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Can Nanomedicines Kill Cancer Stem Cells?

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Abstract

Most tumors are heterogeneous and many cancers contain small population of highly tumorigenic and intrinsically drug resistant cancer stem cells (CSCs). Like normal stem cell, CSCs have ability to self-renew and differentiate to other tumor cell types. They are believed to be a source for drug resistance, tumor recurrence and metastasis. CSCs often overexpress drug efflux transporters, spend most of their time in non-dividing G0 cell cycle state, and therefore, can escape the conventional chemotherapies. Thus, targeting CSCs is essential for developing novel therapies to prevent cancer relapse and emerging of drug resistance. Nanocarrier-based therapeutic agents (nanomedicines) have been used to achieve longer circulation times, better stability and bioavailability over current therapeutics. Recently, some groups have successfully applied nanomedicines to target CSCs to eliminate the tumor and prevent its recurrence. These approaches include 1) delivery of therapeutic agents (small molecules, siRNA, antibodies) that affect embryonic signaling pathways implicated in self-renewal and differentiation in CSCs, 2) inhibiting drug efflux transporters in an attempt to sensitize CSCs to therapy, 3) targeting metabolism in CSCs through nanoformulated chemicals and field-responsive magnetic nanoparticles and carbon nanotubes, and 4) disruption of multiple pathways in drug resistant cells using combination of chemotherapeutic drugs with amphiphilic Pluronic block copolymers. Despite clear progress of these studies the challenges of targeting CSCs by nanomedicines still exist and leave plenty of room for improvement and development. This review summarizes biological processes that are related to CSCs, overviews the current state of anti-CSCs therapies, and discusses state-of-the-art nanomedicine approaches developed to kill CSCs.

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1. Introduction

Tumors are heterogeneous tissues with abundant phenotypically and functionally distinct cell subpopulations each having different capacities to grow, differentiate, develop drug resistance and form metastases [1]. In 1977, Hamburger and Salmon have shown that cancer cells derived from multiple myeloma patients can be cloned in soft agar at a frequency of 1 clone for from 100 to 100,000 cells and hereby suggested that only a small portion of tumor cells are tumorigenic [2]. These rare cells are now sometimes called "cancer stem cells" (CSCs) as they share many characteristics with normal stem cells, such as self-renewal and differentiation. A growing body of evidence suggests that CSCs can play a major role in cancer initiation, progression, resistance, recurrence, and metastasis in selected cancers [1, 3–5]. CSCs are intrinsically drug resistant and often display same phenotypes as multidrug resistant (MDR) cells including expression of drug efflux transporters [6], activation of anti-apoptotic signaling pathways [7, 8], and reprogramming of metabolic processes [5]. Notably, treatment with most commonly used chemotherapeutic drugs often results in an increase of the CSCs fraction in the tumor, making it more likely that these cells survive and spread to distant sites (Table 1). Tumor relapses are often observed after the treatment with those chemotherapies, which kill only the bulk of the more sensitive tumor cells, while allowing more resistant CSCs to evade. As a result, CSCs can even constitute the greatest fraction of the remaining/recurrent tumors (Fig. 1).

Based on these considerations chemotherapeutic approaches targeting CSCs may be more successful in treating cancer. However, tumors display plasticity and therefore elimination and targeting of CSCs without killing other cancer cells (non-CSCs) may not result in the complete cure. It has been shown that CSC phenotype can be "dynamic" as under certain conditions non-CSCs tumor cells can reverse their phenotype and become CSCs. Therefore successful therapy must eliminate both the bulk tumor cells and rare CSCs (Fig. 1). Overall, further preclinical and clinical studies are needed to definitively assess how CSCs respond to therapy. The design of these studies should take into account diverse biomarkers of the CSCs phenotypes and parameters of the CSCs function to provide robust clinical data on the role of such cells in the disease progression and therapy.

Developing simple, effective and robust therapeutic strategies against CSCs is needed to increase the efficacy of cancer therapy. Although some anti-cancer agents proposed recently can efficiently kill CSCs, similar to other anticancer drugs, most such agents have limitations upon translation into clinical studies, such as off-target effect, poor water solubility, short circulation time, inconsistent stability, and unfavorable biodistribution. Nanotechnology has shown significant promise in development of drugs and drug delivery systems that can overcome such limitations and address urgent needs to improve efficacy of diagnosis and therapy of various diseases [15, 16]. There is an increasing number of nanoparticle-based carriers used in drug delivery systems ("nanocarriers"), such as polymeric micelles [17–20], liposomes [21–23], dendrimers [24, 25], nanoemulsions [26],

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gold [27, 28] or metal nanoparticles [29], etc. (Fig. 2). Some nanocarrier-based therapeutic products (also termed "nanomedicines") are already on the market for treatment of cancer, lipid regulation, multiple sclerosis, viral and fungal infections [30, 31] while others undergo clinical and preclinical evaluation. Specifically, in the field of cancer therapy, nanotechnology is applied to improve bioavailability and decrease systemic toxicity of anti-cancer agents [32, 33]. Successful examples of clinically approved nanomedicines for cancer therapy include liposomal doxorubicin Doxil®, albumin-bound paclitaxel Abraxane®, PEG-L-Asparaginase Oncaspar® and others. Doxil®, the first polyethylene glycol (PEG) modified ("PEGylated") liposomal nanomedicine approved by the Food and Drug Administration (FDA) exhibits more than 100 times longer blood circulation half-life than that of free drug and decreases the risk of the cardio toxicity, which is a major side effect of the free drug [34–36].

In the past two decades, examples of nanotechnology-based approaches to tackle the CSCs problem have been accumulating [37, 38]. In general, nanoparticles were applied to target CSCs in three broad and overlapping areas: 1) as "beacons" to label CSCs by their biological signatures [39, 40]; 2) as nanocarriers to deliver "non-druggable" (for example insoluble or unstable) anti-CSCs agents to CSCs [41, 42] and 3) as therapeutic modality on its own to wipe out CSCs without harming normal, healthy stem cells [43–45]. Recently, some groups have successfully applied nanomedicines to target the CSCs to eliminate the tumor and prevent its recurrence. However, the challenges of targeting CSCs by nanomedicines still exist and leave plenty of room for developing future therapies.

CSCs exhibit specific phenotypes that are different from other tumor cells, and can be identified by the overexpression of certain biomarkers, such as, CD133+, CD44+CD24−, aldehyde dehydrogenase (ALDH+) and many others. High expression levels of these markers are associated with poor prognosis in patients [46, 47]. However, using these markers to target anti-cancer agents to CSCs is associated with several pitfalls. *First*, the markers differ from one type of cancer to another and there is no universal marker that can be used for all cancers [48, 49]. *Second*, multiple pools of biologically distinct CSCs can exist within one tumor as was shown both for acute myeloid leukemia [50, 51], as well as solid tumors [52]. *Finally*, CSCs share the markers' expression profiles with normal stem cells and therefore, there is a risk of affecting normal stem cells when targeting chemotherapeutic drug to CSCs. Moreover, since the CSCs phenotype is not a stable trait killing only the CSCs may not be sufficient for eliminating the tumors.

Thus, development of successful therapeutic modalities that can kill CSCs will require a comprehensive understanding of the characteristics of CSCs and relevant mechanisms (Fig. 1) as well as applying modern technologies for drug delivery. In this review we will summarize particular biological processes that are related to CSCs, and overview specific nanomedicine-based therapeutic approaches to ascertain the key question: can nanomedicines make difference in killing CSCs?

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2. Cancer cell heterogeneity and its implications for therapy

2.1 CSCs vs. clonal evolution hypothesis

There is an overwhelming body of evidence suggesting that similar to normal tissues, tumors are comprised of heterogeneous cell populations with varying metastatic potential [53–55], angiogenic potential [56] and drug resistance [57, 58]. M. Gerlinger and colleagues studied the tumor heterogeneity from multiple regions within a single patient's tumor by exome sequencing, chromosome aberration analysis, and ploidy profiling. They observed that about 63–69 % of the mutations found in single biopsies were not uniformly detectable throughout all the regions of the same patient's tumor, which provides clear evidence of tumor heterogeneity and the importance of the multi-region biopsy in cancer diagnosis [59, 60]. However, multi-region biopsy is always limited to the tumors and tumor cells with known and accessible physical location(s). Therefore, an alternative to multi-region biopsy so-called "liquid biopsies" has been developed, which uses single cell profiling of circulating tumor cells (CTCs) from patients' blood by microfluidics-based single cell transcriptional profiling technology [61]. Using this approach Powell and colleagues have identified 87 cancer-associated and reference genes among individual CTCs, which indicates the heterogeneity in the CTCs [61]. Moreover, Rottenberg and colleagues transplanted different parts of original mammary tumor into new host animals and found that these new tumor grafts responded differently to same treatments, displaying variability in times until relapse or rates of tumor growth [57, 62].

While the phenomenon of tumor heterogeneity is frequently observed, the cause of the heterogeneity among the tumor cells is not fully understood and still debated. Partly this is due to incomplete knowledge about cell-of-origin in cancer and factors underlying tumor progression that may vary from one tumor type to another. Still, among all the hypotheses, the theory of CSCs has gradually gained wide acceptance and influenced all approaches to cancer research and therapy [63]. The CSCs hypothesis postulates a hierarchical organization of tumor cells such that only a small population of cancer cells is responsible for sustaining tumorigenesis and establishing the cellular heterogeneity in the primary tumor [64]. At the top of this hierarchy are the CSCs with unlimited dividing ability, that give rise to more differentiated progenies, which are only able to divide certain number of times. CSCs are phenotypically distinct from other tumor cells, which enables their identification using fluorescence-activated cell sorting (FACS). A number of markers have been proven useful for the identification of CSCs in multiple types of leukemia and solid tumors, including CD34, CD133, CD44, epithelial cell adhesion molecule (EpCAM), breast cancer resistance protein (BCRP) and aldehyde dehydrogenase-1 (ALDH1) activity. However, in different types of cancer different molecules may serve as "stem cell markers", and none of these markers can be used exclusively to isolate CSCs in every type of tumor. This greatly complicates the CSCs studies. Furthermore, even using the same type of tumor, the same cell line, and the same CSCs marker, the portion of CSCs reported by different laboratories can be dramatically different. For instance, the ALDH% in SUM159 cells from Dr. Woodward's report is as high as 61.5% [65], while, in Dr. Arteaga's study, the portion of ALDH% in SUM159 is less than 2% [66]. Therefore, CSCs markers alone may not be sufficiently specific to identify CSCs in different tumors. To overcome these limitations, a

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combination of markers has to be used for better isolation and identification of CSCs [67]. However, so far no "perfect" combination of markers has been confirmed to successfully identify CSCs [68]. Similar to normal stem cells, CSCs are capable of self-renewal and differentiation. With the first property, the stem cell has potential to give rise to the new stem cell without differentiating into different cell types. Generally, self-renewal is the process by which stem cells divide to make more stem cells (symmetric division) [69]. With the second property, CSCs can divide and give rise to two distinct cell types – one a copy of the original stem cell and the other a progenitor cell that can differentiate into different cell types after amplification (asymmetric division). It is however characteristic for CSCs to mainly exist in a quiescent non-cycling G_0 state [5, 58]. This makes them significantly less susceptible to chemotherapeutic drugs that kill actively dividing cells [70].

Despite general acceptance of the CSCs model, the question remains which cancers out of the vast variety do actually follow this model? The CSC model was not confirmed for all cancers and was really only tested in a limited number of tumors [71]. It is important to note, that methods used to study the CSCs are rather limited. For example, *in vivo* cell transplantation to immunocompromised mice, which is widely used to study tumorigenicity and to estimate frequencies of tumorigenic cells can severely underestimate the real frequency of tumorigenic cells and results can significantly differ depending on the strain of mice used [72]. In particular, transplantation of melanoma cells into extremely immunocompromised NOD/SCID interleukin-2 receptor gamma chain null (Il2rg(−/−)) mice has shown the frequency of tumorigenic cells to be several orders of magnitude higher compared to the results observed in NOD/SCID mice [72]. Moreover, the ability of a cancer cell to form a tumor does not mean *by default* that it is a stem cell. To qualify, the cell should possess other properties, like drug resistance, specific phenotype, etc. Finally, as mentioned above, one should be very careful using certain markers for CSCs characterization in various tumors since CSCs markers lack specificity and greatly vary between different types of cancers.

