

Dear editor, dear reviewers,

We thank the reviewers and editors for the time and effort that they have put into reviewing the previous version of the manuscript (World Journal of Stem Cells, ID: 46592). Their suggestions have enabled us to improve our work. Based on the instructions provided in the peer review, we have modified the manuscript.

Appended to this letter is our point-by-point response to the comments raised by the reviewers. The comments are reproduced and our responses are given directly afterward.

We would like also to thank you for allowing us to resubmit a revised copy of the manuscript.

We hope that the revised manuscript is accepted for publication in World Journal of Stem Cells.

Sincerely,

Fangyu Du

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Referee: 1 (Reviewer's code: 02566952)

SPECIFIC COMMENTS TO AUTHORS

Q1: The manuscript presents an interesting (and much needed) review about the controversial problem of cancer stem cells especially in the very practical purpose of conceiving improved therapies. Several comments authors may wish to consider would add to an already well composed work. Under the chapter CSC signaling and inhibitors there is a mention about these cells are

deriving from normal cells. Maybe it would be useful for the reader to write a brief phrase about the hypothesis regarding their formation.

Answer: Thank you for this suggestion. We have added phrasing about the formation of CSCs. The detailed phrasing is as follows:

There are two main theories about the possible formation of CSCs: from normal stem cells and from non-stem cells. Studies have shown that CSCs are formed by the transformation of adult stem cells caused by genetic mutations. Normal stem cells have activated self-renewal mechanisms, have longer survival time, and can accumulate more mutations; thus, they have more opportunities to mutate into CSCs [28-30]. Therefore, we hypothesize that gene mutations in normal adult stem cells are caused by endogenous or exogenous stimuli, and then they enter the cell cycle, rapidly divide, and transform into CSCs. Moreover, some differentiated cells may also regain self-renewal capacity before canceration and mutate into CSCs [31-32].

Q2: “The canonical Wnt signaling pathway may be involved in the development of benign or malignant breast cancer” breast cancer= malignant tumors, benign tumors are not cancer.

Answer: We have deleted the word “benign” according to the suggestion.

Q3: The display of pathways known to belong to CSC together with their inhibitors is interesting and for sure industry will greatly profit from reviewing them. Occasionally there is a text mention about adverse effects, however they are not as consequent as other (tabled) description.

Answer: Thank you for this suggestion. We have added this section in manuscript, as follow:

2.5 Adverse effects (AEs) of inhibitors

ETC-159 has shown dose-limiting toxicities, including hyperbilirubinemia and skeletal fragility fractures [136]. The preliminary results of the clinical phase I trial are available, and they showed that PRI-724 results in tertiary reversible hyperbilirubinemia with dose-limiting toxicity. An open phase I/II clinical study involving PRI-724 dose escalation in patients with advanced malignant

myeloid blood diseases is still ongoing ^[51].

For enoticumab, the common severe AEs are fatigue, headache, hypertension and nausea ^[137]. However, six treatment-related serious adverse events were reported in 4 patients: brain natriuretic peptide (BNP) increase, troponin I increase, right ventricular dysfunction and pulmonary hypertension, and left ventricular dysfunction and pulmonary hypertension ^[137]. One patient had a dose-limiting toxicity of grade 3 vomiting and diarrhea ^[138]. Fragility fractures were reported at an ipafricept dose of 20 mg/kg. The hypophosphatemia and decrease in weight are AEs associated with ipafricept grade III treatment ^[139]. A dose-limiting toxicity of grade III mucosal inflammation was observed at the 30 mg dose level of LY-900009 ^[140]. When all-grade patients (20%) were treated with crenigacestat, the most frequent related AEs included diarrhea, vomiting, nausea, decreased appetite, fatigue, asthenia and hypophosphataemia ^[141]. In the phase I clinical trial, dose escalation of LY3056480 confirmed that trans-tympanic injection at the highest dose of 250 µg is safe and well tolerated and safety issues were not observed ^[142]. Patients with glioma were treated with RO-4929097 in combination with radiotherapy and temozolomide, and the results were positive, safe and effective ^[143]. Treatment with BMS-906024 was found to be relatively well tolerated, with minimal diarrhea observed in the subjects ^[144].

