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Review

Targeting cancer stem cells in refractory cancer

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ABSTRACT

Although common cancer therapies, such as chemotherapy and radiation therapy, have recently improved and yielded good results, evaluated as tumor shrinkage, disease recurrence is still a common event for most cancer patients. This is termed refractory cancer. This tumor regrowth following therapy is generally thought to be caused by a small, specific population of tumor cells called cancer stem cells (CSCs). Similar to other stem cells, CSCs have the capacity for self-renewal and multipotent differentiation, and they have been identified in many tumor types based on cell surface protein expression. This specific cell population has stemness characteristics as examined by serial transplantation in animal models. Previous studies have developed a specific signature of cell surface markers and biological functions that can identify CSCs in many solid tumors. In this review, we summarize the characterization of CSCs using new techniques for identifying and quantifying them *in situ*. These techniques and concepts could be valuable for evaluating the effects of therapies on this cell population. Finally, we conclude by discussing several unique preclinical treatment strategies to targets CSCs, such as reprogramming CSCs or inducing attack by immune cells. Therapeutic and diagnostic methodologies that can target and quantify CSCs will be valuable tools for eradicating refractory cancer.

Contents

Abbreviations: CSC, cancer stem cells; ODC, ornithine decarboxylase; LGR5, Leucine-rich repeat-containing G-protein coupled receptor; KLF5, Kruppel-like factor 5; iPS cell: induced pluripotent stem cell, iPC cell: induced pluripotent cancer cell; miRNA, microRNA; PGE2, prostaglandin E2.

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1. Introduction

Cancer is a disease caused by genetic and epigenetic factors that lead to uncontrolled proliferation. It is one of the leading causes of death worldwide [1,2]. Clinically, many cancers show good response to conventional treatments such as chemotherapy and radiotherapy. However, many tumors later recur, even if they initially showed a good response to treatment. This is called refractory cancer. One of the causes of refractory cancer is cellular heterogeneity, which is produced by small populations of cancer stem cells (CSCs). CSCs were first noted in acute myeloid leukemia [3,4] and have since been detected by various *in vitro* assays and *in vivo* animal experiments [5]. As with other types of stem cells, CSCs have both self-renewal and multipotent properties [6,7]. The possible involvement of CSCs in refractory cancer has since been shown in several solid tumors [8–13]. Recent studies have shown that many cancers possess a differentiation hierarchy that arises from malignant CSCs that undergo uncontrolled proliferation and produce daughter cells [6,7] (Fig. 1). It is thought that CSCs are the cause of resistance to conventional treatments [7,14–16] (Fig. 2). Although CSCs account for only a small proportion of tumor cells, they possess persistent proliferative potential. Because of this uncontrolled proliferative potential and the dysregulation of their mechanisms of differentiation, malignant tumors have been proposed to derive from CSCs [3,5].

Here, we review the recent progress made in CSC research, discuss how CSCs maintain their malignant potential, and describe new strategies for targeting CSCs.

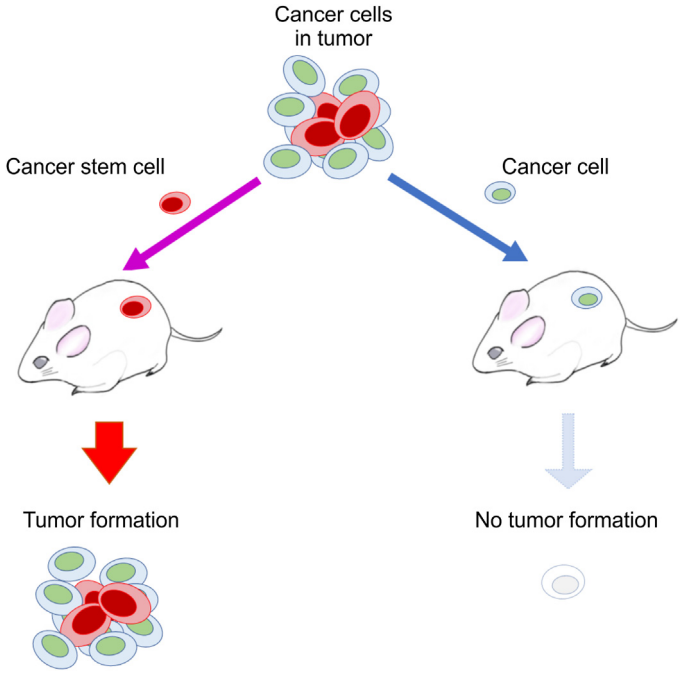

Fig. 1. Cancer stem cells evaluated with xenograft model. Cancers possess a differentiation hierarchy producing heterogeneity. Cancer stem cells are able to form tumors when transplanted into immunodeficient mice.