For certain cancers no distinct cell subpopulation(s) that can be attributed to CSCs was identified so far using existing methodologies. For instance, in a well established engineered mammary tumor mouse model, MMTV–Erbb2 no CSCs subset could be identified using various cell surface markers [73–75]. The heterogeneity and tumor progression in such cases is better explained by a classic "clonal evolution" model, which assumes that tumor heterogeneity is a result of stochastic genetic and/or epigenetic changes in cancer cells and that each cell has a chance to become tumorigenic and/or drug resistant if it accumulates sufficient genetic/epigenetic changes (Fig. 3A). This clone in turn generates phenotypically similar cells with different but close tumorigenic potential without any hierarchy. Moreover, identification of CSCs markers in melanoma to date remains challenging. *In vivo* cell transplantation experiments have shown that very high portion of melanoma cells are tumorigenic (at least 25%) [4, 72] and that these cells produce tumors without any hierarchy. Morrison et al. showed that melanomas from patients have common and phenotypically diverse tumorigenic cells that undergo reversible phenotypic changes and not hierarchically organized *in vivo* [75]. Even though slow-cycling JARID1B-expressing melanoma cells that are required for continuous tumor growth *in vivo* were recently identified, these cells do not

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follow the classical CSCs non-stem cells convert to stem cells was observed for other cancers as well as normal stem cells [77]. This behavior is explained within a so-called “dynamic CSCs model” (Fig. 3B). According to this model CSCs phenotype is much less stable compared to classic CSCs model and non-CSCs can acquire tumorigenicity due to effect of specific microenvironment and/or through genetic/epigenetic changes [49, 64, 78].

Importantly one should be cautious in assigning just one model described above to a certain cancer, as all the models are not mutually exclusive. Thus, in the hierarchically organized cancers the clonal evolution can still occur in CSCs and/or non-CSCs. Therefore, while designing an efficacious and comprehensive cancer treatment strategy one should consider a need for eliminating the CSCs, the non-CSCs as well as impairing the specialized microenvironment housing the CSCs (known as “niche”) (Table 2).

2.2 Drug efflux transporters and drug resistance in CSCs

A very important characteristic of CSCs is the expression of high levels of ATP binding cassette (ABC) transporters (Table 3). Expression of such transporters is commonly associated with MDR as they provide for a unique defense of cells against chemotherapeutic drugs by significantly decreasing the cellular accumulation of cytotoxic agents. (Notably, CSCs are quiescent [70], they spend most of their time in G_0 cell cycle phase [81], and have high DNA repair capacity [82], which is believed to be an additional reason for their chemoresistance.) The classic theory of MDR development posits that MDR cells may repopulate the tumor after the therapy as a result of selection when some of the cancer cells accumulate necessary genetic and/or epigenetic changes that confer drug resistance (Fig. 4A). Such resistant cells acquire selective advantage over the rest of the cells, which allows them to evade the therapy. According to the CSCs theory, tumors already contain small population of intrinsically resistant tumor initiating cells, which similar to normal stem cells overexpress drug efflux transporters, while their more differentiated progenies loose this property. For example, hematopoietic stem cells overexpress ABCG2, while their differentiated progenies do not [83]. Exposure to the chemotherapeutic drugs eliminates the drug sensitive stromal tumor cells, leaving the CSCs. Mutations in the surviving CSCs and their progenies can lead to the development of MDR phenotype (Fig. 4B).

Historically, investigators noticed that a fluorescent vital cell dye Hoechst 33342, which is used to study the cell cycle, could be also used to isolate stem cells [84, 85]. Stem cells are Hoechst dim due to overexpression of the BCRP (ABCG2) drug efflux transporter, which pumps this dye out of the cells. Goodell et al. have demonstrated that display of Hoechst 33342 fluorescence in bone marrow cells (at red and blue emission wavelengths simultaneously) allows distinguishing a small subpopulation of Hoechst dim cells with phenotypic markers of multipotent hematopoietic stem cells. This small subset of cells named “Side Population” was at least 1,000-fold enriched with cells that are capable for repopulation of bone marrow of lethally irradiated mice [86]. From then on, Hoechst 33342 exclusion assay has been performed in many laboratories, and the cells located within the side population have consistently shown CSCs-like properties in many types of tumors, such as breast [84], lung [87], brain [88], pancreatic [89] and others. For example, Yin et al. reported that purified MCF7 side population cells with ABCG2 gene expression that are

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more resistant to ABCG2 substrate, mitoxantrone compared to non-side population cells also have an increased ability to colonize the mouse mammary gland [90]. Inhibition of ABCG2 transporter using low molecular weight inhibitors has been investigated as one of the strategies to sensitize and kill CSCs for more effective therapy. Several inhibitors of ABCG2, including fumitremorgin C (FTC) [91, 92], tryprostatin A [93, 94], tariquidar [95], etc. have been explored. However, toxicities of these compounds to healthy cells observed in *in vitro* and *in vivo* hindered their development in clinical studies. Using a novel ABCG2 inhibitor, a non-toxic FTC analogue KO143 resulted in increased intracellular mitoxantrone accumulation in CD34+CD38− CSCs in the majority of AML patients [96] (Table 3), albeit the clinical benefit of this compound for treatment of cancer remains to be seen. It has been shown recently, that silencing of adenine nucleotide translocator-2 (ANT2), which is involved in glycolytic metabolism and is overexpressed by undifferentiated proliferating cells and CSCs [97] using adenovirus encoding ANT2 shRNA could effectively induce apoptosis in breast CSCs and sensitize these cells to doxorubicin via downregulation of ABCG2 transporter [97].

Drug resistance of CSCs has been also associated with overexpression of other drug efflux transporters. For example, P-glycoprotein (Pgp), also designated ABCB1, is one of the most important efflux transporters related to CSCs resistance. Studies have shown that higher expression of CD133 in CSCs or transfected cells was accompanied with an elevated ABCB1 efflux activity. For example, Angeastro et al. found that Pgp/ABCB1 was upregulated and displayed functional drug efflux activity in 62% of CD133+ glioma cells in comparison with the parental cells [98]. The mechanism behind the increase in transporter expression and activity in CSCs is not clear, however, recent study pointed out that cisplatin treatment selects for MDR CD133+ cells by activating Notch signaling pathway [10]. Treatment of lung cancer cells with low doses of cisplatin enriched CD133+ cells and upregulated ABCG2 and ABCB1 transporters expression in these cells. These effects were also mediated through the Notch signaling pathway, as the co-administration of the Notch signaling inhibitor remarkably reduced levels of CD133, and increased the sensitivity of tumors to the chemotherapy [10].

Pgp cousin, ABCB5 is another ABC transporter implicated in the drug resistance in CSCs in different tumor types. For example, Frank and colleagues noted that ABCB5 can serve as biomarker for purification and characterization of adult skin-associated stem cells, while also functioning as a MDR transporter and conferring resistance to chemotherapy *in vitro* in human malignant melanoma [99–101]. ABCB5 was found to be specifically expressed on CD133+ melanoma cells [102]. Monoclonal antibodies (mAb) against ABCB5 were also shown to sensitize melanoma cells to anti-cancer drug, doxorubicin [100]. Therefore ABCB5 inhibition may represent an attractive strategy for the development of new melanoma therapies.

Even though MDR has been known for over 30 years (since discovery of Pgp in 1976) the success in overcoming it clinically is rather limited. Several strategies were proposed to overcome drug resistance [103–106]. Much effort has been devoted to development of new cytotoxic drugs and the use of combination chemotherapy regimens to improve cure rates. Unfortunately these strategies have produced only limited success in MDR tumors [107].

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Dose-intensified and high-dose regimens are active in certain cases, however treatment-related morbidity and mortality are usually high [103, 107]. Thus, alternative strategies using chemosensitizing agents to amplify drug activity in resistant tumors have been evaluated in both laboratory and clinical investigations [106, 108]. Since the 1990's a variety of agents that inhibit drug efflux transporters have been attempted in clinical studies in different cancers. Initial studies have focused on agents, which inhibit the Pgp efflux pump. Several generations of such Pgp-modulating agents have been developed and tested in clinical trials [58, 105, 109]. The first generation of MDR reversal agents, such as cyclosporine A and verapamil had relatively low affinity to Pgp and their use was limited by unacceptable toxicity and low tolerability. The more potent and less toxic agents of the second generation (valspodar, biricodar and others) have shown some success but they also affected non-targeted proteins and failed due to the unpredictable pharmacokinetic interactions. Notably some of these agents inhibited cytochrome P450, and this resulted in increased drug levels in the blood and the risk of drug overexposure [109]. The even more potent and specific third generation agents were expected to have less effect on drug pharmacokinetics [105, 106] and are currently in clinical trials. But some of these trials have already reported negative results and study design issues, regarding either dosing regiments or patient selection [110]. Overall, targeting of ABC transporters with low molecular weight inhibitors have not been very successful in overcoming general MDR in cancers, and therefore, it is likely that in the case of CSCs this approach at present stage will not prove effective. Thus, broad-spectrum inhibition, rather than modulation of single mechanisms, is likely to be required to circumvent drug resistance and eradicate CSCs. As was mentioned above, there have been attempts to directly (using low molecular weight inhibitors or mAb) or indirectly (inhibition of cell signaling pathways or regulatory RNA, etc.) target ABC transporters in CSCs, however, the number of reports using ABC transporters as a target for CSCs therapy is rather limited. Possibilities of using polymer therapeutics and polymeric nanocarriers to circumvent drug resistance of CSCs are discussed in **Section 3** below.

2.3 Abnormal cellular metabolism in CSCs

It is known that the mitochondrial metabolism in normal stem cells is different from that in differentiated cells [120, 121]. Stem cells are characterized with higher rate of glycolysis, higher amount of ATP/cell and more pronounced perinuclear localization of mitochondria [120]. Moreover, the integrity and function of mitochondria are important for life span, differentiation, and overall maintenance of the stem cells [120, 121]. Similar to normal stem cells the CSCs display different metabolism compared to the rest of the tumor cells. For example, the mitochondrial membrane potential within a tumor mass is heterogeneous and these differences can be used as a tool to identify, isolate and enrich CSCs populations [122]. Specific properties of lung CSCs, that distinguish them from the bulk tumor cells have also been described, such as low quantity of mtDNA, high mitochondria membrane potential, low oxygen and glucose consumption, and a low intracellular concentration of ATP and reactive oxygen species (ROS) [122].

Recently, Lagadinou and colleagues reported that leukemia CSCs have lower rate of energy metabolism and lower cellular oxidative status (termed "ROS-low") compared to non-CSCs [123]. These authors have also shown that ROS-low leukemia CSCs aberrantly express

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BCL-2, which provides a potential targeting strategy for CSCs therapy. Similar observation was made in the glioma stem cells (GSCs). Vlashi and colleagues evaluated the metabolic state of GSCs and compared basal oxygen consumption rates and extracellular acidification rates in differentiated tumor cells and GSCs derived from tumorspheres [124]. They found that GSCs consumed less glucose, produced less lactate, and had higher ATP levels compared to their differentiated counterparts. Interestingly, GSCs tolerated very well the inhibition of either glycolysis or mitochondrial respiration, and only simultaneous inhibition of both metabolic pathways induced significant ATP depletion, indicating that GSCs utilize multiple pathways to produce energy [124]. Notably, the ability of GSCs to tolerate oxidative stress was different in different cell lines and correlated with the intrinsic radioresistance of GSCs. In other studies a direct correlation between mitochondria membrane potential and tumorigenicity was found [125]. Specifically, the cells with higher mitochondria membrane potential also had higher tumorigenicity while depletion of the membrane potential in those cells with rapamycin significantly decreased their tumorigenicity [125]. In conclusion targeting tumor metabolism and reprogramming it to the “normal” status can potentially impair growth of CSCs, induce differentiation of CSCs and finally sensitize CSCs to the therapy. Some applications of nanomedicines in this direction are discussed below in **Section 3.4**.

2.4 Embryonic signaling pathways in CSCs

It is widely accepted that tumors often develop and progress due to deregulated self-renewal pathways. During the last decade, a better understanding of these aberrant signaling pathways allowed to propose new therapeutic strategies aiming to suppress the tumor regrowth. Specifically, Wnt, Notch, and Hedgehog embryonic signaling pathways have been implicated in self-renewal and differentiation in CSCs in many types of cancer [126, 127]. These signaling pathways related to CSCs are of interest as potential targets for therapeutics development [126]. Several therapeutic agents and cell-based therapeutics targeting these signaling pathways have reached various stages of clinical trials (Table 4).

For example, Wnt/β-catenin signaling pathway was shown to play a pivotal role in the maintenance of tissue homeostasis by regulating self-renewal of normal stem cells as well as proliferation or differentiation of progenitor (transit-amplifying) cells in embryogenesis [8, 128]. The β-catenin is a transcription factor, which in un-activated cells is phosphorylated and localized in the cytoplasm. Upon activation of the Wnt signaling the β-catenin becomes dephosphorylated and translocates into the nucleus where it activates the target genes [129]. Chronic upregulation of Wnt signaling was observed in several cancers and appears to play an important role in CSCs [8]. This pathway is involved in malignant transformation, and associated with tumor aggressiveness, metastases and poor clinical outcome [130–132]. One example of therapeutic agent targeting the Wnt/β-catenin pathway is PRI-724, a specific small molecule CREB-binding protein (CBP)/β-catenin antagonist, which is in Phase I and II clinical trials in patients with acute myeloid leukemia (AML), chronic myeloid leukemia (CML), pancreatic cancer and advanced solid tumors [133]. Another example is Vantictumab (OMP-18R5) a mAb that interacts with the extracellular domain of 5 Frizzled receptors (1, 2, 5, 7, 8) and blocks canonical Wnt signaling [134]. Preclinical xenograft

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studies have shown OMP-18R5 inhibits tumor growth, reduces tumor-initiating cell frequency and exhibits synergistic activity with conventional therapy [134].