Moreover, the AEs commonly observed in vismodegib-treated patients, including muscle spasms, ageusia/dysgeusia, alopecia, weight loss, and fatigue, lead to poor clinical outcomes because of the decreased quality of life and treatment discontinuation ^[145], and the more severe AEs can lead to death ^[146]. In some cases, treatment with vismodegib resulted in the development of squamous cell carcinoma ^[147], amenorrhea ^[148], and persistent alopecia ^[149]. Patient treated with vismodegib for one month can develop severe nausea, jaundice, and cholestasis with significantly elevated BUN, creatinine and liver enzymes ^[150].

Sonidegib shows the same issues of drug resistance and AEs as vismodegib.

However, a phase I trial demonstrated that sonidegib treatment is well tolerated and effective. The maximum tolerated dose is 800 mg administered once daily [151]. Common grade 3/4 hematological AEs are thrombocytopenia (91%), followed by neutropenia (84%) and anemia (77%). The ClinicalTrials.gov Identifier is NCT02129101, and the treatment under examination. Patients treated with glasdegib develop some adverse events, such as dysgeusia, muscle spasms, alopecia, decreased appetite, increased blood creatinine phosphokinase, constipation and diarrhea [152].

Treatment with patidegib results in fatigue, muscle cramps and rash [153]. A phase I study demonstrated that BMS-833923 administered to patients with cancer was safe and well tolerated at all doses [154]. Adverse events related to napabucasin administration have been mostly mild, although some patients have experienced grade 3 gastrointestinal adverse events. More severe adverse events can be alleviated by dose reduction, discontinuation of napabucasin or by medication to reverse or manage symptoms [155]. Patients developed fatigue, hyperglycemia, nausea, rash hypertriglyceridemia, mucositis, hypophosphatemia, decreased appetite, and diarrhea after treatment with GDC-0084 [156].

Q4: This is an important topic and it would be good to insert the side effects (that are potentially a lot due to the fact these inhibitors interfere with pathways that are crucial for any tissue renewal mechanism. Basically, we have here information about the existence of cancer stem cells (ascertain in some cancers but not in all) about the biochemical pathways active in such cells followed by a very thorough description of this pathway inhibition that could act as treatment. A chapter about side effects is missing as well as a proof whatsoever this inhibitors actually DO target CSCs. Are the authors aware about studies reporting the precise targeting of CSCs using (at least some) of the molecules proposed?

Answer: We have added a chapter about the adverse effects of inhibitors as

well as information about the precise targeting of CSCs as follows:

Adverse effects:

ETC-159 has shown dose-limiting toxicities, including hyperbilirubinemia and skeletal fragility fractures [136]. The preliminary results of the clinical phase I trial are available, and they showed that PRI-724 results in tertiary reversible hyperbilirubinemia with dose-limiting toxicity. An open phase I/II clinical study involving PRI-724 dose escalation in patients with advanced malignant myeloid blood diseases is still ongoing [51].

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and treatment discontinuation ^[145], and the more severe AEs can lead to death ^[146]. In some cases, treatment with vismodegib resulted in the development of squamous cell carcinoma ^[147], amenorrhea ^[148], and persistent alopecia ^[149]. Patient treated with vismodegib for one month can develop severe nausea, jaundice, and cholestasis with significantly elevated BUN, creatinine and liver enzymes ^[150].

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Precise targeting of CSCs

Among this class of therapeutics, for example, vantiactumab ^[66], ipafricept ^[67] and ETC-159 ^[68], which show anti-CSC effects in preclinical model experiments, are in clinical trials to treat cancer patients ^[69].

Pretreatment of multiple myeloma (MM) CSCs with the γ -secretase inhibitor RO4929097, a Notch pathway inhibitor, can reverse bruceantin-induced effects on MM-CSCs proliferation [93].

Treatment of HCC38 cells, a triple-negative breast cancer (TNBC) stem cell line, with vismodegib significantly decreased TNBC cell proliferation, cell invasion and mammosphere formation while inducing cell apoptosis by inhibiting the protein expression or phosphorylation of downstream signaling molecules. Tumor formation and growth of HCC1806 cells (another TNBC stem cell line) pretreated with vismodegib are effectively suppressed in xenograft mouse models. Treatment with vismodegib may provide a novel alternative therapeutic strategy against TNBC that targets breast cancer stem cells (BCSCs), and it could provide promising insights for clinical applications in patients with TNBC [109].

