter CSCs or an asymmetrical self-renewing cell division into one daughter CSC and one differentiated progenitor cell, resulting in number expansion of CSCs as the tumor grows and to expand the tumor[7].

Recent evidence suggests that CSCs may arise from normal stem cells, progenitor cells, or more differentiated cells[7,33] through multiple mutations of genes as a result of their genomic instability[34] or oncogene-induced plasticity[35]. The genetic and epigenetic instability of these cells may result in the accumulation of mutations that enable them to acquire the ability of self-renewal and tumorigenicity. The epithelial-mesenchymal transition (EMT)

is one mechanism to generate CSCs endowed with an invasive and metastatic phenotype[36]. EMT is a series of steps, resulting in the transformation of epithelial cells into fibroblast-like and motile[36,37] cells, and eventually the cancer cells acquire the ability to invade, migrate, and disseminate[38,39]. CSCs and normal stem cells share many similarities in terms of self-renewal, production of differentiated progeny, expression of specific surface markers and oncogenes, utilization of common signaling pathways, and the importance of the stem cell niche[2,32]. CSCs are not synonymous with normal stem cells. CSCs differ significantly from normal stem cells in their tumorigenic activity. CSCs can form tumors when transplanted into animals, but normal stem cells cannot[7].

Thus, we can define CSCs through these four key characteristics: (a) self-renewal—the CSCs subpopulation can be serially transplanted through multiple generations, indicating the self-renewing capacity; (b) differentiation—pluripotent CSCs can not only form tumorigenic daughter CSCs by symmetrical cell division but also generate bulk populations of non-tumorigenic cells by asymmetrical cell division; (c) tumorigenicity—a small subpopulation of CSCs have tumorigenic potential when transplanted into animals; and (d) specific surface markers, by which the CSCs subpopulation can be separated from the non-stem cells[5,16,40]. Therefore, according to the definition and characteristics of CSCs, we can conclude that the two hallmark features of CSCs are self-renewal and lineage capacity.

3. CSCs: isolation and identification

The isolation of the minority of CSCs from mass tumor tissues or cell lines and the identification of the stem-like CSCs by diverse methods will be quite important to researches of tumor initiation, tumor development, and tumor diagnostics and therapeutics. Since CSCs and normal stem cells have much in common, we can also use the stem cell properties, such as the expression of specific surface markers, to isolate and identify CSCs. Up to now, we usually take advantage of these features, namely, the sphere forming ability in non-adherent medium, dye exclusion ability which is because of the over-expression of efflux transporters, expression of specific cell surface markers and signaling pathways, intracellular enzyme activity, and clonogenicity, to isolate the CSCs[3,36,41,42]. Thus, there are several *in vitro* assays to identify CSCs, such as sphere forming assays[41,43], Hoechst dye exclusion (SP cells)[16,33], detection enzymatic activity of aldehyde dehydrogenase1 (ALDH1)[44,45], detection of surface markers[3,7,16], signaling pathway identification[46], serial colony-forming unit assays (replating assays)[33], lable-retention assays[7], and migration assays[39,47]. However, *in vitro* assays alone are not enough to demonstrate that the cells we detect are CSCs, for normal stem cells or progenitors may have the same characteristics as CSCs and these assays cannot show tumor propagation. Thus *in vivo* assays are regarded as the gold standard, including serial transplantation in animal models, which can complement and enhance the ability of *in vitro* assays to identify CSCs[7]. However, improved and optimized methods need to be developed to identify CSCs.

3.1. Stem cell markers

The main markers used for isolation, identification and purification of CSCs include surface cell-adhesion molecules (*e.g.*, CD133, CD24, hyaluronic acid (HA) receptor CD44), cytoprotective enzymes (such as aldehyde dehydrogenase, ALDH), transcription factors (*e.g.*, OCT-4, SOX-2), and drug-efflux pumps (*e.g.*, ATP-binding cassette (ABC) drug transporters and multidrug resistance transporter1, MDR1). Flow cytometry analysis, fluorescence-activated cell sorting (FACS) analysis, polymerase chain reaction (PCR) analysis and immunofluorescent staining analysis are based on these specific markers of CSCs and are widely used to isolate, purify, and characterize the CSCs[16,21,43,48,49].