Notch signaling pathway is one of the most intensively studied putative therapeutic targets in CSCs. Notch as well as most of its ligands are transmembrane proteins, and therefore Notch pathway is usually activated through a direct cell-cell contact. Upon ligand–receptor binding the Notch receptor undergoes a conformational change that exposes previously protected sites to proteolytic cleavage by metalloprotease and γ-secretase and results in release of extracellular and intracellular fragments [126]. Interestingly, Notch signaling pathway appears to play some role in drug resistance. Thus the side population isolated from breast adenocarcinoma cells MCF7 overexpress Notch protein [135]. The down-regulation of Notch expression enhances drug sensitivity of the cancer cells, inhibits tumor regrowth and reduces migration and invasion of cancer cells. Inhibition of γ-secretase-mediated Notch cleavage is a primary focus for the development of Notch pathway targeting therapeutics [136]. For example, a phase I clinical trial (NCT00100152) evaluated a small molecule γ-secretase inhibitor, MK-0752 in relapsed or refractory T-cell acute lymphoblastic leukemia (T-ALL) and advanced breast cancer patients [137]. The Phase I/II Study of MK-0752 followed by docetaxel in advanced or metastatic breast cancer has been also completed (Table 4). OncoMed Pharmaceuticals, Inc. has been developing several mAb-based therapeutic agents that target Notch signaling pathway (Table 4). For example, Demcizumab (OMP-21M18) is a humanized mAb against Delta-like ligand 4 (DLL4) in the Notch pathway that inhibits CSCs growth and promotes cell differentiation. It has shown broad-spectrum activity in pancreatic [138], breast and colon tumors [139]. In combination with standard chemotherapy OMP-21M18 is currently in Phase I studies in patients with non-squamous non-small cell lung cancer (NSCLC), metastatic colorectal cancer and pancreatic cancer (Table 4). Another agent believed to target CSCs is OMP-52M51, a humanized mAb against the cell surface Notch 1 receptor, which is currently evaluated in Phase I studies for patients with relapsed or refractory lymphoid malignancies and solid tumors. OMP-59R5 is a humanized mAb screened against Notch2 that binds both the Notch2 and Notch3 receptors. This agent is capable of downregulating the Notch pathway signaling and affecting pericytes, hereby impacting stromal and tumor microenvironment. Currently, OMP-59R5 is evaluated in Phase 1 dose escalation study in patients with previously treated solid tumors, for which there is no remaining standard curative therapy and no therapy with a demonstrated survival benefit. Additionally, two Phase Ib/II studies of 1) OMP-59R5 in combination with Nab-paclitaxel (Abraxane) and gemcitabine in subjects with previously untreated stage IV pancreatic cancer ("ALPINE"), and 2) OMP-59R5 in combination with cisplatin and etoposide in patients with small cell lung carcinoma (SCLC) ("PINNACLE") are recruiting participants.

Hedgehog (Hh) signaling pathway has also been recognized as one of the most important pathways in control of the patterning maintenance, tissue polarity, and stem-cell maintenance during stem cell and embryonic development. The Hh pathway receptor, Patched (Ptch1), in un-activated state actively suppresses the activity of a G-protein coupled receptor-like protein, called Smoothened (SMO). In mammals three ligands, Sonic Hh (SHh), Desert Hh (DHh), and Indian Hh (IHh) can activate the Hh pathway. Binding of one of these ligands to Ptch1 receptor releases the inhibition of SMO, what leads to downstream

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activation of three transcription factors Gli1, Gli2 and/or Gli3 [140, 141]. Deregulation of this pathway leads to tumorigenesis and metastasis [142]. It was first discovered in Gorlin syndrome patients, which have somatic mutations in Ptch1 gene and develop multiple basal cell carcinomas (BCCs) in their lifetime [143, 144]. Activating and inhibiting mutations in Hh have been later reported in other BCCs and medulloblastoma [72, 145–147]. Recent studies suggest that Hh signaling regulates CSCs in human cancers including pancreatic cancer, breast cancer, multiple myeloma, glioblastoma and CML [7, 87, 148–151]. Several Hh inhibitors were identified in recent studies (Table 4). For example, Vismodegib (GDC-0449) a small molecule inhibitor of SMO decreased cell viability and induced apoptosis in pancreatic CSCs [152]. This agent is now evaluated in combination with gemcitabine in a pilot study treating patients with advanced pancreatic cancer. Another example is BMS-833923 (XL139), an oral small molecule inhibitor of SMO capable of inhibiting the expression of downstream effectors in the Hh pathway (Gli1 and Ptch1), shown to reduce clone formation and fraction of ALDH+ CSCs [153, 154]. BMS-833923 has been evaluated as a single agent in Phase I trial in patients with advanced or metastatic cancers, as well as in combination with other agents such as an ongoing Phase II study of BMS-833923 in combination with Dasatinib (BMS-354825) in newly diagnosed Chronic Phase (CP) CML subjects.

Other than Wnt, Notch, and Hh embryonic signaling pathways, Focal Adhesion Kinase (FAK), a cytoplasmic tyrosine kinase that mediated signal transduction by integrins and growth factor receptors also plays an important role in the tumor initiating capability of CSCs [155]. There are two small molecule drugs, VS-6063 and VS-4718, which target FAK signaling pathways and inhibit CSCs that are currently in early stages of clinical development (Table 4). The first one is evaluated as a single agent in malignant pleural mesothelioma and in combination with paclitaxel in patients with advanced ovarian cancer. The second one is evaluated in metastatic non-hematologic malignancies.

In addition to the new therapeutic agents some currently approved drugs have also shown ability to affect embryonic signaling pathways and displayed activity against CSCs. For example, mAb-based anticancer drug, Trastuzumab (Herceptin) has shown ability to decrease subpopulation of CD90+ cells in gastric cancer [156] and suppress tumorsphere formation in breast cancer [157]. The studies suggest that this antibody affects ERBB2 signaling [156] and inhibits Notch1 signaling [157] in cancer cells. Trastuzumab was also recently evaluated in combination with the Natural Killer (NK) cells to develop therapeutic modality against breast cancer [158]. This work demonstrated the depletion of the $CD44^{high}$/$CD24^{low}$/$HER2^{low}$ cells and antibody-dependent NK cells–mediated cytotoxicity (ADCC) in breast cancer model [158]. Altogether new therapeutic agents and current drugs that target embryonic signaling pathways in CSCs alone and in combination with other existing and novel anti-cancer modalities have shown great promise in treatment of cancer.

2.5 Use of natural compounds and repurposing of existing drugs to treat CSCs

In attempts of developing anti-CSCs therapies some investigators explored effects of natural compounds and nutraceuticals (Table 5). For instance, Li et al. reported that sulforaphane, a dietary component of brocconi/broccoli sprouts, can decrease fraction of ALDH+ cells by

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65% to 80% (from 3.01% to 1.47% or 0.49%) in human breast cancer SUM 159 cell line [45]. Sulforaphane was shown to prevent NF-kappaB binding, downregulate apoptosis inhibitors and induce apoptosis, along with preventing clonogenicity in pancreatic CSCs [159]. It was also shown to potentiate the efficacy of imatinib against chronic leukemia CSCs through enhanced abrogation of Wnt/β-catenin function [160]. A pilot study will determine whether broccoli sprouts rich in sulforaphane and quercetin as nutritional supplement can positively affect the overall survival of surgically non-treatable patients with pancreatic ductal adenocarcinoma that receive conventional cytoreductive (radio-) chemotherapy (NCT01879878). Another example of nutraceutical with anti-CSCs activity is curcumin and its analogues contained in Indian spice turmeric. One analog GO-Y030 was shown to decrease phosphorylation of the signal transducer and activator of transcription 3 (STAT3), cell viability and tumorsphere formation in ALDH+ and CD133+ colon CSCs [161]. Curcumin separately and in combination with another black pepper derived compound an alkaloid piperine have shown to inhibit the tumorsphere formation, serial passaging, and fraction of ALDH+ cells in normal and malignant breast cells [162]. These compounds were also inhibiting the Wnt signaling pathway and suppressing breast stem cells self-renewal but did not cause toxicity to differentiated cells. Other study suggested that curcumin induced cell death in esophageal cancer cells through modulating Notch signaling. Altogether, these studies suggest that select nutraceuticals could serve as potential cancer preventive agents.

Along with nutraceuticals some well known drugs that were not originally intended for cancer use has shown considerable anti-CSCs activity (Table 5). For example, metformin, a first-line drug for type II diabetes has been reported to selectively target CSCs with CD44+CD24− phenotype in breast cancer [163]. Based on this finding the effects of metformin on CSCs are evaluated in clinical trial in patients with colorectal cancer (NCT01440127). Moreover, metformin is evaluated in combination with standard systemic chemotherapy in relapsed ALL that has a dismal outcome (NCT01324180) as well as with chemotherapy to women with advanced ovarian, primary peritoneal or fallopian tube cancer (NCT01579812).

Silencing of E-cadherin using shRNA produces stabilized mesenchymal-like state that captures many CSCs properties and provides novel model for CSC therapy screening [9, 164]. Using this method salinomycin, an antibacterial drug was selected via high-throughput screening as a potential anti-CSCs agent that can kill CSCs 100 times more effectively than the anti-cancer drug paclitaxel [9]. It reduced the $CD44^{high}CD24^{-/low}$ cell portion and decreased the tumorigenicity and lung metastases *in vivo* in breast cancer model [9]. Unfortunately, severe toxicity prevented the translation of salinomycin to the clinic for human cancer patients, however, this example presents another case of repurposing the existing drugs for anti-cancer therapy.

The search for the "old" drugs that can be repurposed for the new function of cancer treatment or prevention based on their anti-CSCs activity continues. For example, in a surprising finding upon screening a library of known compounds an antipsychotic drug thioridazine was shown to selectively target the neoplastic cells and impair the human

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somatic CSCs capable of *in vivo* leukemic disease initiation, while having no effect on normal blood stem cells [165].

These nutraceuticals and repurposed drugs that have shown anti-CSCs activity are summarized in Table 5. Like regular chemotherapeutic drugs many of them are hydrophobic and poorly soluble and/or have other problems after administration to the body such as poor absorption, rapid metabolism, fast systemic elimination and low plasma stability, which results in poor systemic bioavailability. Furthermore, as many of the anti-CSCs agents are "old" drugs that have been used for treatment of other diseases, potential side effects (for instance, neural and muscular toxicity of salinomycin [166], lactic acidosis of metformin [167] etc.) limits advancement of these agents to clinics.

2.6 CSCs niche

The niche is specialized microenvironment housing the stem cells that is essential for their life and function [180, 181]. The niche regulates stem cells' capacity to self-renew and differentiate and prevents stem cells from depletion [182]. It is a complex environment and includes various types of cells, such as immune and mesenchymal cells, extracellular matrix and vascular network. Extensive research implies that CSCs also reside in a specific niche that controls their differentiation, proliferation and self-renewal [73, 183, 184]. Notably, CSCs can also be generated by the microenvironment through induction of CSCs features in more differentiated tumor cells [185]. Therefore, CSCs niche represent a potential target for cancer therapy, although it is not sufficiently well studied and its role in cancer is still being debated [186].

2.7 CSC isolation and characterization methods

As discussed above CSCs are functionally and phenotypically distinct from other tumor cells. Based on these properties a number of assays have been developed to isolate/characterize the CSCs. This section describes some of the main methods used to study CSCs. The self-renewal and lineage capacity are fundamental properties of any stem cell. Thus, CSCs can also be identified by their ability to self-renew, as well as form and propagate tumors [144]. Therefore, the *first* functional approach used to study CSCs is serial transplantation into immunocompromised (typically NOD/SCID) mice. It was initially designed to study normal stem cells, specifically hematopoietic stem cells. In this assay the cells are orthotopically transplanted into immunocompromised mice and mice are observed for tumor formation over certain period of time. Moreover, the frequency of tumor initiating cells in the sample can be estimated using a series of limiting dilution transplantations [145, 146, 187]. For this purpose graded dilutions of cells are transplanted into mice, and the animals are further observed for tumor formation. The extent of the cell dilution should vary from sufficiently low to ensure that each mouse within a group develops tumors (positive response) to sufficiently high to ensure that the positive response is observed very rarely. The response can be quantified as a function of the dose of the transplanted cells. In this approach the tumor initiating cell frequency is determined from the semi-logarithmic plot of fraction of negative responses as a function of cell dose, and corresponds to the cell dilution, which produces 37% of negative responses. The analysis is carried out under assumption that only one tumor initiating cell is sufficient to produce the tumor and that other cancer

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cells are present in excess (for more detailed information and its limitations please refer to [146]). To evaluate self-renewal, the tumor cells are isolated and transplanted into new host animal. The ability to form serially transplantable tumors suggests a capacity for self-renewal while the number of serial transplants can serve as a measure of self-renewal potential [147]. This method is considered a "gold standard" to demonstrate two major properties of stem cells (i.e. the ability to recapitulate the original tumor and self-renew), but it also has some major disadvantages. On the one hand, orthotropic cell transplantation simulates the organ-of-origin microenvironment, but on the other hand, the effect of microenvironment is very complex and can significantly alter the results. Therefore, the strain of the mice used for the assay can strongly affect the result and tumor initiating cell frequencies can be drastically differ for different mice strains [72]. This approach has also some technical shortcomings and limitations. For instance, the use of Matrigel® for *in vivo* serial transplantations may affect tumor formation and thus alter the "apparent" frequency of tumor initiating cells. Additionally, preparation of viable single-cell suspensions can be challenging for solid tumors and may accidentally lead to a loss of the CSCs populations.