2. Detecting CSCs

Previous studies that have isolated CSCs provide great insights into how they promote resistance to treatment [17–20]. CSCs have multiple properties that protect against cytotoxic drugs and DNA damage. Several reports have shown that cancers with higher expression of stem cell markers are associated with an increased risk of recurrence and poorer prognosis [21–23]. CSCs identified from solid tumors usually express the specific markers of the site of origin. In breast cancer, CSCs tend to be $CD44^{+}CD24^{-/low}Lin^{-}$ [10], and as few as 100 cells with this phenotype are able to form tumors in transplanted mice, whereas tens of thousands of cells with alternate phenotypes fail to form tumors. Furthermore, in serial passaging experiments in which the extracted cells from one mouse are transplanted into another, this tumorigenic subpopulation could generate new tumors containing $CD44^{+}CD24^{-/low}Lin^{-}$ cells within each passage. These results indicate that these tumorigenic cells behave like CSCs. CD44 is an adhesion molecule, and the variant isoforms containing v8-v10 (CD44v9) have been identified as CSC surface markers [24]. Similar results have also been reported in brain tumors, and brain tumor stem cells were exclusively isolated using the neural stem cell surface marker CD133 [9,25,26]. In gastrointestinal cancers, several CSC surface markers have been reported [27–31]. Although CSCs are a small proportion of cancer cells, they have low cell turnover, which allows for isolation of CSCs for gene expression profile analysis. Additionally, this population has the ability to extrude dye, which allows for the isolation of CSCs [32,33].

Isolation of CSCs is performed using flow cytometry focusing on cell surface markers, which allows for the study of tumor profiles. Although this procedure can detect the CSC population and target specific clusters, the manipulation stresses them and may alter their biology. Therefore, visualizing CSCs *in situ* would be preferable, and it is necessary to develop a system to analyze CSC behaviors within their natural environment. Focusing on the characteristics of CSCs, such as their dormancy, low protein turnover rate, and decreased 26S proteasome activity, has great advantages for cancer research [34]. A fusion of green fluorescent protein (ZsGreen) and the C-terminal degron of ornithine decarboxylase (ODC) is retained (green fluorescence-positive) in CSCs with low 26S proteasome activity due to decreased protein degradation. In several solid tumors, these fluorescent (ZsGreen-ODC-positive) cells demonstrate features of stemness, such as

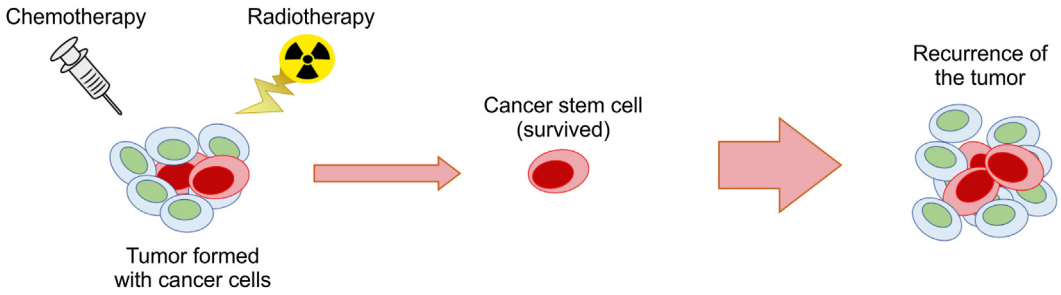

Fig. 2. Conventional cancer treatments and the stem cells.