The most widely used method for identifying CSCs is based on specific cell surface markers[3,7,37], such as CD133, CD24 and CD44. However, the surface markers of CSCs or normal stem cells in one organ or tissue are frequently not completely shared with the markers of CSCs or normal stem cells in other organs or tissues. For example, Al-Hajj et al.[12] demonstrated that in human breast cancer, $CD44^{+}CD24^{-}$ phenotype cells were tumorigenic, but the alternate phenotypes were unable to form tumors. While Li et al.[50] showed that in human pancreatic cancer cells, $CD44^{+}CD24^{+}ESA^{+}$ (ESA: epithelial-specific antigen) phenotype was much more tumorigenic than nontumorigenic cancer cells. Yang et al.[19] also suggested that $CD45^{-}CD90^{+}CD44^{+}$ could be used as a marker for human liver cancer and as a target for the diagnosis and therapy of this malignancy. Furthermore, the surface markers that are used to isolate or identify CSCs from many malignant tissues are not expressed by CSCs exclusively, because many normal stem cells and even some normal tissues can express the same markers. Fox et al.[51] demonstrated that normal human tissues could express different CD44 isoforms, such as the normal epithelial tissues as well as some tumors could express a wide range of variants at high levels. Moreover, whether a surface marker can be used to isolate or identify stem cells from a specific tissue is environment dependent, meaning that the marker expression may change with the context of the stem cells[7].

Thus, the use of surface marker expression solely is insufficient to identify CSCs. Detection of surface markers must be associated with other functional assays, such as the sphere-forming assay in serum-free medium or soft agar medium, detection of enzymatic activity of ALDH1, and measurement of the expression of specific CSC genes to provide persuasive evidence for the existence of CSCs.

3.2. Sphere forming assays

One important property of CSCs, as well as normal stem cells, is to form spheres or grow into colonies in serum-free medium or in soft agar medium. To show aggregation and proliferation of stem cells *in vitro*, cells are harvested from tumor specimens and suspended at a low density in serum-free medium supplemented with epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), and the colony-forming capacity can be determined in soft agar media. Qiu et al.[21] demonstrated that small cell lung cancer (SCLC) cell lines H446 cells were proliferative and capable of self-renewal in a defined serum-free medium, and then they found that the sphere-derived cells had increased *in vitro* clonogenic and *in vivo* tumorigenic potentials as well as drug-resistance properties, suggesting the CSC populations in H446 cells. Zhang et al.[42] and Ghani et al.[41] also took advantage of

the property of stem cells to form spheres or colonies in non-adherent culture to determine whether the cells obtained from tumors were stem-like cells with self-renewal capacity. Dou et al.[43] used both serum-free culture assay and colony-forming assay to measure the proliferative activity and clone-forming capability of tumor cells.

However, despite these merits, these *in vitro* assays have many limitations. For example, it is difficult to confirm the clonality (signal cell origin) of *in vitro* assays. Moreover, the cells are under selection pressure exerted by the culture conditions, leading to selection of only the cell populations that are able to survive and proliferate under such specific conditions[33]. In addition, *in vitro* assays measure *ex vivo* proliferation instead of true self-renewal and they cannot show the tumor formation ability of CSCs. To overcome these drawbacks, the results of *in vitro* assays must be confirmed by *in vivo* assay.

3.3. Transplantation assays

The hallmarks of any stem cell are the ability to self-renew and the capacity to differentiate; thus, assays that measure CSCs activity should emphasize self-renewal as well as tumor propagation, and the gold standard assay which fulfills these two criteria for identifying CSCs is serial transplantation in animal models[7]. To identify CSCs in human tumors using serial transplantation assays, tumor cells are transplanted into the immunocompromised (typically NOD/SCID) mice, monitoring the mice at various time points for tumor growth; then, xenograft tumors or primary human tumors must be isolated from the mice and implanted into other immunocompromised mice to show self-renewal and tumor formation capacities[7,45,50,52].

However, it is reported that a relatively large number of cancer cells are required to initiate tumor growth when xenotransplanted into animal models[5,30,36]. This may be because only a minor population of cancer cells are capable of self-renewal and tumor-formation; these cancer cells may be inefficient to initiate tumor growth, and, in addition, it is likely that the cancer cells are in a foreign microenvironment that lacks the specific signals for the survival and development when xenotransplanted into immunodeficient mice. It is thus necessary to sort the cancer cells based on the specific markers of CSCs, and select stem-like cells for higher tumor obtaining rates.

4. CSC-targeted mechanisms and implications

The traditional and mainstream therapies to treat cancer are chemotherapy and radiotherapy, although they face many obstacles such as systemic or local toxicity and drug resistance. The most popular anti-cancer agents consist of paclitaxel, doxorubicin and cisplatin, and so on. Though these agents are capable of high cytotoxicity that kills the bulk of tumor and are commonly used in clinic, they are non-targeting and often result in tumor recurrence because of drug resistance[1,53]. For many cancers, ionizing radiation represents the best non-invasive therapy with benefits in overall survival, but it may also cause therapy failure owing to the existence of CSCs[30,54,55]. Although CSCs account for only a small part of the bulk tumors, they are the cardinal reason leading to therapeutic resistance. The mechanisms of CSCs that contribute to therapeutic resistance include relative dormancy/slow cell cycle kinetics, high capacity for DNA repair, high expression of multiple drug resistance membrane transporters (*e.g.*, ABC transporters), high expression of anti-apoptosis proteins, and the microenvironment (hypoxia, acidosis, etc.)[1,29,37,56,57]. Both tumor recurrence and serious side effects contribute to the failure of traditional therapies. Thus, traditional chemo-radiotherapy should be combined with new strategies targeting CSCs to prevent tumor relapse and to provide a high-efficient and low-toxic treatment for cancer therapy.