The second functional approach to CSCs isolation and characterization involves *in vitro* anchorage-independent growth and colony-forming unit (CFU) assay. This approach is considered a surrogate for *in vivo* transplantation and is fast, quantitative and high-throughput. Briefly, culturing CSCs under non-adhesive conditions (ultra-low attachment culture system) in serum-free medium supplemented with adequate growth factors and cultured can produce so-called "tumorspheres" [188]. Regular tumor cells in these conditions undergo "anoikis", a suspension-induced apoptosis, while CSCs survive and form three-dimensional tumorspheres on the one cell-one sphere (colony) basis. Moreover culturing in suspension encourages the display of the CSCs properties, and thus, the resulting tumorspheres contain higher portions of CSCs than the parental tumor cells [189]. Similar to *in vivo* limiting dilution assay, the frequency of tumor initiating cells, or colony-forming units, can be determined using *in vitro* limiting dilution. It should be noted however, that these *in vitro* and *in vivo* assays are not self-excluding, but rather complementary to each other. Low cost and high throughput capacity of the *in vitro* assay makes it very convenient for screening, but the results should be ultimately confirmed by a regular *in vivo* serial transplantation assay.

The third functional method for isolating CSCs is based on overexpression by these cells of drug efflux transporters, specifically BCRP. We have discussed above that Hoechst 33342 excluding side populations were shown to maintain CSCs properties in many types of cancers and currently this method is widely used to isolate CSCs [87, 148, 149].

The *phenotypic method* for CSCs isolation by flow cytometry using fluorescently labeled antibodies that target distinct phenotypes of CSCs. Over past several years numerous CSCs markers and their combinations have been identified in many cancers as described above. However, since the marker specificity varies from one type of cancer to another and even within the same tumor such markers may not be exclusive to CSCs, one should be very careful in using such markers for CSCs isolation and characterization. In this regard using a combination of two or more markers in CSCs analysis is always recommended. Moreover, any analysis using CSCs surface markers must be supplemented with one or several

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functional assays described above to validate the self-renewal and tumor initiating capacity of the cells with identified phenotype.

Finally, increased activity of cytoplasmic enzyme aldehyde dehydrogenase-1 (ALDH-1) has been reported to be characteristic for CSCs in many cancers [150, 151, 190]. Based on this property an assay has been developed using ALDH substrate, which becomes highly fluorescent after enzymatic transformation. As a result, the ALDH-overexpressing cells can be identified and sorted by flow cytometry. However, similar to other phenotype-based sorting assays, the identified cells must to be functionally characterized to confirm their stem cell-like properties.

Overall, a good set of tools to study CSCs is currently available. Various *in vitro* assays must be used in combination and while they can be very useful in the beginning of the study due to low cost and efficiency, the results have to be carefully validated *in vivo*.

2.8 Summary of molecular and cellular targets for CSCs therapeutics development

As mentioned before, many researchers agree that the truly curative therapies should target both CSCs and non-CSCs [1, 49]. A lot of effort has recently been put towards developing anti-CSCs therapies. There are several drugs in clinical trials, and many more in preclinical development that are designed to specifically eliminate or suppress CSCs. The possible therapeutic approaches to kill CSCs are summarized in Fig. 5. Briefly, the use of the CSCs surface markers could be used both to "recognize" CSCs as well as deliver therapeutic agents to CSCs rather selectively. Abolishing drug efflux functionality using either low molecular mass inhibitors of drug efflux pumps or siRNA to downregulate MDR in CSCs can increase efficacy of conventional therapies using drug-efflux dependent therapeutic agents. Targeting abnormal metabolism in CSCs may deplete energy in these cells and suppress their function, by impairing their growth, inducing differentiation and, finally, sensitizing them to the therapy. Inhibiting embryonic signaling pathways in CSCs can also affect the life cycle of the CSCs and inhibit their tumorigenic potential. Last but not the least, impairing the CSCs niche might deprive CSCs of an essential microenvironment that controls their differentiation, proliferation and self-renewal. These approaches are in different stages of development. Novel investigational agents targeting the Wnt, Notch, and Hh pathways are currently in late preclinical development stages, and some of them are in early phase 1–2 clinical studies. Other approaches like using drug efflux pump inhibitors in combination with chemotherapeutic agents have been evaluated rather extensively with relatively little success. The knowledge of CSCs and their niche microenvironments still remains a nascent field. But all strategies to treat CSCs have serious pitfalls associated with off target effects and high toxicity, poor bioavailability, poor cellular delivery, low efficacy and other problems that have hindered more conventional therapeutics. Some of these pitfalls can be addressed using drug delivery and nanomedicine approaches as discussed in the next section.

3. Nanomedicine-based therapies against CSCs

Despite being noted as one of the most important developments in the treatment of tumor relapse [191], nanocarrier-mediated drug delivery systems to overcome tumor drug

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resistance leave plenty of room for improvements, especially for targeting drug resistant CSCs. Nanomedicines offer a fundamental advantage over current therapeutic agents that are limited in use due to problems of degradation, solubility, rapid clearance from the body and poor cellular uptake. Nanomedicines commonly display longer circulation time, higher stability, greater bioavailability and activity. Modern nanocarriers can deliver more than one drug and/or imaging agent at the same time. Nanoparticles can passively accumulate at the tumor site due to enhanced permeability and retention (EPR) effect, where the drug can be released passively or by a triggered mechanism. The surface of the nanoparticles can be modified with targeting moieties for increased specificity and cellular uptake in target cells. Overall, nanomedicines can serve as high-capacity carriers for therapeutic drugs and genes or imaging agents. Additionally, nanoparticle itself can act as biological modulator or transducer of energy to the cell surrounding, which is used for example in magnetic hyperthermia to kill cancer cells as well as in some other approaches.

3.1 Application of nanocarriers to deliver chemotherapeutic agents to CSCs

One major application of nanocarriers is to deliver “non-druggable” molecules or existing drugs having serious problems, such as poor solubility, stability and/or high toxicity. This application can be useful for qualifying therapeutic agents and drug candidates that aim to eliminate CSCs. For instance, an antibiotic salinomycin discussed above (Table 5) has shown high potency in killing CSCs, but displayed poor aqueous solubility and severe toxicity that hinders its clinical application. Incorporating salinomycin in nanocarrier can address these problems. For example, Wei and colleagues conjugated salinomycin to a hyaluronic acid-based nanogel to target CD44-positive drug resistant cells [41]. CD44 receptor is expressed at the surface of many cancer cells and it binds hyaluronic acid, which allows to target such nanogels to CSCs [192]. This approach enhanced the bioavailability, delivery and cytotoxic activity of salinomycin in both drug resistant cancer cell culture and multicellular spheroids.

Nanocarriers were also used to deliver therapeutic agents that affect embryonic signaling pathways in CSCs. As discussed above a number of these agents have been evaluated clinically (Table 4), however, in certain cases their use was hindered by poor solubility, off-target drug delivery and severe side effects. Recently Zhou and coauthors described a N-(2-hydroxypropyl)methacrylamide (HPMA) conjugate of cyclopamine, a Hh pathway inhibitor, that efficiently eliminated CD133+ cells within a prostate cancer epithelial cell line RC-92a/hTERT [44]. Conjugation to HPMA improved the solubility of the drug and possibly decreased its systemic toxicity. In another study Chenna and colleagues used poly(D,L-lactide-co-glycolide) (PLGA)-PEG nanoparticles to encapsulate HPI-1, a potent antagonist of Hh transcription factor [193]. HPI-1 has shown promise in inhibition of Hh pathway *in vitro*, however, *in vivo* translation of this agents was hindered due to its highly lipophilic nature and poor aqueous solubility. The nanofomulated HPI-1 (NanoHHI) had significantly improved the systemic bioavailability compared to HPI-1 alone and showed strong antitumor activity *in vivo*. NanoHHI also decreased the population of CD133+ cancer cells and in combination with gemcitabine significantly impeded the growth of orthotopic pancreatic and hepatocellular cancer xenografts [42].

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One important property of nanocarriers is possibility to simultaneously incorporate multiple therapeutic agents. For example, polymeric micelles of stearate-grafted chitosan oligosaccharide were used for co-delivery of paclitaxel and doxorubicin [194]. Another study employed PLGA nanoparticles for simultaneous delivery of vincristine and verapamil [195]. A liposomal delivery formulation for quercetin and vincristine was also developed [196]. Additionally, Kwon et al. reported that PEG-block-poly(D,L-lactic acid) (PEG-b-PLA) polymeric micelles can deliver multiple drugs including combinations of paclitaxel and 17-allylamino-17-demethoxygeldanamycin (17-AAG), etoposide and 17-AAG, docetaxel and 17-AAG as well as paclitaxel, etoposide and 17-AAG [197]. Most recently Han et al. described several synergistic drug combinations in very high capacity (nearly 50 % wt/wt) poly(2-oxazoline) polymeric micelles [198]. Such multidrug combinations can act upon multiple signaling pathways in cancer cells resulting in synergistic anticancer effect and increased efficacy of the cancer treatment.

Likewise, the nanocarriers that combine MDR modulators and cytotoxic drugs could simultaneously sensitize CSCs and deliver their chemotherapeutic payloads. The use of such dual load nanocarriers may contribute to anti-CSCs therapy in future clinical trials [63]. Moreover, incorporating several MDR modulators within one nanocarrier can allow for broad-spectrum inhibition of drug efflux transporters in CSCs. One can also use antibodies against the drug efflux transporters to inhibit drug efflux and increase drug accumulation in the CSCs [113]. For example, Yang and colleagues employed combination therapy of anti-ABCG2 mAb and paclitaxel loaded iron oxide magnetic nanoparticles, coated with Pluronic F68 against CSCs in multiple myeloma [113]. The combination therapy led to significant reduction of tumor growth *in vivo* and was shown to be more effective than either the paclitaxel, iron oxide nanoparticles or anti-ABCG2 mAb. The synergistic effect can be explained by simultaneous inhibition of ABC transporter by antibodies and delivery of paclitaxel to the CSCs via magnetic nanoparticles.

3.2 Application of nanocarriers to deliver nucleic acid therapeutics to CSCs

Aside from solubilization of poorly soluble drugs nanocarriers can increase stability and cell transport of biomacromolecules, in particular, nucleic acid therapeutics that have shown some promise for treatment of cancer [199]. Recent studies have uncovered the regulatory role of microRNAs (miRNA) in CSCs [200] and provided rationale for using miRNAs both as diagnostic and prognostic biomarkers of tumor progression and anti-cancer agents. For example, recent findings suggest that miRNA-34a interferes with the viability of CSCs, creates a barrier to metastasis, and abolishes chemoresistance by directly repressing CD44 in pancreatic [201], gastric [202], prostate [203] and brain [204] cancers. However, miRNA-based therapy is hampered by several challenges including lack of tissue specificity, poor cellular uptake and risk of systemic toxicity [205]. Nanocarrier-based miRNA delivery systems have shown clear advantages in CSCs. Thus, Shi et al. described solid lipid nanoparticles for delivery of CD44 protein repressor miRNA-34a into CD44+ lung cancer cells [206]. This nanocarrier not only protected miRNA from degradation, but also enhanced the miRNA accumulation in lungs. In May 2013 Mirna Therapeutics, Inc. announced that the first miRNA clinical study had been initiated in patients with unresectable primary liver cancer or metastatic cancer with liver involvement (NCT01829971). In this trial MRX34, a

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liposome-formulated mimic of the tumor suppressor miRNA-34 is given to patients intravenously two times per week for three weeks. In the pre-clinical studies, miRNA-34 mimic has shown the ability to induce the cell cycle arrest, senescence and apoptosis. Recently, this agent was also shown to interfere with the viability of CSCs, decrease metastases formation, and abolish chemoresistance [203].

Another interesting example is using nanocarriers for siRNA delivery to silence genes encoding the drug efflux transporters and thereby sensitize these CSCs to chemotherapeutic drugs. For instance, to increase stability and delivery of MDR1 silencing siRNA in MDR1-expressing human colon CD133+ CSCs, Liu et al. used cross-linked PEI-Lipid$_{1:16}$ nanoparticles [207]. The nanoformulated siRNA effectively reduced the expression of MDR1 in CSCs, and chemosensitized these cells to paclitaxel.