In 2015, Li et al. demonstrated that BBI608 has strong anti-CSC effects *in vitro* and *in vivo* in a broad range of cancer types [120]. Zhang et al. confirmed that BBI608 is able to kill prostate CSCs, inhibit CSC properties, and inhibit the expression of stemness-related genes [121]. Currently, BBI608 is being assessed in clinical trials, and the results suggest that BBI608 is a potent anti-tumorigenic and anti-CSC drug in different tumor types [122-125].

Q5: As it is a review about CSCs and not about potential pharmacological inhibitors, do authors are aware about other methods of targeting CSCs (such as hyperthermia, nanoparticles, antibodies and so on). Even though it is obviously not within the authors field of interest their existence should be at least mentioned for making such review complete.

Answer: This comment was highly appreciated. We have added this important section according your comment as follows:

3.1 Nano-drug delivery system targeting CSCs

A NDDS with a variety of properties is often used for targeted delivery and sustained release drugs [157]. Due to the strong phagocytic ability of tumor cells, abundant blood vessels in diseased tissues and large gaps in vascular endothelial cells, NDDSs are more likely to enter the capillary wall of a tumor and become enriched in tumor tissues, which provides a new method for eradicating CSCs. The nanocarrier are small sized, have a large specific surface area and present high surface activity, degradation *in vivo*, good biocompatibility and no immunogenicity; moreover, the drug loading and pharmacokinetic properties can be improved by surface modifications to achieve targeted delivery and sustained release of drugs [158-159].

Liu and coworkers prepared the paclitaxel-loaded nanoparticles modified by cell-penetrating peptide R8, which specifically targets CSCs and blocks angiogenesis, reduced nutrient and blood supplement, thus leading to the suppressed proliferation of glioma CSCs [160]. Han et al. delivered gemcitabine (GEM) to BCSCs using hyaluronic acid (HA)-modified liposomes, and this modification improved the drug stability, prolonged the half-life, enhanced cell toxicity and anti-tumor metastasis and inhibited cell clone formation [161].

Cui et al. synthesized a gelatinase-sensitive polymer nanocarrier that was loaded with miR-200c and acted on gastric cancer cells, and their results indicated that gastric cancer cells were significantly sensitized to radiotherapy. In addition, the expression of CD44⁺ was down-regulated, the number of BGC823 cells (CD44⁺) was decreased, the cell invasion, metastasis and anti-apoptosis ability were weakened, and the resistance characteristics of CSCs were decreased [162]. Moreover, other nano drug-loading systems have been used for targeted therapy of CSCs, such as nano-loaded genes [162], nano-loaded siRNA [163], and nano-both drugs [164].

3.2 Targeting mitochondrion inhibiting CSCs

Changes in the mitochondria in CSCs, including morphological changes, abnormal activation of signaling pathways, dysfunction, reactive oxygen species (ROS) generation, and mitochondrial autophagy, are key to regulating

the proliferation and apoptosis of CSCs and represent one of the causes of anticancer therapy failure. CSCs exhibited significant anaerobic glycolysis characteristics, such as increased expression of glycolytic enzymes, increased production of lactic acid and decreased or rested mitochondrial function [165]. These characteristics are similar to that of stem cells, suggesting that the mitochondrial glycolysis pathway played a key role in regulating the proliferation and apoptosis of CSCs.

Liu et al. reported that CSCs could utilize the glycolytic pathway to supply energy induced by glucose and increase the expression of hexokinase 1 (HK-1), HK-2 and pyruvate dehydrogenase kinase 1 (PDK-1), which can prolong the life of CSCs [166]. Dichloroethyl ester can inhibit PDK phosphorylation of pyruvate dehydrogenase (PDH) and promote the conversion of pyruvate to Acyl-CoA, which converted mitochondrial metabolism from glycolytic to oxidative phosphorylation, thereby reducing cell proliferation, promoting apoptosis and inhibiting tumor growth [167]. In addition to producing ATP, mitochondria are the main site for ROS production. BCSCs have been reported to have a higher ability to scavenge ROS than the corresponding tumor cells, thereby maintaining ROS at a relatively low level, although BCSCs die when the ROS levels are too high [168]. Kawano et al. found that knocking out the CD44 variant gene could reduce the defense ability of CSCs against ROS and enhance the killing ability of CD44 variant strongly positive cells treated with cisplatin but not CD44 variant weakly positive cells [169]. Increasing the ROS level and destroying the protective effect of ROS on CSCs may also offer an alternative method for cancer treatment [170].