4.1. Targeting the molecular signaling pathways

Signaling pathways are essential for normal stem cells related to self-renewal, proliferation and differentiation; however, the dysregulation or aberrant activation of these key pathways may result in the formation of CSCs which induce tumorigenesis. These important signaling pathways consist of Hedgehog (Hh), Notch, Wnt/β-catenin, high mobility group AT-hook 2 (HMGA2), Bcl-2, Bmi-1, etc. The most studied and characterized pathways are Hh, Notch, Wnt/β-catenin, which are responsible for the formation of CSCs[46,58–60]. Therefore, targeting these aberrant signaling pathways that are important for the formation of CSCs offers a new strategy for cancer therapy.

4.1.1. The Hedgehog signaling pathway

The Hedgehog (Hh) pathway is essential for the maintenance of stem cells and plays a crucial role in development and patterning during mammalian embryogenesis[61]. When Hh protein binds to the transmembrane protein called patched (Ptch), the Hh pathway is activated[59], resulting in the regulation of target genes which are involved in many cellular functions including proliferation, survival, metastasis, and pathway auto-regulation[58,61,62]. The aberrant activation of the Hh pathway may lead to deformations in development as well as contribute to tumorigenesis in various human cancers[46,63].

Recent researches have suggested that the Hh pathway is essential for the maintenance of CSCs in various human cancers including pancreatic cancer, gastric cancer, colorectal cancer, and so on[60,64–66], and it is also responsible for treatment resistance of cancer cells[67]. Thus, inhibitors that obstruct any step of the Hh signaling pathway may result in depletion of CSCs and overcome the treatment resistance. Xia et al.[68] showed the signaling pathways in pancreatic CSCs include the Hh pathway, and introduced inhibitors targeting Hh pathway for cancer therapy. Most drugs for Hh pathway therapeutics inhibit the signaling molecule smoothened (SMO), like cyclopamine and GDC-0449 (Vismodegib)[69], but they would be ineffective against tumors that harbor molecular lesions that lie downstream of SMO. So other agents such as arsenic trioxide (As_2O_3) which inhibits glioma-associated oncogene homolog (Gli) proteins are used in combination with SMO inhibitors[63]. Tang et al.[66] found that (−)-epigallocatechin-3-gallate (EGCG) inhibited the components of sonic hedgehog (SHh) pathway (SMO, Ptch, Gli1 and Gli2) and Gli transcriptional activity, and the combination of quercetin with EGCG had synergistic inhibitory effects on self-renewal capacity of CSCs through attenuation of TCF/

LEF and Gli activities. They suggested that therapeutics targeting SHh pathway might improve the therapeutic outcomes of patients with pancreatic cancer by targeting CSCs.

4.1.2. The Notch signaling pathway

The Notch signaling pathway plays crucial roles in cell-cell communication and in multiple cell fate decisions during embryonic development and adult life[70,71]. The Notch pathway is activated through ligand-receptor interactions of four receptors (Notch-1–Notch-4) and five Notch ligands (Delta-like1, 3, 4 and Jagged1, 2)[71], resulting in the expression of multiple target genes[2,59]. The Notch pathway is involved in stem cell proliferation, differentiation, and apoptosis, but its role in tumorigenesis is context-dependent and can be either oncogenic or oncosuppressive[71]. Specifically, Notch functions as an oncogenic protein in most human cancers including cervical, lung, colon, head and neck, prostate, pancreatic cancer, etc., while it may act as tumor suppressor in skin cancer, hepatocellular carcinoma, and SCLC[68,72–74].

The Notch pathway is often over-activated in a variety of cancers, and it is believed to be a target to eliminate CSCs[75–77]. Blocking the proteolytic process which is crucial for the formation of Notch intracellular domain (NICD) is one of the most efficient methods to inhibit Notch signaling pathway[59]. Fan et al.[78] used γ-secretase inhibitors (GSIs) to block Notch pathway in glioblastoma, resulting in the reduced neurosphere growth and clonogenicity *in vitro*; the reduced expression of putative CSCs markers, in addition, the reduced tumor growth *in vivo*. Thus, they suggested that GSIs which block Notch pathway might be useful chemotherapeutic reagents to target CSCs in malignant gliomas. Kondratyev et al.[79] also reported that GSI MRK-003 eliminated cancer stem-like cells and inhibited the self-renewal and proliferation of breast CSCs. However, GSIs are nonselective drugs because they are able to block the cleavage of all four Notch ligands and various γ-secretase substrates; thus, 'natural agents' that are non-toxic to humans are needed to overcome the limitations of GSIs[75].