Altogether, nanocarriers are of considerable interest for delivery of siRNA and other nucleic acid based therapeutics in CSCs. However, so far much of the siRNA therapeutics using nanocarriers (mostly lipid based nanoparticles) are limited to delivery of siRNA to the liver as it is one organ where the systemic delivery has proven to be relatively easy. It remains to be seen whether the nanocarrier technology can target siRNA to distal CSCs located in other organs.

3.3 Targeting of nanocarriers to CSCs

In addition to small molecule drugs and nucleic acid-based therapeutics, antibodies have also shown potential for eliminating CSCs. Antibodies have long been used in clinical practice to target cancer cells and treat cancer [208, 209]. Most popular targets include human epidermal growth factor receptor 1 (EGFR1, HER1), human epidermal growth factor receptor 2 (EGFR2, HER2, Neu, ErbB-2, CD340, p185), membrane-spanning 4-domain subfamily A member1 (CD20), vascular endothelial cell growth factor (VEGF) and Interleukin-6 (IL-6). These target antigens are certainly important for cancer development and progression, but search for new targets is still ongoing. Some of the approved and new anti-cancer mAb and antibody constructs have shown efficacy against CSCs and induced tumor regression in clinical trials as discussed above (Table 4).

Another application of antibodies is targeting of the therapeutic agents to CSCs. Antibody-drug conjugates and antibody-modified nanocarriers have been explored for selective targeting of CSCs to develop anti-CSCs therapeutics [208, 210]. Few attempts have been made to modify the surface of nanocarriers with mAb. Since CSCs express specific cell surface biomarkers it was natural to employ antibodies against these biomarkers for targeted drug delivery. For example, Swaminathan et al. developed paclitaxel-loaded polymeric PLGA nanoparticles conjugated with anti-CD133 mAb [40]. Although, the accumulation of the targeted nanoparticles in the CD133 overexpressing Caco2 cells was 9 times higher than that of non-targeted nanoparticles, *in vitro* tumorsphere and soft agar colony formation assays in MCF7 cells has shown their similar activity. (Notably, the paclitaxel alone increased the number of colonies formed in soft agar to 122% of untreated control.) Nevertheless, the targeted paclitaxel-loaded nanoparticles have induced 70% tumor volume reduction compared to untreated control in *in vivo* MDA-MB 231 xenograft tumor model,

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whereas the non-targeted nanoparticles or paclitaxel in Cremophor EL®/ethanol solution resulted in 43% and 33% tumor inhibition respectively [40].

One common advantage of nanocarriers is possibility of incorporating multiple modalities in one carrier system, allowing for delivery of either multiple therapeutic agents or imaging and therapeutic agents at the same time. For example, Gao et al. have developed a bi-functional nanomedicine for *in vivo* imaging and therapy of cancer that targeted prostate stem cell antigen (PSCA), a cell surface antigen expressed in the CSCs. This nanomedicine comprised PLGA nanoparticles loaded with superparamagnetic iron oxide nanoparticles (SPIONs) and docetaxel. The surface of these nanoparticles was modified with the anti-PSCA mAb. The targeted nanoparticles provided a negative MRI contrast enhancement and effective tumor inhibition *in vivo* [211].

Despite these and some other examples all mAb-based nanomedicines targeting CSCs are in the early preclinical development stages. One common limitation of such approaches using mAb is potential toxicity to normal stem cells in the body. Preclinical studies used mAb against human antigens that may not cross-react with some murine antigens. This could mask potential systemic toxicity and other side effects in the mouse models. Moreover, the antibodies used in preclinical and clinical studies must be very well characterized. For instance, the use of different CD133 antibody clones that recognize different CD133 splice variants and epitopes of different glycosylation status, has led to inconsistent results [212].

In addition to mAb, non-immunogenic synthetic “antibodies”, aptamers, are composed of oligonucleotides or peptides, have shown potential for development of targeted CSCs therapeutics and molecular imaging systems. For example, a CD133 specific aptamer 25–32 times smaller than mAb has shown superior tumor penetration and retention compared to anti-CD133 antibodies in a 3-D tumorsphere model [213]. Due to their chemical versatility aptamers can be easily conjugated to nanoparticles comprising diagnostic and/or therapeutic agents.

However, independently of the targeting moiety one fundamental limitation of the CSCs targeted nanocarriers could be poor extravasation and exposure of the circulating nanocarriers to their targets. It has been shown that CSCs can localize in the necrotic areas of the tumors, which is particularly hard to reach [214]. Therefore, the targeted nanocarrier approaches may be more suitable to treat cancers where the CSCs are more readily accessible, such as hematological malignancies.

3.4 Using nanoparticles to target metabolism in CSCs

Targeting metabolism in drug resistant cancers and CSCs has proven to be a challenging task. For example, CSCs in various cancers, including breast [215, 216], leukemia [123], gastrointestinal [217] and brain [218], are characterized by relatively low levels of ROS, which in combination with higher DNA repair results in radioresistance of CSCs. The lower levels of ROS are attributed to the higher content of antioxidant ROS-scavenging compounds [217, 219]. Also, CSCs are often localized in the hypoxic areas of the tumors. Thus, it is suggested that by enhancing tumor oxygenation it is possible to make CSCs more susceptible to current treatments. For example, hyperthermia is used to promote tumor

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reoxygenation [220–223], radiosensitization [224] and heat shock [225, 226] in cancer cells. However, current clinical implementations of hyperthermia have been limited due to the nonspecific heating to the normal tissues and organs and consequent treatment-limiting side effects [227, 228].

Several approaches using nanomedicines to enhance ROS generation and induce morphological and functional changes and enhancement of apoptosis and necrosis in CSCs have been investigated. In one recent example the authors designed the mitochondria targeting PEGylated liposomes incorporating anticancer drug, daunorubicin and mitochondrial regulator, quinacrine [229]. The liposomes displayed long circulatory effect *in vivo* and selectively accumulated within the tumor sites presumably due to the EPR effect. The mitochondrial regulator dequalinium was attached to the liposome surface to obtain the mitochondrial targeting effect. These targeted liposomes selectively accumulated into mitochondria which induced activation of the pro-apoptotic Bax protein, depleted the mitochondrial membrane potential, opened the mitochondrial permeability transition pores, released cytochrome C, and initiated a cascade of caspase 9 and 3 reactions. These effects ultimately led to apoptosis in of MCF-7 CSCs.

Other studies have focused on killing CSCs by delivering in them the energy dissipating magnetic nanoparticles or carbon nanotubes that are responsive to external physical fields. For example, Sadhukha and colleagues discovered that the magnetic hyperthermia transduced by SPION in the alternating current (AC) magnetic field can reduce CSCs population [45]. They have shown that SPION-transduced hyperthermia decreases side population cells, ADLH expression, tumorsphere formation, and *in vivo* xenotransplantation, suggesting that magnetic hyperthermia reduced or, even eliminated the CSCs in treated A549 and MDA-MB 231 breast cancer cells. Notably, ROS generation was increased upon magnetic hyperthermia proportional to the time of the magnetic field exposure. At the same time, conventional hyperthermia induced by placing the cells in the warm (46°C) water bath did not affect the CSCs and did not induce ROS production. Interestingly ROS generation was observed upon conventional hyperthermia in the cancer cells that were first incubated with magnetic nanoparticles. The authors speculated that magnetic hyperthermia has potential for lower rates of tumor recurrence compared to conventional cancer therapies.

In an earlier study by Rinaldi et al. [230] the SPION conjugated to epidermal growth factor (EGF) were shown to target EGFR in the breast cancer cells, resulting in delivery of these nanoparticles to lysosomes. Upon application of the AC magnetic field the EGF-targeted SPION disrupted the lysosomal membranes, induced production of ROS and killed the EGFR-overexpressing cancer cells but not the cancer cells with lower level of EGFR expression [231]. Interestingly, the non-targeted SPION did not produce such strong effects in the cancer cells. Needless to say, the nature of the cytotoxic effects of the magnetic nanoparticles in the field is not fully understood. On one hand, it could be induced by local dissipation of heat (local heating) in the vicinity of the SPION surface, which results in protein denaturing and disruption cell membranes, especially in the case of the EGF-targeted nanoparticles. On the other hand, some authors suggested that there could be induced mechanical effect due to realignment of the magnetic nanoparticles along the field, which

can disrupt or denature proteins and structures joining the nanoparticles and thereby affect the cell signaling [232, 233].

Using triple-negative breast cancer model, Burke et al. recently demonstrated that breast CSCs are markedly resistant to traditional hyperthermia and become enriched in the surviving cell population following the treatment [234]. The mechanism may be related to the elevated expression levels of heat shock protein 90 (HSP90) in breast CSCs. In contrast, breast CSCs appeared to be sensitive to PEG coated multi-walled carbon nanotube-mediated (MWCNT) thermal treatment stimulated by near infrared irradiation, and lost their long-term proliferative capacity. Moreover, MWCNT-mediated hyperthermia promoted complete tumor regression and long-term survival of mice with CSCs-driven breast tumors. However, non-CSCs displayed same sensitivity to the MWCNT-mediated hyperthermia as the CSCs. It appeared that in contrast to SPION-induced hyperthermia, which induced ROS production in cancer cells, MWCNT-mediated hyperthermia induced critical plasma membrane damage, which is not selective to a specific cell phenotype. Altogether the study of the effects of electromagnetic field-responsive nanoparticles on CSCs is in its infancy but the existing examples suggest that such effects may be of interest for developing novel anti-CSCs therapies.

3.5 Pluronic block copolymers as multifaceted sensitizers of MDR and CSCs

Pluronic block copolymers (also known as “poloxamers”) represent a class of polymers with triblock structure consisting of hydrophobic poly(propylene oxide) (PPO) block, flanked from both sides with hydrophilic poly(ethylene oxide) (PEO, same as PEG) blocks (PEO-PPO-PEO) (Fig. 6A). Pluronic micelles carrying solubilized drugs were the first polymeric micelles reported as nanocarriers for drug delivery in animal models [235] and the first-in-class polymeric micelle drug, which reached the clinical stage in human patients [236]. In addition to the drug carrier function of the polymeric micelles, Pluronic molecules alone have unique ability to chemosensitize MDR tumors by 1) inhibiting ABC transporter–mediated drug efflux [237, 238], 2) inhibiting mitochondrial respiration and ATP synthesis in MDR cells [239, 240], and 3) enhancing pro-apoptotic signaling in the MDR cells in response to treatment with drug [17, 241] (Fig. 6B). The Phase II human clinical trial of doxorubicin incorporated into mixed micelles of Pluronic F127 and L61 (SP1049C) has shown high objective response rates (43%) and increased median survival (10 months) in patients with inoperable metastatic adenocarcinoma of the esophagus and gastro esophageal junction [242].

Treatment of MDR cells with doxorubicin/Pluronic formulation significantly enhanced the pro-apoptotic signaling compared to the drug alone and inhibited the antiapoptotic defense mechanisms *in vitro* [241]. Similar effects were observed *in vivo* [238]. It was demonstrated that doxorubicin/Pluronic treatment of tumor-bearing mice significantly increased levels of caspases 8 and 9 compared to doxorubicin alone. Additionally, depletion of major intrinsic cellular antioxidant GSH would increase cell sensitivity to the ROS. Therefore when combined with doxorubicin Pluronic not only drastically increases the drug accumulation in the cells, but also strongly induces early, as well as late stages of proapoptotic signaling in

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MDR cells *in vitro* and *in vivo*, which in combination with the doxorubicin cytotoxic effect results in significantly increased cell death.

One of the most important biological effects of Pluronic block copolymers is ATP depletion in tumor cells. The copolymers that induce the strongest ATP depletion (e.g. Pluronic P85 and Pluronic L61) were also shown to be the most potent chemosensitizers of MDR cells [243]. There is a clear correlation between the ATP depletion and chemosensitization effect of Pluronic [244]. Moreover, both *in vitro* and *in vivo* tumor models the remarkable ATP depletion effect of Pluronic was selective for MDR phenotype, while non-MDR cells were not as much responsive [238]. This is supported by previous findings that modulation of energy metabolism in drug resistant cells increases drug accumulation [245]. The reason for Pluronic selectivity in MDR cells is uncertain, but may be in part due to differences in energy metabolism in drug sensitive and drug resistant cells [239].

Our previous studies have shown that Pluronic reaches mitochondria and inhibits complexes I and IV of mitochondria respiratory chain [239]. Furthermore, it stimulates the production of ROS and release of cytochrome c, which are the early signs of mitochondrial apoptotic pathway. Similar to ATP depletion and inhibition of respiration, Pluronic induced the ROS formation and cytochrome c release selectively in MDR cells, while non-MDR cells did not respond in that manner. In addition to induction of ROS production and cytochrome c release in MDR cells, Pluronic promotes drug-induced apoptosis [241]. Inhibition of mitochondria respiratory chain complexes is most likely the main reason for increased ROS production in MDR cells after treatment with Pluronic.