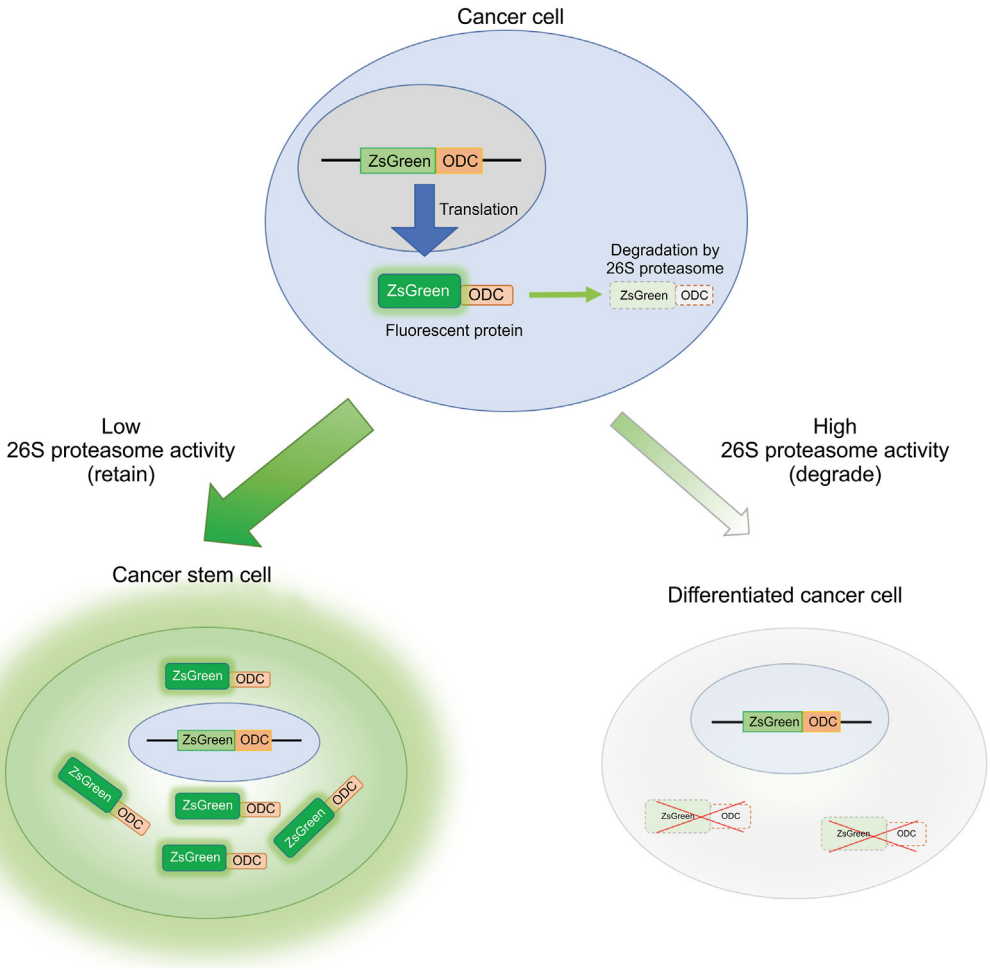

Fig. 3. Visualization of cancer stem cells. The technique is based on the decreased activity of the 26S proteasome in cancer stem cells. Ornithine decarboxylase (ODC) is normally destroyed by the proteasome. Using a vector encoding a fusion of green fluorescence protein (ZsGreen) and the C-terminal degron of ODC, cancer stem cells with low 26S proteasome activity can be visualized.

tumor formation in xenotransplant models and asymmetric cell division (Fig. 3) [18–20]. The fluorescent cells are also more chemo- and radioresistant than non-fluorescent cells [19,20]. The ZsGreen-ODC system has been used in various solid tumors [35,36]. Visualizing CSCs using this system not only allows for stem cell research but also for drug screening to search for novel agents.

A recent report proposed marking revived stem cells based on high clusterin expression, which is found in cells with damage-induced quiescence [37]. The revived stem cells undergo yes-associated protein 1-dependent transient expansion and reconstitute the leucine-rich repeat-containing G-protein coupled receptor (LGR5)-positive crypt-base columnar cells, regenerating a functional intestine. Another report shows the plasticity of LGR5-negative cancer cells, which drive metastasis in colorectal cancer [38]. It is necessary to think about CSCs as unique entities, not relying solely on the cell surface markers of the original stem cells, because of their plasticity, particularly when focusing on the treatment course of the refractory cancer.

Morimoto and colleagues reported a novel target in CSCs, Kruppel-like factor 5 (KLF5) [39]. In that study, therapeutic microRNA (miRNA) was used to suppress the cancer stemness, specifically focusing on KLF5 expression [39]. KLF5 acts as a core regulator in the process of intestinal oncogenesis, which has been demonstrated in a genetically manipulated mouse model and LGR5-positive intestinal stem cells [40]. KLF5 controls the stemness of embryonic stem cells [41].