4.1.3. The Wnt signaling pathway

The Wnt signaling pathway is another developmental pathway involved in multiple biological processes including embryogenesis, development, cell proliferation, survival and differentiation[68,80]. The canonical Wnt/β-catenin signaling pathway is by far the best characterized among Wnt pathways[58]. Wnt/β-catenin signaling is initiated when a Wnt ligand (secreted glycoprotein) binds to the cell membrane co-receptors, resulting in the activation of target genes[80]. The canonical Wnt signaling pathway plays an important role in self-renewal and maintenance of stem cells and CSCs of tissues such as skin, intestine and mammary gland[81,82]. However, oncogenic mutations of β-catenin, or inactivating mutations of APC (adenomatous polyposis coli) tumor suppressor may result in the dysregulation of Wnt/β-catenin pathway in cancer cells or CSCs, which induces neoplastic proliferation[83].

The Wnt signaling pathway can be inhibited by Wnt inhibitory factors, Wnt antagonists and conditional knockout of β-catenin. Extracellular molecules antagonize the Wnt signaling pathway by preventing ligand-receptor interactions[84]. Inhibitors of the Wnt/β-catenin signaling pathway can be divided into two classes: small-molecule inhibitors and biologic inhibitors[85]. Small-molecule inhibitors include existing drugs such as nonsteroidal anti-inflammatory drugs (NSAID) or natural compounds, and molecular-targeted agents such as the cAMP response-element binding protein binding protein (CBP)/β-catenin antagonist ICG-001. These inhibitors can interfere with Wnt pathway by inhibiting the Wnt target enzyme cyclooxygenase 2 (*e.g.* aspirin, indomethacin), by activating E-cadherin (*e.g.* vitamins A and D derivatives), or by promoting degradation of TCF (*e.g.* celecoxib). Biologic inhibitors include monoclonal antibodies, small interfering RNAs (siRNAs), and recombinant proteins against Wnt1/2, WIF1(Wnt inhibitory factor 1) and SFRPs (secreted frizzled-related proteins)[80,81,85]. Takahashi-Yanaga et al.[85] showed that CBP/β-catenin antagonist ICG-001 was able to target and eliminate drug-resistant leukemic stem cells both *in vivo* and *in vitro*. Teng et al.[86] knocked down the expression of β-catenin using RNA interference technology to inhibit Wnt signaling, resulting in down-regulation of many CSC properties, such as the Wnt target gene cyclin D1, proliferation, clone formation, migration, drug resistance, as well as the expression of OCT-4. They demonstrated that canonical Wnt signaling plays an important role in lung CSC properties. Therefore, targeting Wnt signaling pathway in CSCs is another approach for cancer therapy.

Although dysregulation of these signaling pathways is found in CSCs, they express normally in normal stem cells. Thus, agents targeting these signaling pathways not only can target the CSCs but also influence normal stem cells, inducing unwanted effects. In this regard, targeting agents should be modified or should be combined with other CSC-targeting therapies to improve their specificity.

4.2. Targeting CSCs markers

The markers used to isolate, identify and enrich CSCs are also ideal targets for cancer therapy. Targeting cytotoxic drugs to CSCs with the help of stem cell surface markers provides a useful method to treat cancer. Also, the use of inhibitors targeting drug-detoxify enzymes, drug-efflux pumps, or transcription factors of CSCs represents a novel approach to target the CSCs and reduce cancer recurrence and metastasis.

4.2.1. Targeting surface markers

CSCs in various tumors highly express specific surface markers, such as CD133 in hepatocellular and gastric cancer[87], $CD44^+CD24^+ESA^+$ in pancreatic CSCs[50], $CD9^+CD24^+CD26^+$ in human malignant mesothelioma CSCs[41]. Among these markers, CD133 is considered the most important CSC-associated marker identified so far[88].

CD133 (prominin-1, PROM1) which was discovered as a marker of primitive haematopoietic and neural stem cells, is a pentaspan transmembrane glycoprotein overexpressed in both humans and mice tumors[89]. Some evidence has suggested that $CD133^+$ CSCs display strong resistance to chemotherapy and radiotherapy[54,90]. Todaro et al.[18] identified and characterized stem-like cells from colon carcinomas using CD133, and revealed that $CD133^+$ cells grew in undifferentiated tumor spheroids *in vitro* and initiated tumor growth in immunodeficient mice. In addition, the $CD133^+$ stem-like cells survived standard chemotherapeutic treatment with oxaliplatin and 5-fluorouracil (5-FU). Rappa et al.[91] investigated that down-