The ability of Pluronic block copolymers to sensitize MDR tumors have been used in many studies attempting to design a drug delivery system and overcome drug resistance in cancer. For example Pluronic P123/F127 mixed micelles were designed for delivery of paclitaxel to drug resistant tumors [246]. In this work the authors prepared multifunctional folate-modified Pluronic micelles to utilize the folate-mediated endocytosis for enhanced uptake of the micelles in combination with Pluronic ability to sensitize the MDR cells. They have shown that these micelles significantly increase the bioavailability of paclitaxel and display strong anti-tumor effect in drug resistant tumor xenograft model. More recently the same group demonstrated that paclitaxel-loaded Pluronic P123/F127 mixed micelles significantly enhanced the anti-cancer activity of paclitaxel in lung cancer resistance protein (LRP) overexpressing human lung adenocarcinoma *in vivo* [247].

In another study the mixed micelles of methoxyPEG–poly(lactide) block copolymer (MPEG–PLA or MPP) and Pluronic (L61, L62 and P85) were prepared to enhance the stability of Pluronic micelles upon dilution in blood stream for delivery of docetaxel to MDR tumors [248]. Pluronic significantly affected the drug loading, drug release, and pharmacokinetic profile of these micelles, which resulted in more effective inhibition of tumor growth and overcoming the MDR by the mixed micelles.

Pluronic block copolymers have been also used in design of drugs for oral administration as it has been shown that selected copolymers can greatly increase permeability of multiple drugs in intestinal cells by inhibiting the drug efflux transport systems [249]. For example

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Hosseinzadeh and co-authors have designed and characterized chitosan-Pluronic nanoparticles for oral delivery of gemcitabine [250]. These nanoparticles have shown high loading efficiency and good mucoadhesion properties that can be used to treat colon cancer [250]. Moreover, it was demonstrated in another study that Pluronic P123 effectively inhibited the Pgp efflux in intestine but had no effect on intestinal CYP450 metabolism. This suggested that the copolymer can improve oral bioavailability of Pgp substrates without interfering with the metabolism of the drug [251].

We have shown recently that treatment of the mice bearing the leukemia ascitic cells with SP1049C reduces the tumor aggressiveness, *in vivo* tumor formation frequency and *in vitro* clonogenic potential of the leukemia cells derived from the treated animals [252]. SP1049C also prevented the overexpression of BCRP and activation of Wnt/β-catenin signaling observed with doxorubicin alone, significantly altered the DNA methylation profiles of the cells and decreased CD133+ cells populations, which displayed CSCs-like properties and were more tumorigenic compared to CD133− cells [252]. Moreover in the same *in vivo* leukemia ascitic cells model, the treatment of animals with Pluronic P85 and doxorubicin prevented the development of MDR observed upon long-term treatment of the animals with doxorubicin alone [253]. The cell culture studies also demonstrated that Pluronic P85 inhibits development of MDR in leukemia cells [253] and breast cancer cells [254] induced by exposure of these cells to the increased concentrations of doxorubicin. This suggests that Pluronic-based drug formulations may have potential in eliminating CSCs, thereby providing rationale for evaluating SP1049C as an anti-CSCs agent in the clinical trials.

Pluronic block copolymers were also shown to enhance the radiofrequency ablation induced hyperthermia in solid tumors and sensitize cancer cells to hyperthermia *in vitro* and *in vivo* [255]. Consistent with the prior finding of the structure-functional relationships in MDR cancer cells [243] the copolymers with lower molecular weights and hydrophilic-lipophilic balance (HLB) < 8 demonstrated the highest thermosensitizing activity [256]. Notably, in these Pluronics the number of PO units was even multiple of the length of 16 C-C bond length, which is a typical length of mammalian fatty acid acyl chain. Therefore the thermosensitization ability of Pluronic copolymer may be directly linked to its ability to interact with plasma membrane and induce membrane fluidzation. It has been shown that the more fluid the cell membrane becomes upon hyperthermia, the higher the cell sensitivity to the treatment is [257]. Furthermore, in another study it was shown that Pluronic in combination with low-grade hyperthermia depleted the intracellular ATP and decreased the expression of heat shock protein 70 (HSP70) [258]. It is known that heat shock proteins, in particular HSP70, increases the thermoresistance of the tumors [259]. Notably, the reduction of HSP70 expression by Pluronic L61 was observed both *in vitro* and *in vivo* [258]. Overall, while having relatively low inherent toxicity Pluronic block copolymers display unique effects on cancer cells and act as potent chemo- and thermosensitizers, which have shown clear potential to enhance anti-CSCs therapeutics.

4. Generalizations and future directions

The overview of the current state of the art suggests that there is a clear rationale and promise in application of the nanomedicines and drug delivery technologies to develop

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novel anti-CSCs therapies. Among all different kinds of drug delivery systems, polymer-based nanomaterials and nanocarriers are of special interest. They include polymeric micelles, polyelectrolyte complexes with DNA, RNA, proteins and peptides, soft polymer nanogels, solid polymer nanoparticles, polymer coated inorganic materials (including SPION), polymer coated liposomes and others. Properly designed polymeric nanoparticles generally have narrow size distribution, good batch to batch reproducibility and controllable drug-release profiles [260]. Furthermore, the polymer-based nanocarriers also offer great synthetic versatility that allows particles to be tailored for specific needs including modification of the particle surface with targeting moieties for site-specific delivery to cancer cells or tumor vasculature [260]. However, application of nanomedicine technologies in anti-CSCs therapies is in a relatively early stage and along with the great promise of these technologies many issues remain unresolved or unknown. While selected nanomedicines have been approved clinically and more nanomedicine-based therapeutics are in human clinical trials, to advance this technology for anti-CSCs clinical therapies more basic and applied research is needed.

First of all, as is true for all nanoparticles that are designed to reach their target cells in the organism, nanomedicines targeting CSCs need to penetrate to the sites where their target cells are located. Indeed, poor extravasation of macromolecular objects with sizes exceeding 5–10 nm within interstitial spaces severely limits the ability of these objects to reach their cellular targets within a normal tissue or tumor [261]. Hence, targeting of nanoparticles may be restricted mainly to those sites readily accessible to the circulatory system, such as blood components, endothelial cells, sub-endothelial structures, etc. Despite expectations associated with the EPR effect in cancer, it is possible and even likely that in many cases this effect will not be sufficient to reach CSCs, especially those cells located in the necrotic sites of the tumors, or otherwise deeply embedded in their niche. This may be a paramount limitation in cases when the nanoparticles need to come in contact and internalize in the CSCs, such as approaches using energy dissipation from external field into the cells through magnetic nanoparticles or carbon nanotubes, intracellular delivery of siRNA or other similar technologies.

Another, set of problems arises in the cases when there is an intention to target nanoparticles to the surface of the CSCs using antibodies or other targeting moieties. The concept of targeted delivery of polymeric drug carriers to disease sites in the body is very popular and on a surface simple. Unfortunately, it was rarely successfully realized in relevant animal models and, to the best of our knowledge, never taken to clinic. We already discussed above the difficulties in developing antibodies or other ligand groups that can selectively recognize and target CSCs but not the normal stem cells or other off-target cells. Identifying good surface markers is always an uphill battle as very few if any as such markers are available that exhibit sufficient selectivity in the body. Moreover, as also already noted the targeted nanomedicines must be able to reach the surfaces of the CSCs to interact with these cells through the targeting moieties, and this is not always possible. Some nanomedicines display elevated circulation times, which may to some extent mitigate the effect of their poor extravasation into tissues due to increased exposure of the therapeutics in the body. However, existing examples such as PEGylated liposomes also suggest that increased circulation times may come at a cost of deposition of liposomes to skin resulting in skin

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toxicity effects [261]. In addition, an increase in the "cargo space" of the nanocarrier is usually associated with an increase in the volume and surface area of this carrier. As a result, nonspecific surface interactions of the nanocarriers with biological objects in the body (cell surfaces, biomacromolecules, etc.) may elevate to the extent that they become comparable with the specific binding of the targeting moieties with the receptors. At this point the advantages of specific targeting may drop due to nonspecific binding of carriers with cells and tissues, such as monocytes or macrophages. One obvious remedy could be, firstly, to modify the surface of the carrier with "inert" polymer that would have minimal interactions with the cells, and, secondly, to attach one or multiple targeting moieties at the inert layer to allow for specific binding of the targeting moieties with their receptor. Some soft materials like water-swollen cross-linked negatively charged block polyelectrolyte nanogels were recently described that have very little if any detectable nonspecific binding to surfaces, yet exhibit very good accumulation in tumors after surface modification with ligands that bind to tumor specific-receptors [262, 263]. The problem, however, is in appropriate design of an "inert" polymer shell that could minimize interactions of diverse nanomaterials with non-targeted cells. The current "gold standard" of inert polymers is PEG but there are clear indications that even this polymer attached to nanomaterials can activate complement, and otherwise engage the body immunity [15].

Another "out-of-the-box" approach to site-specific delivery of nanomedicines that was developed recently consists in engaging antigen presenting cells to design better therapies [264]. It has long been known that macrophages and microglia as well as other mononuclear phagocytes can entrap colloidal nanomaterials as exhibition of natural protective response aimed to eliminate alien bodies from the organism. Hence, the goal of drug delivery was to avoid or minimize such interactions. In contrast, the recent works proposed to use immunocytes as carriers for nanoformulated drugs. In such approach, the drug nanoparticles are engineered to maximize entrapment into the circulating immunocytes. These cells are known to migrate to sites tissue injury, infection, or disease, where they can deliver and release the encapsulated drug. The proof-of-concept was shown using nanosuspensions of antiretroviral drugs [265], magnetic nanoparticles [266] polyion complex micelles incorporating enzymes ("nanozymes") [267] and even plasmid DNA [268]. Moreover, our studies have shown that such cells can efficiently transfer incorporated in them nanoparticles [269] or gene expression vectors [268] to other cells in the sites of inflammation and disease. A possibility of targeting nanoparticles to macrophages for example through the folate receptors with a subsequent goal of using these macrophages as "Trojan horses" to deliver their payloads to disease sites has also been demonstrated [270]. Although this approach has not been used so far for delivery of anticancer and anti-CSCs therapeutics, it is seems to be promising since macrophages often comprise the CSCs niche.

The use of nanocarriers for multifaceted and combination anti-CSCs therapies is clearly in the very beginning. Although as discussed above the conventional inhibitors of drug efflux transporters after extensive work and clinical evaluation did not show sufficient promise, the search for novel chemosensitizers of drug resistant cells is currently underway. In this regard Pluronic block copolymers and in particular doxorubicin-Pluronic drug, SP1049C is of considerable interest, due to its multiple activities in killing and preventing development of drug resistant and metastatic cancers [18]. Notably, SP1049C is of the few so far one

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clinically evaluated nanomedicine that has also shown anti-CSCs activity. In some cases, nanocarriers with very high capacity for drug incorporation, such as for example poly(2-oxazoline) polymeric micelles, can be used to decrease carrier associated toxicities that are dose limiting for some conventional formulations such as Cremophor EL®/ethanol solution [271]. As a result the maximal tolerated dose of the reformulated nanocarrier may decrease allowing use for treatment high drug doses that may be more efficacious in eliminating CSCs. In other cases such carriers can allow simultaneously mixing different drugs in one carrier particle, and this in principle can open very broad possibilities in designing synergistic drug combinations that could affect interrelated cell signaling pathways in CSCs. Such "drug discovery by (nano) formulation" is in early stages of its development but it could have very far-reaching consequences. While, certain drug combinations might be more efficient with respect to regular non-CSCs, it could not be excluded that their effect on the CSCs might be different, for example antagonistic, and *vice versa*. Therefore, one should explore how various drug combinations affect not only regular cancer cell populations, but also CSCs.

Altogether CSCs provide an attractive target for therapy of cancers. However, targeting drug efflux transporters, gene silencing, affecting embryonic cells pathways and metabolism in CSCs alone will most likely not be able to provide the single "weapon", a "magic bullet" that will irradiate cancer. Comprehensive therapeutic agents affecting both CSCs and non-CSCs are needed. A successful therapy also requires a thorough understanding of cross-talk between CSCs characteristics and innate microenvironment and ability to intervene in functioning of this environment to derail the cancer initiation, progression, resistance, recurrence, and metastasis. In the near future, personalized therapy approaches for treating patients based on their CSCs characterization may hold great potential to increase the outcome of therapy and improve the quality of patients' lives. We certainly hope that nanomedicines will provide additional benefits for the personalized therapy and contribute to the existing range of therapeutic modalities to safe, extend and ease life of cancer patients.

5. Conclusions

Numerous drugs and treatment regimens have emerged since 1950s when chemotherapy was first applied to treat cancer patients. However in many cancers the treatment efficiencies of chemotherapy have rapidly leveled off. The current success in fight against cancer has been mostly accounted for the better screening and early detection methods rather to more effective treatments available. In this review we summarized the current views on CSCs models and described attempts to develop anti-CSCs therapies using conventional and nanotechnology-based therapeutics. Understanding tumor biology is critical for the development of successful therapies. Therefore, we focused on describing the major properties of CSCs as possible targets for therapy development. Development of a "magic bullet" that will eliminate CSCs within the tumor and therefore prevent the development of drug resistance, metastasis and relapse does sound appealing. However the complexity of the tumor organization as well as its (still) very limited understanding hinders the progress in this direction. It is also important to realize, that every cancer is different, every patient is different and even within the same patient primary and metastatic tumors are different and can respond differently to the treatments. Thus, discovery and development of the

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personalized combinational therapies might be a key to successful treatments. We believe that nanomedicine can provide unique advantages in development of such therapies.