3.3 Autophagy and CSCs

Over the past several years, autophagy has emerged as a requirement for the maintenance of stemness in both normal tissue stem cells [171] and CSCs [172-173]. Autophagy plays a vital role in promoting and inhibiting two different effects in different stages of tumor and tumor development; therefore, activation and inhibition of autophagy could improve the therapeutic effects

for tumors [174]. Autophagy maintains the function of different types of stem cells, such as microenvironmental homeostasis and stem cell characteristics [175].

Some autophagy-related genes promote the survival of CSC in the autophagy process. Gong et al. discovered that Beclin1 and autophagy are also essential for CSC maintenance and tumorigenesis *in vivo* [176]. A key role for autophagy in maintaining BCSCs has been identified according to two different shRNA screens, with Beclin-1/ATG6 identified from a shRNA screen for genes that modulate the plasticity of BCSCs [177] and ATG4A identified from a screen for genes required for mammosphere formation [178]. Damage-regulated autophagy modulator 1 (DRAM1) and P62 are abundantly expressed in adult glioblastoma, and glioma stem cells are regulated in biological metabolism, tumor migration and invasion by specific siRNA acting on DRAM1 and P62 proteins [179]. Knocking out LC3 and ATG12 genes, the CD44⁺/CD24^{-/low} BCSCs are inhibited. The autophagy flux of non-adherent cells (CD44⁺/CD24^{-/low}) in TNBC is higher than that of adherent cells, and the expression of CD44⁺/CD24^{-/low} in TNBC is decreased after treatment with the autophagy inhibitor chloroquine (CQ) [180]. Jiang et al. found that CSCs from malignant gliomas cause cell death due to the accumulation of autophagy-related proteins and autophagosomes [181]. Similarly, the activation of autophagy under hypoxic conditions promotes the dedifferentiation of non-pancreatic CSCs into stem-like cells, suggesting that autophagy can affect the source transformation process of CSC [182].

Because autophagy changes the sensitivity of CSCs to conventional treatment or directly destroys cells using the toxicity produced by CSC autophagy, autophagy could be used as a treatment for CSCs. Conventional therapy presents difficulty in the specific killing of tumor cells and shows many negative effects in patients. Although autophagy therapy has milder effects on normal cells than conventional therapy, it may also have unknown effects based on cells remain to be explored.

3.4 Hyperthermia for CSCs

As a curative treatment for cancer, hyperthermia is attracting increasing attention and recognition from doctors and patients. In hyperthermia treatment, tumor tissue is directly heated through ultrasound, microwave, radio frequency, infrared, visible light, alternating magnetic fields, or heat-generating substances. This therapy does not include ionizing radiation and thus avoids the damage from radiation to patients and operators and pollution to the environment.

Tumor cells are more sensitive to heat, and the tumor area radiates more slowly; therefore, the temperature in the tumor area can be changed by hyperthermia, which leads to the death of tumor cells. At present, the temperature range of the hyperthermia method is 42~45°C and the temperature of the thermal ablation is above 65°C [183-184]. Hyperthermia can enhance the expression of apoptotic genes, such as P53 and FAS, thereby blocking the cell cycle, inhibiting tumor cell proliferation, and leading to tumor cell apoptosis [185]. The expression of heat shock protein (HSP) is increased during hyperthermia, which stimulates the body's anti-tumor immune response [186]. The endothelial cells of the tumor microvessels proliferate and are more sensitive under heat; thus, the microvessels in the tumor tissue are more susceptible to heat damage than those of normal tissues.

Hyperthermia enhances immune system responses to cancer, such as by up-regulating the homing of immune cells and the function of adhesion molecules on both immune cells and endothelial cells, activating cytotoxic T cells (CTLs), dendritic cells (DCs), and natural killer cell (NK), and inhibiting immune suppression [187]. As an adjunct to cancer therapy, hyperthermia plays an increasingly important role in the treatment of tumors. [188]. By using hyperthermia in combination with chemotherapy [189], radiotherapy [190], immunotherapy [191] or surgery [192], the dose of these therapies may be reduced to ease their side-effects without reducing their therapeutic effects.

The adverse effects of acute or chronic periods of regional hyperthermia do not develop often and are usually minor, and they include skin burns and skin

pain; however, these events usually heal spontaneously ^[193]. Overall, hyperthermia is considered an alternative therapeutic method when it is used appropriately.