regulation of CD133 using short hairpin RNAs in human metastatic melanoma leading to slower cell growth, reduced cell motility, and decreased ability to form spheroids, and reduced capacity of metastasis, particularly to the spinal cord. Then they used monoclonal antibodies directed against two different epitopes of the CD133 protein to treat FEMX-I cells (human malignant melanoma cells), and found a specific, dose-dependent cytotoxic effect in FEMX-I cells. It was concluded that CD133 was not only a CSC marker but might also be an important therapeutic target for many CD133-expressing cancer types including metastatic melanoma. In another study, Smith et al.[87] conjugated a murine anti-human CD133 antibody (AC133) to a potent cytotoxic drug (monomethyl auristatin F, MMAF), and found that the conjugates effectively inhibited the growth of Hep3B hepatocellular and KATO III gastric cancer cells *in vitro*. It suggests that CD133 is a potential therapeutic target for antibody-drug conjugates, and anti-CD133 antibody-drug conjugates may be a therapeutic method to eliminate $CD133^+$ tumors.

4.2.2. *Targeting drug-detoxify enzymes*

ALDH are a group of $NAD(P)^+$-dependent enzymes that catalyze the oxidization of aldehydes into carboxylic acids[2]. Certain isoenzymes (*e.g.* ALDH1) of ALDH superfamily not only act as markers for both normal and CSCs but may also play important functional roles in self-protection, differentiation and expansion. Thus ALDH can act as drug-detoxifying enzymes and be responsible for therapeutic resistance[45,92,93]. In the Aldefluor assay, ALDH-activated fluorescent substrate is used as a marker for measuring and isolating normal and CSCs with high ALDH activity[33]. Croker et al.[94] identified a subpopulation of stem-like $ALDH^{hi}CD44^+$ cells in human breast cancer cell lines. They demonstrated that $ALDH^{hi}CD44^+$ cells were more resistant to standard cancer therapy such as chemotherapy (doxorubicin/paclitaxel) or radiotherapy, and that inhibiting ALDH activity of cell populations through specific ALDH inhibitor diethylaminobenzaldehyde (DEAB) or all-trans retinoic acid (ATRA) sensitized these cells to treatment. However, only DEAB had a long-term sensitization effect, indicating that selectively blocking ALDH activity played a key role in targeting the resistant cells and revealed a promising target for cancer treatment.

4.2.3. *Targeting drug-efflux pumps*

ABC drug transporters are overexpressed in both normal stem cells and many TSCs as efflux pumps to protect stem cells from xenobiotic toxins[22,95–97]. ABCG2 (also known as BCRP), an important member of ABC transporter family, is regarded as a potential marker of CSCs as well as a mechanism in multidrug resistance (MDR)[2,33]. ABCG2 is an important determinant of the side-population (SP) phenotype. SP cells show many features of CSCs with regard to self-renewal, lineage capacity, tumorigenicity, and the expression of CSC markers and stem cell genes, and can be found in various tumor cell lines; thus, SP cells can be assumed as CSCs[33,98]. Xia et al.[99] developed an image-based high-content screening (HCS) system to specifically identify 12 potent high drug efflux cancer cell (HDECC) inhibitors from 1280 pharmacologically active compounds. Then through *in vitro* assays and *in vivo* assays, they showed that these inhibitors were able to overcome MDR by inhibiting SP and increase the efficacy of chemotherapy, or reduce the tumorigenicity of lung cancer cells possibly by affecting stem-like cancer cells. Fong et al.[100] used Hoechst 33,342 dye and flow cytometry to examine the inhibition effect of curcumin on the rat C6 glioma cell line. The dye-exclusion assay indicated the activity present in SP cells, and they observed a decrease in SP cells after daily treatment of curcumin, indicating that curcumin might be active against brain CSCs and that phytochemicals could offer therapeutic potentials for targeting CSCs. It is worth noting that ABC transporters are highly expressed not only in CSCs but also in normal stem cells; moreover, ABCG2 and ABCB1 are important in maintaining the blood-brain barrier, so ideal therapy using inhibitors should be designed to specially target ABC transporters of CSCs but spare normal stem cells. Therefore, the combined use of inhibitors which specially target ABC drug transporters of CSCs and chemotherapy drugs offers a powerful and selective strategy to eradicate CSCs[95].

As we have mentioned above, different kinds of CSCs may not share the same markers, and these markers are expressed not only by CSCs but also by other cells such as normal stem cells. Therefore, strategies targeting these markers should consider these conditions, and find more specific targeting methods.