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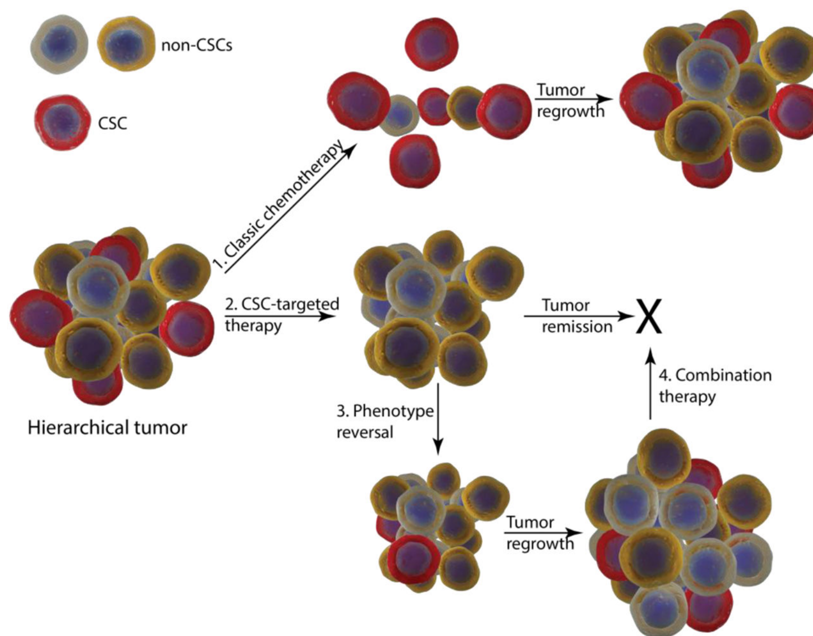

Fig. 1. Treatment of hierarchical tumors

(1) Treatment with most commonly used chemotherapeutic drugs (classic chemotherapy) often results in reduction in tumor volume, but drug-resistant CSCs can survive, repopulate the tumor and spread to distal sites ("tumor regrowth"). **(2)** Treatment of hierarchical cancers with therapeutic agents that target CSCs can kill CSCs and result in tumor remission. **(3)** According to the dynamic CSCs model differentiated cancer cells can reverse their phenotype and acquire CSCs properties ("phenotype reversal"). Therefore even if CSCs are selectively eliminated **(2)** the remaining cancer cells that reverse CSC phenotype will result in the tumor regrowth. **(4)** Combination treatment with the CSCs targeting drugs and conventional chemotherapies that reduce both CSCs and stromal cells may be an ideal regimen to achieve durable response.

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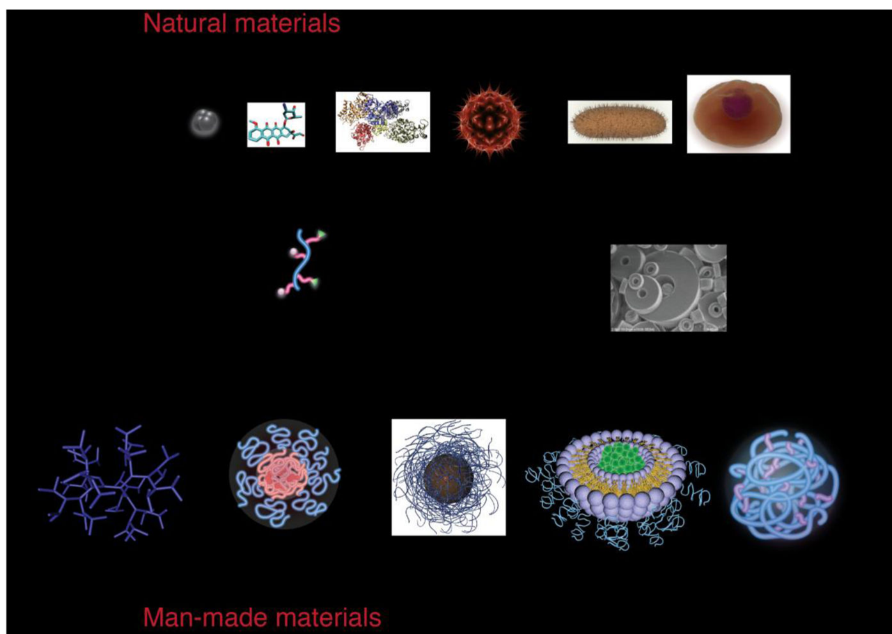

Fig. 2. Schematic showing the main nanoparticles and microparticles investigated in the drug delivery applications

Microphotograph insert presents images of PRINT® (“Particle Replication In Non-Wetting Templates”) microparticles from the laboratory of Prof. DeSimone at the University of North Carolina at Chapel Hill provided by his graduate students T. Shen and C. Fromen.

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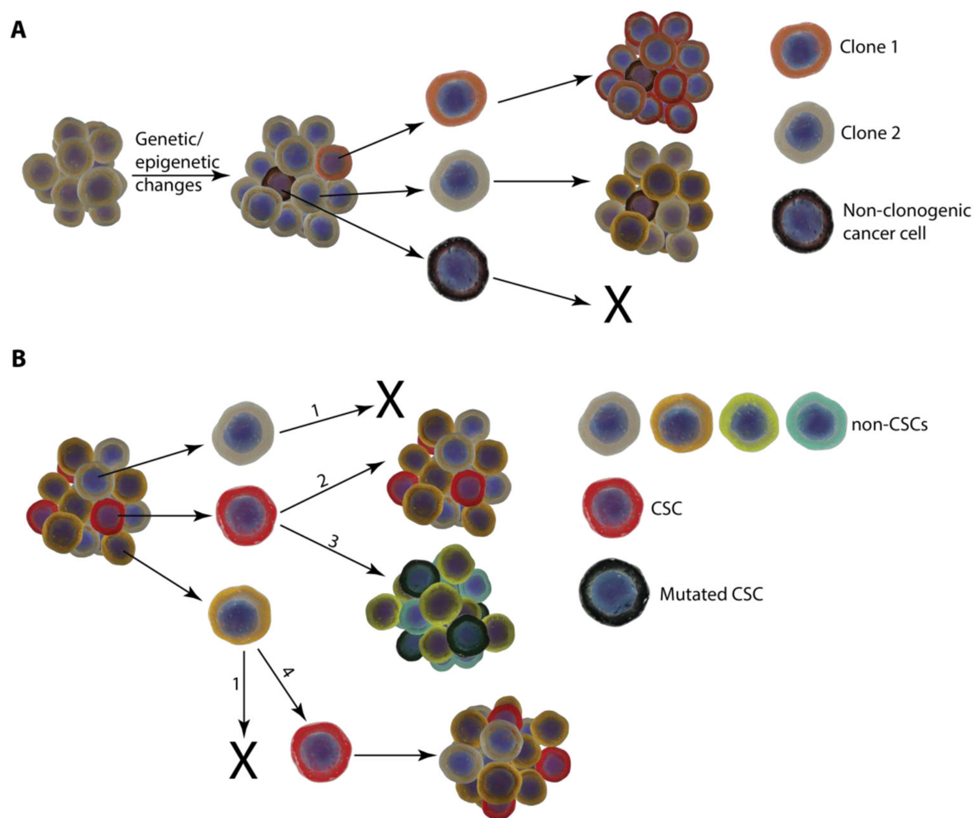

Fig. 3. Clonal evolution model (A) vs. CSC model (B)

(A) Clonal evolution model is a non-hierarchical model where stochastic genetic and/or epigenetic changes confer growth advantage as well as heritable phenotypic and functional differences. Clones can be different in their tumorigenicity and will produce cells with similar tumorigenic potential (e.g., Clone 1 and Clone 2). However some cells may lose their tumorigenic capacity due to non-favorable genetic/epigenetic changes or microenvironment (non-clonogenic cancer cells). **(B)** CSCs model is a hierarchical model: **(1)** non tumorigenic cancer cell cannot generate new tumor; **(2)** CSCs have the ability to generate a tumor, based on its self-renewal and tumorigenic properties; **(3)** clonal evolution in CSCs due to genetic and/or epigenetic changes in CSCs leads to expansion of CSCs pool, higher heterogeneity and possibly development of drug resistance; **(4)** recent studies demonstrated that CSCs phenotype is dynamic and non-stem cells may acquire CSCs properties (phenotype reversal), which eventually results in tumor recurrence.

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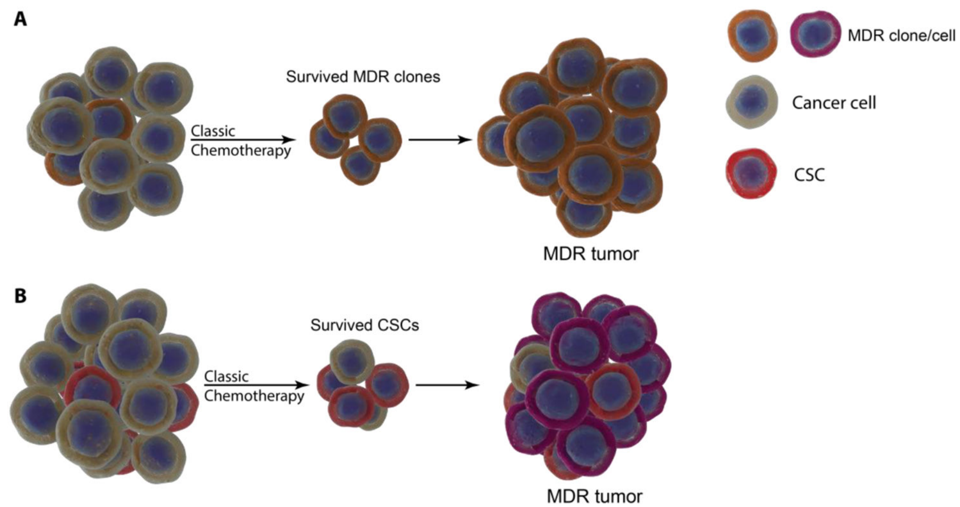

Fig. 4. Models of MDR development

(**A**) Conventional model: rare cells in the tumor have accumulated significant genetic and/or epigenetic alterations that confer MDR. These cells will survive the chemotherapy and proliferate in MDR tumor. **(B)** CSCs model: tumor contains small population of CSCs and their differentiated progenies. The chemotherapy eliminates the differentiated cells, while the MDR CSCs survive and produce drug resistant heterogeneous tumor, consisting of CSCs and various differentiated (drug resistant) cells.

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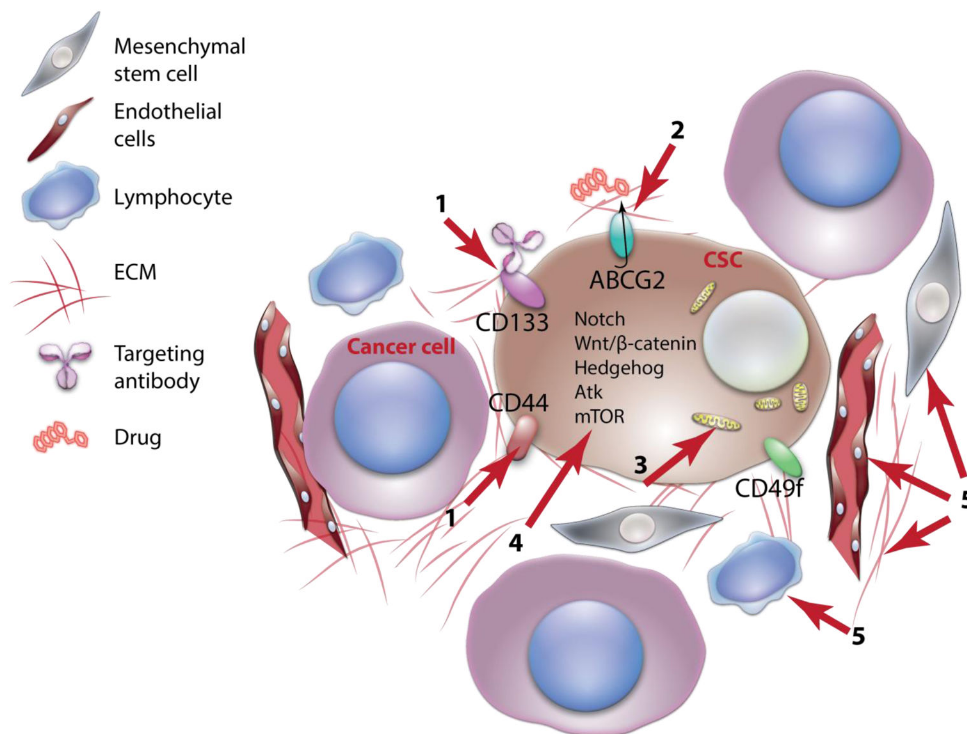

Fig. 5. Possible therapeutic strategies that can eliminate CSCs

(1) Targeting cell surface proteins (for example, CD133, CD44, et al.) to develop site-specific therapeutics against CSCs. **(2)** Inhibiting the drug efflux transporters to sensitize drug resistant CSCs to chemotherapeutic agents. **(3)** Altering CSCs metabolism by “reprogramming” it to “normal” status to impair growth of CSCs, induce differentiation of CSCs and, finally, sensitize CSCs to the therapy. **(4)** Inhibiting cell signaling pathways that are critical for the CSCs survival and proliferation to develop anti-CSCs therapeutic agents. **(5)** Affecting the vascular niche of the CSCs to impair the specialized microenvironment housing the CSCs.