3.5 Immunotherapy targeting CSCs

The CD44 receptor is a specific tumor antigen located on the surface of CSCs, and hyaluronan (HA) is the ligand for all CD44 receptors or subtypes. CD44 receptor-specific antigens play an important role in CSCs. Utilizing the monoclonal antibody MEN-85 that binds to the C-terminus of the hyaluronate-binding domain (HABD) of CD44 causes a conformational rearrangement that results in the CD44 receptor detaching from the surface of CSCs, thereby blocking the signaling pathway of HA-CD44 ^[194]. CD44 is a known marker of CSCs, and the CD44 gene splice isoforms are CD44s and CD44v. Li et al. reported that the CSC marker CD44s is up-regulated in human pancreatic tumors and associated with patient survival time. CD44s is necessary for the initiation, growth, metastasis, and postradiation recurrence of xenograft tumors in mice. Antibodies targeting the CD44 receptor can eliminate bulk tumor cells and CSCs from the tumors ^[195]. CD44s is the predominant isoform expressed in BCSCs. Elimination of the CD44s isoform impairs CSC traits. However, manipulating the splicing regulator epithelial splicing regulatory protein 1 (ESRP1) to shift alternative splicing from CD44v to CD44s leads to the induction of CSC properties ^[196]. These results suggest that alternative splicing provides functional gene versatility that is essential for different cancer cell states and thus cancer phenotypes. CSCs can also express a variety of specific antigens; for example, the CD44 receptor can also be expressed in lung cancer cells as well as BCSCs ^[197]. BCSCs not only express CD44 receptor but also express aldehyde dehydrogenase (ALDH) ^[198].

The Notch signaling pathway is abnormally active in the tumor microenvironment. Inhibitor targeting that blocks signal transmission can reduce the number of CSCs and inhibit the development of tumors ^[199]. Blocking Wnt signaling can reduce the expression of CD44 and ALDH on the

CSC surface and inhibit tumor self-renewal and metastasis [200]. ALDH1 is widely distributed in humans and highly expressed in stem cells of normal tissues as a marker of normal stem cells. Notably, the activity of ALDH1 is increased in multiple myeloma and myeloid leukemia [201]. Moreover, ALDH1 is also expressed in most cancer tissues at different levels, such as breast cancer, lung cancer, colon cancer [202].

In 2019, Chen et al. demonstrated that the depletion of ubiquitin-specific protease 9X (USP9X) markedly downregulated ALDH1A3, thereby resulting in the loss of the self-renewal and tumorigenic capacity of mesenchymal (MES) glioblastoma stem cells (GSCs). Furthermore, the USP9X inhibitor WP1130 induces ALDH1A3 degradation and showed marked therapeutic efficacy in MES GSC-derived orthotopic xenograft models [203].

Humanized anti-CD47 antibody Hu5F9-G4 can safely and effectively treat 5 invasive childhood brain tumors in mice [204]. Hu5F9-G4 demonstrated therapeutic efficacy *in vitro* and *in vivo* in patient-derived orthotopic xenograft models; notably, Hu5F9-G4 showed minimal activity against normal human neural cells. Advani et al. confirmed that the macrophage checkpoint inhibitor Hu5F9-G4 synergized with rituximab can safely and effectively eliminate aggressive and indolent lymphoma, and no clinically significant safety events were observed in this study [205]. Liu and coworkers indicated that CpG oligodeoxynucleotide, a toll-like receptor 9 agonist, combined with CD47 inhibitors can rapidly induce tumor shrinkage and prolong survival in mice [206]. It is worth noting that a number of new drugs targeting CD47 are undergoing clinical trials, such as AO-176, CC-90002, NI-1701, IBI118, TI-063. Therefore, macrophage immunological checkpoint blocking therapy (e.g., CD47 antibody) is expected to provide a new cancer immunotherapy strategy.

3.6 Targeting the CSC microenvironment

Although tumors initiate from oncogenic changes in a cancer cell, subsequent tumor progression and therapeutic responses depend on interactions between the cancer cells and the tumor microenvironment (TME) [207-208]. The cells and

molecules in the TME are in a dynamic process that leads to a large number of immunosuppressive cells (e.g., myeloid-derived suppressor cells (MDSCs), regulatory cells (Tregs), and tumor-associated macrophage (TAM)), and a large number of inflammatory related factors (e.g., chemokines, transforming growth factor- β (TGF- β), interleukin) assemble together in the TME. Then, they jointly promote tumor immune escape, tumor growth and metastasis [209].