4.3. *Targeting CSC niche and the quiescent state*

Normal stem cells reside in a ‘‘stem cell niche’’ which provides necessary signals for the maintenance of stem cell properties[101]. Likewise, CSCs require a similar microenvironment, termed CSC niche, which provides appropriate signals (necessary signaling pathways) to regulate self-renewal and the normal homeostatic processes such as inflammation, EMT, hypoxia and angiogenesis[101]. Vermeulen et al.[102] found that Wnt activity functionally designated the colon CSC population, and proposed that the ‘‘stemness’’ of colon cancer cells was in part orchestrated by the microenvironment. Calabrese et al.[103] proposed that the brain tumor microvasculature formed a niche that was critical for the maintenance of CSCs, and the vascular niches were important targets for therapeutic approaches. Hypoxia plays a key role in tumor progression and hypoxic tumor microenvironment also controls CSCs[104]. Morrison et al.[29] speculated that antiangiogenic therapies might induce CSC niche hypoxia, conferring radioresistance to the CSCs. Conley et al[105]. demonstrated that antiangiogenic agents such as sunitinib and bevacizumab could drive breast CSC stimulation by generating intratumoral hypoxia. Thus, they suggested that these antiangiogenic agents should be combined with CSC-targeted drugs. In addition, Zhong et al.[106] suggested that the inhibitors such as LY294002 and rapamycin for hypoxia-inducible factor1α (HIF-1α) could provide a basis for therapeutic efficacy. Therefore, targeting the CSC niche in combination with chemotherapy can provide a promising strategy for eradicating CSCs.

CSCs are resistant to traditional chemotherapy, because most of the current anticancer drugs target tumor growth by inhibiting DNA synthesis or cell division of actively dividing cancer cells; however, CSCs are frequently in a quiescent state[107]. Quiescence physiologically protects adult stem cells from harmful insults and prevents the exhaustion of their replicative potential[58]. CSCs are postulated to contribute to tumor dormancy and usually have a slow cell cycle kinetics which protects CSCs from chemo-radiotherapy[108]. Thus,

maintaining the cells in a quiescent state by blocking specific receptors and signaling pathways within the CSC niche can inhibit CSC functions of tumor initiation and metastasis[101]. Inducing dormant CSCs to enter the cell cycle provides an alternative way to restore chemo- and radio-sensitivity. Recent studies have revealed that some cytokines such as interferon-α (IFNα) and granulocyte-colony stimulating factor (G-CSF), or As_2O_3 can efficiently promote the cycling of normal haematopoietic stem cells (HSCs) and LSCs[109]. Therefore, combination of IFNα, G-CSF, As_2O_3 with chemotherapeutic agents may effectively target the dormant LSCs[109].

Targeting the stem cell niche can exhaust the source of nutrition and change the essential signals needed by CSCs. On the other hand, it can also influence the normal stem cell niche, or disrupt the levels of signals for normal cells. Thus, more sophisticated strategies are needed to overcome the shortcomings of the existing methods.

4.4. Manipulation of miRNA expression

MicroRNAs (miRNAs) are approximately 21-nucleotide long non-coding RNAs that regulate self-renewal, differentiation, and division of cells *via* post-transcriptional gene silencing[110]. miRNAs can act as both tumor suppressors and oncogenes, both of which are deregulated in cancers[111,112]. For example, microRNA-34a (miR-34a) is a direct target of tumor suppressor gene p53 and down-regulated in many cancers[113]. MiR-34a is a tumor suppressor that acts by targeting multiple oncogenes such as c-Met, Notch-1, Notch-2 and CDK6 and by inducing the differentiation of CSCs in brain tumors and glioma stem cells[114,115]. Liu et al.[116] showed that CD44 was a direct and functional target of miR-34a and miR-34a was a key negative regulator of $CD44^+$ prostate cancer cells, suggesting that miR-34a was a novel therapeutic agent against prostate CSCs. miR-21 and miR-205 are highly expressed and predicted to act as oncogenes by targeting the tumor suppressor genes in head and neck cancer cell lines[111,117]. A powerful technique for therapeutic targeting of miRNAs, as well as for miRNA functionalization both *in vitro* and *in vivo* is antisense oligonucleotide (ASO) inhibition[118]. Shi et al.[119] prepared solid lipid nanoparticles (SLNs) loading with anti-miRNA oligonucleotide (AMO) for suppressing microRNA-21 functions. Then they demonstrated that the cationic AMO-loaded SLNs had high antisense efficiency of miR-21 and subsequently decreased the proliferation, migration and invasion of human lung cancer A549 cells. Nozawa et al.[120] showed that siRNA could downregulate the expression of epidermal growth factor receptor (EGFR) and inhibit cell growth of head and neck squamous cell carcinoma (HNSCC), and EGFR siRNA significantly enhanced the chemosensetivity of HNSCC to cisplatin, 5-FU and docetaxel. Therefore, microRNA-based therapeutics that can rectify the aberrant transcript levels of cancer cells and especially target CSCs are of great potential in cancer therapy.