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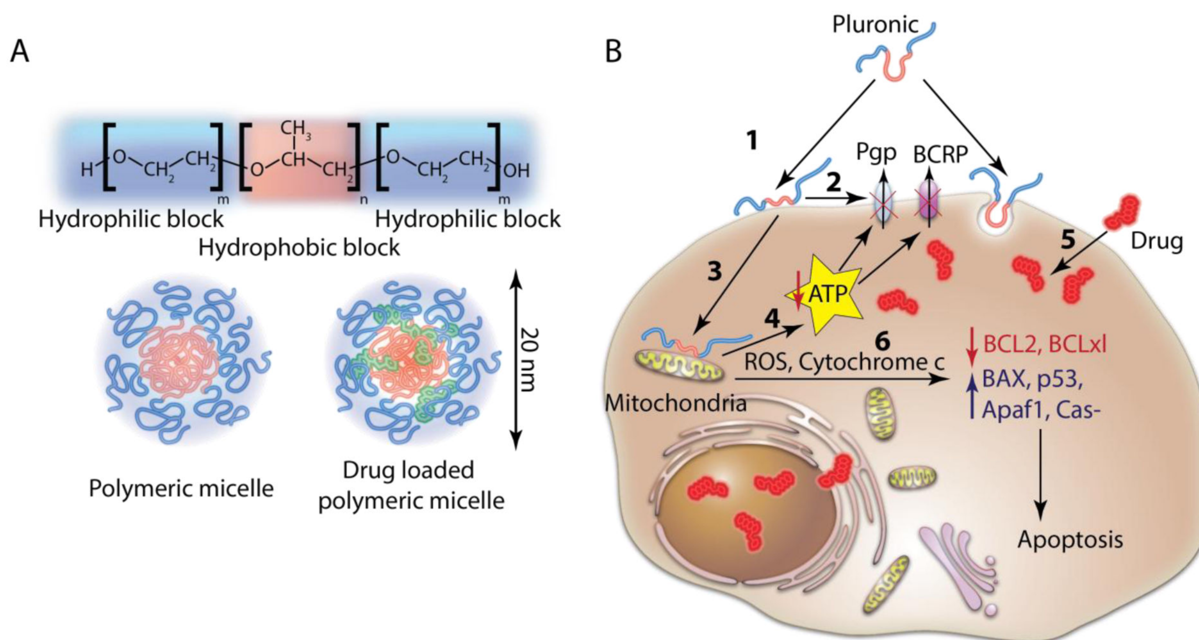

Fig. 6.

(A) Structure of Pluronic block copolymers along with empty and drug-loaded polymeric micelles; and (B) schematic representation of multiple effects of Pluronic block copolymers in MDR cells. Pluronic molecule binds with the cholesterol-rich domains in the cell membranes ("lipid-rafts") **(1)** and perturbs their structure. This results in inhibition of the ATPase activity of the drug efflux pumps, Pgp and BCRP (**2**). Pluronic translocates into mitochondria, decreases mitochondria membrane potential and inhibits respiration **(3)**. This leads to inhibition of the mitochondrial H^+-ATPase and ATP depletion **(4)**. The ATP depletion **(4)** along with inhibition of the ATPase activity of the Pgp and BCRP (**2**) results in the impairment of the drug efflux and increased accumulation of the drug in cells (**5**). The interaction of Pluronic in mitochondria also releases cytochrome C, increases ROS production, and shifts the cell signaling in response to the drug towards apoptosis (**6**). The Pgp inhibition, ATP depletion, accumulation of ROS and enhanced pro-apoptotic signaling vs. anti-apoptotic defense are usually observed in MDR cells but not in their sensitive counterparts. Based on references [17, 18, 239].

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Table 1

Therapeutic agents reported to enrich tumors with CSCs.

Therapeutic agent or combination treatment	CSCs detection method or biomarker	Mechanism of tumor enrichment with CSCs	Source	Assays used to determine effect of therapeutic agent(s) on CSCs
Paclitaxel [9]	Tumorsphere assay, CD44+CD24−	Increased mesenchymal trans-differentiation	HMLER, MCF7Ras, 4T1	*In vivo* tumorigenesis assay, tumorsphere assay
Cisplatin [10]	CD133+	Activation of the Notch signaling pathway	H460 and H661, human patients	Sphere-forming assay, soft agar assay and in vivo anti-tumor growth assay
Sunitinib and bevacizumab [11]	Aldefluor+, ALDH1+	Activation of the Akt/β-catenin CSCs regulatory pathway	MDA-MB 231, SUM159	*In vivo* TIC enrichment assay and tumorigenesis assay
Combination therapy (FEC, FAC, CMF)# [12]	Tumorsphere assay, CD44+CD24−	Development of ABCG2, reduction of let-7	Biopsy from breast tumor patients, pleural fluid samples from patients, SK-3rd developed from SKBR-3 NOD/SCID mice	*In vitro* tumorsphere assay, in vivo tumorigenesis and metastasis assay
Paclitaxel, epirubicin [13]	ALDH1+	-	Biopsy from breast tumor patients	-
Endocrine therapy (letrozole), chemotherapy (docetaxel) [14]	CD44+CD24−, tumorsphere assay	Increase in mesenchymal and tumor-initiating features	Biopsy from breast tumor patients	IHC, AQUA, RT-PCR

#Common designations of the combination therapies: FEC: 5-fluorouracil 500 mg/m^2, epirubicin 100 mg/m^2, cyclophosphamide 500 mg/m^2 every 3 weeks; FAC: 5-fluorouracil 500 mg/m^2, doxorubicin 50 mg/m^2, cyclophosphamide 500 mg/m^2 every 3 weeks; CMF: cyclophosphamide 600 mg/m^2, methotrexate 50 mg/m^2, 5-fluorouracil 500 mg/m^2 every 3 weeks.

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Table 2

Models explaining cancer cell heterogeneity and possible treatment strategies.

Model	Hierarchy	Origin of heterogeneity	Treatment strategy	Example
Classical CSCs	Yes	Different subpopulations of cancer cells as result of CSCs differentiation.	Eliminating small CSCs subpopulation	Hematopoietic cancer [79]
Clonal evolution	No	Genetic, epigenetic and/or microenvironment-driven phenotypic changes	Elimination of all tumor cells	High-grade B cell lymphoblastic leukemia [75]
Dynamic CSCs	Not necessary	Genetic, epigenetic and/or microenvironment-driven phenotypic changes	Targeting CSCs, non-CSCs and CSCs niche	Melanoma [49, 80] [1]

[1] No specific markers identified so far that can distinguish melanoma CSCs. Melanoma tumors from patients were not hierarchically organized and many markers appeared to be reversibly expressed by tumorigenic melanoma cells.

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Table 3

Some examples of drug efflux transporters expression in CSCs.

Drug efflux transporters	CSCs biomarkers	Cancer type
ABCG2	CD44+CD24− [97]	Breast cancer cell lines
	CD34+CD38− [96]	Acute myeloid leukemia (AML) primary tissue
	CD133+ [111]	Human glioblastoma cell lines
	CD133+ [112]	Primary non-small cell lung cancer (NSCLC)
	CD138−CD34− [113]	Multiple myeloma
ABCB1	CD133+ [98]	Glioma cells
	CD133+ [10]	Lung adenocarcinomas cell lines
	CD133+ [114]	Human hepatocellular carcinoma cells
	$ALDH^{low}$ [115]	Human colorectal cancer
ABCB5	CD133+ [99, 100, 116]	Human malignant melanoma
	CD44+ [117]	Oral squamous cell carcinoma specimens and cell lines
	CD49f+, CD90+ [118]	Nodular melanoma specimens
	CD133+, Epcam+ [119]	142 clinical samples from liver tumors, adjacent non-tumorous liver tissue, and liver tissue from patients who did not have cancer

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Table 4

Examples of currently evaluated new therapeutic agents that target pathways implicated in CSCs.[1]

Therapeutic agent, & Company	Type	Target	Clinical trial identifier	Cancer types	Status
PRI-724 Prism Pharma Co., Ltd	Small molecule	Wnt, CBP/β-catenin	NCT01606579	AML	Phase I
				CML	Phase II
			NCT01764477	Advanced pancreatic cancer, metastatic pancreatic cancer, pancreatic adenocarcinoma	Phase I
			NCT01302405	Advanced solid tumors	Phase I
OMP-18R5 (Vantictumab) OncoMed Pharmaceuticals, Inc.	mAb	Wnt	NCT01345201	Solid tumors	Phase I
MK-0752 University of Michigan Cancer Center	Small molecule	Notch, γ-secretase	NCT00645333	Advanced or metastatic breast cancer	Phase II completed
OMP-21M18 (Demcizumab) OncoMed Pharmaceuticals, Inc.	mAb	Notch, DLL4	NCT01189968	Non-squamous NSCLC	Phase I
			NCT01189929	Advanced pancreatic cancer	Phase I
			NCT01189942	Advanced colorectal cancer	Phase I
OMP-52M51 OncoMed Pharmaceuticals, Inc.	mAb	Notch1 receptor	NCT01703572	Relapsed or refractory lymphoid malignancies	Phase I
			NCT01778439	Relapsed or refractory solid tumors	Phase I
OMP-59R5 OncoMed Pharmaceuticals, Inc.	mAb	Notch2, Notch3	NCT01277146[2]	Solid tumors	Phase I
			NCT01647828[2]	Stage IV pancreatic cancer	Phase I b/II
			NCT01859741[2]	Stage IV SCLC	Phase Ib/II
GDC-0449 (Vismodegib) National Cancer Institute	Small molecule	Hh, SMO	NCT01195415	Stage IV pancreatic cancer	Pilot study
BMS-833923 (XL139) Bristol-Myers Squibb	Small molecule	Hh, SMO	NCT00670189	Advanced or metastatic cancers	Phase I
			NCT01357655	CP CML	Phase II
VS-6063 Verastem, Inc.	Small molecule	FAK	NCT01870609[2]	Malignant pleural mesothelioma	Phase II
			NCT01778803[2]	Advanced ovarian cancer	Phase I/Ib
VS-4718 Verastem, Inc.	Small molecule	FAK	NCT01849744[2]	Metastatic non-hematologic malignancies	Phase I

[1] Based on available on-line information and descriptions at the clinical trial website (http://clinicaltrials.gov/) as of September 2013.

[2] The effect of the agent on the CSCs in patients is not evaluated in this trial; however, the company designates this agent as anti-CSCs therapeutic.

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Table 5

Nutraceuticals and repurposed drugs that decrease fraction of CSCs.

Therapeutic agent & main use	Tumor and/or CSCs source	CSCs detection method or biomarker	Proposed mechanism of CSCs depletion	Limitation/Toxicity
Sulforaphane, an extract from cruciferous vegetables	Pancreatic [168], prostate [168] and breast cancers [169]	$ALDH^{high}$ [168, 169]	Inhibits Wnt signaling [169]; prevent NF-κB binding	Low solubility [170]
Curcumin and its analogues, Indian spice turmeric	Colon [161], breast [162], and esophageal cancers [171]	ALDH+/CD133+ [161]; ALDH+ [162]	Inhibits STAT3 [161]; affects Wnt [162], and Notch signaling [171]	Poor solubility and bioavailability [172]
Metformin, an anti diabetic drug	Breast [163] and pancreatic cancers [173, 174];	CD44+CD24– [163]; CD133+ [173]; Epcam, EZH2, Notch-1, Nanog & Oct4 [174]	Inhibition of cellular transformation [163]	Poor cell membrane permeability [175]; lactic acidosis [167]
Salinomycin, an antibacterial ionophore drug	Breast [9] and lung cancers [176], CLL [177]	CD44+CD24– [9], $ALDH^{high}$ [176]	Increases epithelial differentiation [9], inhibits Wnt signaling [177]	Poor solubility [178], neural and muscular toxicity [166]
Thioridazine, an anti-psychotic drug	Pluripotent CSCs, breast cancer [165]	CD34+ hematopoietic progenitor cells; CD44+CD24– [165]	Targets dopamine receptor of CSCs [165]	Fatal narcoleptics malignant syndrome [179]