Among the identified CXC chemokine receptors (CXCRs), CXCR4 is most closely related to tumor cells, and it was first discovered as a co-receptor of HIV. Until now, CXCR4 has been found in a variety of cancers, including breast, prostate, lung, colon and multiple myeloma [210]. The overexpression of CXCR4 is associated with poor prognosis in glioblastoma multiforme (GBM). Wu et al. indicated that anti-CXCR4 and anti-programmed cell death protein 1 (PD-1) combination immunotherapy can modulate tumor-infiltrating populations of the glioma microenvironment [211]. Some CXCR4 antagonists (e.g., BL-8040 and X4P-001-IO) are used in combination with PD-(L)1 drugs, such as Keytruda, Tecentri and Opdivo. CXCL12/CXCR4-mediated desmoplasia in metastatic breast cancers (mBCs) promotes immunosuppression; therefore, it is a potential target for overcoming therapeutic resistance to immune checkpoint blockade in mBC patients [212-213].

Neutrophils are first-responders to sites of infection and tissue damage [214]. Coperchini et al. demonstrated that the chemokine receptor CXCR1 promotes neutrophil recruitment, CSC proliferation, and neoplastic mass formation. Therefore, neutrophil recruitment signaling pathways, such as CXCL8-CXCR1, have the potential for use as targets for anti-cancer therapies [215].

Danhier et al. utilized the acidic TME as a target of nano-theranostics to enable cancer-specific imaging and therapy, and this approach showed advantages over conventional tumor targeting strategies [216]. However, the acidic TME not only plays an essential role in the initiation, progression, and metastasis of tumors but also participates in the induction of treatment resistance. The TME is related with oncogenesis, host genetics, the microbiome,

and immune cell activity ^[217]. More novel therapies targeting CSCs and the TME require further exploration, comprehensive analysis and consolidation of both clinical and experimental data.

Referee: 2 (Reviewer's code: 02446215)

SPECIFIC COMMENTS TO AUTHORS

This is an interesting paper that summarize the therapeutic options related to cancer stem cells. Some suggestions are provided below to improve the manuscript:

Q1: I suggest to add a paragraph related to autophagy and cancer stem cells focus on therapeutic applications in this field.

Answer: Thank you for this suggestion. We have added the suggested section (3.3 Autophagy and CSCs) in the revised manuscript as follows:

3.3 Autophagy and CSCs

Over the past several years, autophagy has emerged as a requirement for the maintenance of stemness in both normal tissue stem cells ^[171] and CSCs ^[172-173]. Autophagy plays a vital role in promoting and inhibiting two different effects in different stages of tumor and tumor development; therefore, activation and inhibition of autophagy could improve the therapeutic effects for tumors ^[174]. Autophagy maintains the function of different types of stem cells, such as microenvironmental homeostasis and stem cell characteristics ^[175].

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tumor migration and invasion by specific siRNA acting on DRAM1 and P62 proteins [179]. Knocking out LC3 and ATG12 genes, the CD44⁺/CD24^{-/low} BCSCs are inhibited. The autophagy flux of non-adherent cells (CD44⁺/CD24^{-/low}) in TNBC is higher than that of adherent cells, and the expression of CD44⁺/CD24^{-/low} in TNBC is decreased after treatment with the autophagy inhibitor chloroquine (CQ) [180]. Jiang et al. found that CSCs from malignant gliomas cause cell death due to the accumulation of autophagy-related proteins and autophagosomes [181]. Similarly, the activation of autophagy under hypoxic conditions promotes the dedifferentiation of non-pancreatic CSCs into stem-like cells, suggesting that autophagy can affect the source transformation process of CSC [182].

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Q2: Please define acronymus the first time the term is used.

Answer: We have defined and checked all of the acronyms throughout the text according to your suggestions.

Referee: 3 (Reviewer's code: 02909322)

SPECIFIC COMMENTS TO AUTHORS

This review aims to introduce the field of cancer stem cells and the important signaling pathways in cancer stem cells and to present the application of approved inhibitors in the clinical stage o patients. These are some issues should be concerned as below:

Q1: One main question: The review mainly discussed the targeting signaling pathway (including Wnt, Hedgehog, Notch, BMP, PI3K/Akt, etc) and related inhibitions in CSCs. However, these complex pathways have cross-talk to regulate the CSC phenotype. Moreover, non-CSC populations also exhibit overlapped signaling pathways. So, the authors have to discuss other issues about targeting CSCs ① Targeting surface markers expressed by CSCs (for example, CD47, ALDH1, CD44, etc), ② Targeting the CSC microenvironment (for example, CXCR4, CXCR1).