4.5. Induction of CSCs apoptosis

Apoptosis governs tissue development and homeostatic balance through a complex network of molecules that mediate death and survival signals, and it is also critical for maintaining normal cell physiological processes[57,121]. Dysregulation of apoptotic mechanisms contributes to cancer development, progression, as well as CSC resistance. These mechanisms include impaired apoptotic machinery, increased DNA damage repair after radiotherapy and chemotherapy, altered cell cycle checkpoint control, and upregulation of MDR proteins[57]. Therefore, manipulating the apoptotic machinery to induce apoptosis of CSCs shows great potential to eradicate CSCs for cancer therapy. Many compounds induce apoptosis by targeting the intrinsic and extrinsic apoptosis pathways. For example, NK-κB is a transcription factor that inhibits apoptosis by increasing the expression of survival factor[121]. Hexum et al.[122] synthesized several bicyclic cyclohexenones which were able to inhibit NK-κB signaling by inhibiting NK-κB-induced IL-8 expression and exhibit antiproliferative activity against A549 cells (a human lung adenocarcinoma epithelial cell line), CCRF-CEM cells (a human T cell lymphoblast-like cell line) and DU-145 cells (a human prostate carcinoma cell line). Tumor necrosis factor-related apoptosis induced ligand (TRAIL) is one of the pro-apoptotic proteins that induce apoptosis in a wide range of cancer types[121]. The combined treatment with recombinant TRAIL and the Akt signaling inhibitor perifosine showed a synergistic pro-apoptotic activity against AML cells[123]. The delivery of TRAIL *via* mesenchymal stem cells (MSCs) is a new targeting therapy. MSCs are emerging as promising anti-cancer agents with the property of inherent tumor-trophic migratory which allow them to serve as vehicles for anticancer gene delivery[124]. Loebinger et al.[125] showed that TRAIL-expressing MSCs migrated to tumors and reduced the growth of primary cancers and metastasis, and TRAIL-expressing MSCs combined with mitoxantrone chemotherapy had a synergistic effect in apoptotic induction of putative CSCs. The expression of many antiapoptotic proteins is also responsible for CSC resistance. Todaro et al.[18] revealed that $CD133^+$ CSCs from colon carcinomas could produce and use IL-4 to protect them from apoptosis, and treatment with an IL-4Rα antagonist or anti-IL-4 neutralizing antibody significantly enhanced the sensitivity of $CD133^+$ cells to standard chemotherapeutic drugs (oxaliplatin and 5-FU).

Improperly activated DNA damage repair pathways enable cancer cells to survive chemo- and radiotherapy. DNA repair pathways compete with apoptotic signaling to determine the fate of damaged cells[58]. However, CSCs prone to enhanced DNA repair capacity and anti-apoptosis pathways to avoid cell death. Chen et al.[126] demonstrated that esophageal cancer stem cells (ECSCs) employed attenuated DNA damage response (DDR) and decreased DNA repair potential to handle severe genomic insults when treated with DNA damaging agents. Marie et al.[127] also found that when exposed to ionizing radiation, epidermoid carcinoma cells showed rapid DNA repair mediated by fibroblast growth factor 2 (FGF2). Thus, agents that interfere with DNA repair have great therapeutic potential. Poly(ADP-ribose) polymerase (PARP) inhibitors potentiate the activity of DNA-damaging agents and radiation for the treatment of many cancers[58,128]. The combined use of novel inhibitors of DNA-dependent protein kinase and PARP-1 can inhibit DNA repair and act as potent radiosensitizers[129]. In addition, the pharmacological abrogation of checkpoint kinase (Chk) 1 could selectively kill cancer cells with p53 defects[58]. Bao et al.[130] suggested that a specific inhibitor of the Chk1 and Chk2 could reverse the radioresistance of $CD133^+$ glioma stem cells.

Despite the effectiveness of inducing apoptosis, therapies should avoid inducing apoptosis in normal cells, and should improve the specificity and effectiveness in inducing CSCs apoptosis.