Answer: Thank you for these valuable comments. We have added these points (3.5 Immunotherapy of targeting CSCs) in the revised manuscript as follows:

3.5 Immunotherapy of targeting CSCs

The CD44 receptor is a specific tumor antigen located on the surface of CSCs, and hyaluronan (HA) is the ligand for all CD44 receptors or subtypes. CD44 receptor-specific antigens play an important role in CSCs. Utilizing the monoclonal antibody MEN-85 that binds to the C-terminus of the hyaluronate-binding domain (HABD) of CD44 causes a conformational rearrangement that results in the CD44 receptor detaching from the surface of CSCs, thereby blocking the signaling pathway of HA-CD44 ^[194]. CD44 is a known marker of CSCs, and the CD44 gene splice isoforms are CD44s and CD44v. Li et al. reported that the CSC marker CD44s is up-regulated in human pancreatic tumors and associated with patient survival time. CD44s is necessary for the initiation, growth, metastasis, and postradiation recurrence of xenograft tumors in mice. Antibodies targeting the CD44 receptor can eliminate bulk tumor cells and CSCs from the tumors ^[195]. CD44s is the predominant isoform expressed in BCSCs. Elimination of the CD44s isoform impairs CSC traits. However, manipulating the splicing regulator epithelial splicing regulatory protein 1 (ESRP1) to shift alternative splicing from CD44v to CD44s leads to the induction of CSC properties ^[196]. These results suggest that alternative splicing provides functional gene versatility that is essential for different cancer cell states and thus cancer phenotypes. CSCs can also express a variety of specific

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clinical trials, such as AO-176, CC-90002, NI-1701, IBI118, TI-063. Therefore, macrophage immunological checkpoint blocking therapy (e.g., CD47 antibody) is expected to provide a new cancer immunotherapy strategy.

Q2: In recent years, the existence of CSCs in solid tumors including breast cancer, prostate cancer and pancreatic cancer, etc. In fact, according to head neck cancer, Wu A had indicated that aldehyde dehydrogenase 1(ALDH1) could be a functional marker for identifying cancer stem cells in human nasopharyngeal carcinoma (NPC). (Cancer Lett. 2013). The authors should add this finding in the Introduction section.

Answer: Thank you for this comment. We have added this finding and reference in the revised manuscript.

Q3: Regarding to Fig. 1. Targeting Wnt, Hedgehog, Notch, BMP, Bmi, PI3K/Akt and STAT signaling pathways and the characteristics of cancer stem cells. The description of CSCs features is not somewhat correct. For example, "Apoptosis" should be rewritten as "Apoptosis resistance".

Answer: **We have changed this text according to your comment.**

Q4: What optimal time is for the use of a CSC-specific therapy ? before or soon after diagnosis, before or concurrently with neoadjuvant chemo/radiotherapy? The authors should discuss it in the Conclusion section.

Answer: Thank you for this comment. We have added this discussion in Conclusion section, as follow:

As for the optimal time is for the use of a CSC-specific therapy, there is no related reference to clarify this key issue. However, once the related cancer is identified, the targeting CSCs therapy can be concurrently proceeded with neoadjuvant chemo/radiotherapy. Generally, conventional chemotherapy can only inhibit tumor growth and lead to drug resistance, but cannot kill CSCs. However, the key to cancer treatment is how to deracinate CSCs. Targeting CSCs therapy can effectively remove CSCs and avoid drug resistance. Moreover, synergy therapy with chemo/radiotherapy may reduce the dosage

and AEs while do not change the physiological effect. However, when and how to use targeting CSCs to treat related cancers requires clinical trials to determine.

Referee: 4 (Reviewer's code: 00007461)

SPECIFIC COMMENTS TO AUTHORS

Q1: The manuscript/review is generally very well done and informative. An effort can be made to improve the review by including the most recent findings/most recent publications on these topics.

Answer: Thank you for this comment. We have added some of the most recent findings and most recent publications in the revised manuscript.