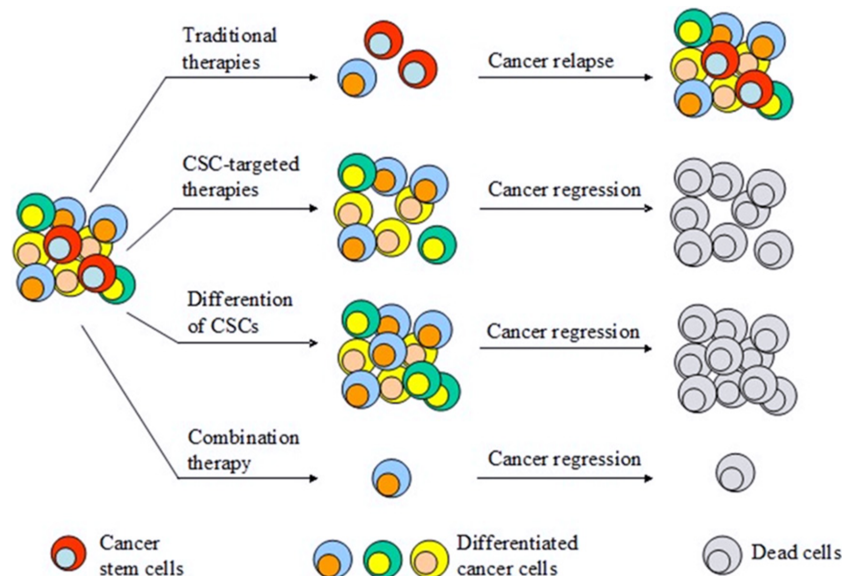

Fig. 1 Therapeutic implications. The traditional cancer therapies kill differentiated cancer cells but fail to target CSCs, resulting in cancer relapse. However, CSC-targeted therapies can eliminate or differentiate the CSCs, and the remaining and resulting differentiated cancer cells will die thereafter. But it is promising to combine CSC-targeted therapies and traditional therapies for depleting CSCs as well as killing differentiated cancer cells, this combination therapy may have the benefits of increased efficacy and quick action.

4.6. *Induction of CSC differentiation*

Apart from elimination therapies noted above that increase the efficacy of cancer therapy, another way to control tumor progression is to induce differentiation of CSCs (Fig. 1). Differentiation therapy could force CSCs to differentiate terminally and lose their self-renewal property[70]. Although many agents have been studied in differentiation therapy, only two kinds of anticancer drugs can affect cancer cell differentiation: retinoic acids and drugs targeting tumor epigenetic changes[131]. Retinoic acid (RA, Vitamin A), and its analogs (retinoid) can subvert the malignant progression process through signal modulation mediated mainly by retinoid receptors[70,131]. Campos et al.[132] revealed that ATRA induced differentiation of stem-like glioma cells (SLGC) and showed an antitumor effect both *in vitro* and *in vivo*. Their research demonstrates that differentiation therapy by retinoic acids may target the CSCs in glioblastoma. Ginestier et al.[133] demonstrated that modulation of the retinoid signaling might be sufficient to promote self-renewal or induce differentiation of breast CSCs. They also indicated that ATRA treatment induced the differentiation of breast CSCs, resulting in a significant decrease of the breast CSC population. These results suggested that ATRA might be a therapeutic strategy for targeting breast CSCs. Histone deacetylase (HDAC) inhibitors, suberoylanilide hydroxamic acid (SAHA) can cause growth arrest, differentiation, and/or apoptosis of many tumor types *in vivo* and *in vitro*, and has been used exprerimentally in cancer differentiation therapy[70,134]. Butler et al.[135] found that hydroxamic acid-based hybrid polar compounds such as SAHA suppressed the growth of human prostate cancer cells, and suggested that these compounds could be useful and relatively nontoxic agents for the treatment of prostate carcinoma. The combined use of differentiation-inducing agents and chemotherapy represents an effective approach to eliminate the CSCs. Lombardo et al.[136] demonstrated that bone morphogenetic protein 4 (BMP4), which could promote normal colonic stem cells differentiation, was able to promote terminal differentiation, to induce apoptosis of chemoresistant colorectal cancer stem cells (CRC-SCs), as well as enhance the chemosensitization of CRC-SCs to 5-fluorouracil and oxaliplatin, suggesting a therapeutic target against CSCs in advanced colorectal tumors.

Inducing CSC differentiation provides an alternative way to deplete cancer cells, while agents such as ATRA, SAHA may cause normal stem cells differentiation or other side effects. Therefore, toxicity studies should be done before the further application of the drugs.

5. Conclusion and perspectives

According to the CSC theory, CSCs are responsible not only for tumor initiation, development, and metastasis, but also for therapeutic resistance. Standard oncology treatments such as chemotherapy and radiotherapy can only shrink the tumors by killing the active tumor cells but miss the quiescent CSCs that lead to resistance and relapse, and may even enrich CSCs for a more resistant state[30,53]. These traditional approaches usually include systemic or local toxicity. Thus, new treatments targeting CSCs are necessary for improving patient survival rate and elongating life span. In this review, we discussed some strategies for cancer therapy which can directly eliminate or differentiate CSCs. These targeting strategies provide novel and promising approaches for CSC-targeted cancer therapy. Furthermore, the combination of targeted therapies directly eliminating or differentiating CSCs with established therapies may have a synergistic action and increased efficacy in cancer treatment. There is great need to develop new methods or improve the existing methods to isolate and identify CSCs. Since CSCs and normal stem cells share many properties, targeting CSCs may unfortunately affect normal stem cells. Thus, more precise targeting therapies which can selectively target CSCs but spare normal stem cells are greatly needed.

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