3. Treatments based on cancer cell reprogramming

The investigation of embryonic stem cell development, from zygote to blastodermic vesicle, has helped elucidate the molecular mechanisms specifying pluripotent differentiation [42,43]. In multipotent stem cells, several transcription factors cooperate to regulate pluripotency through strict epigenetic regulation [44–47]. In our previous study, we found that the transcription factors necessary to create induced pluripotent stem (iPS) cells have similar effects as cancer-related oncogenes and tumor suppressor genes. The transcription factors OCT3/4, SOX2, KLF4, and c-MYC were introduced into cancer cell lines. The cells generated in our study were similar to iPS cells in morphology and displayed embryonic stem cell-like gene expression and epigenetic modifications [44–47]. The introduction of these transcription factors into gastrointestinal cancer cells resulted in the reprogramming of the cells to the pluripotent state and also sensitized them to differentiation [48]. The reprogrammed cancer cells could be differentiated into various cells, such as epithelial, mesenchymal, neural, or adipose lineages, under varying conditions [48]. As tumor suppressor genes extend the lifespan of embryonic stem cells, their repression

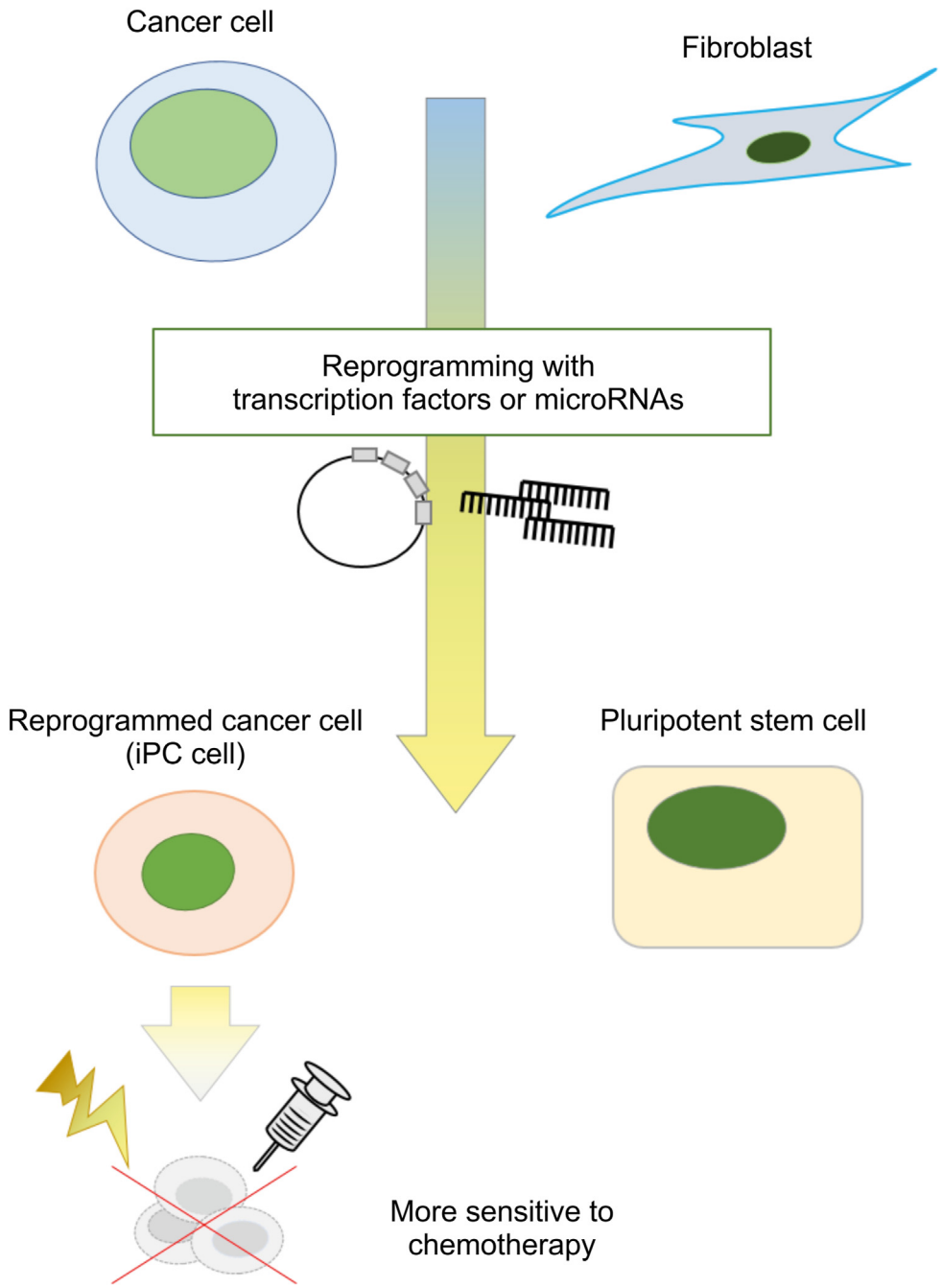

Fig. 4. Strategy of cancer treatment based on reprogramming. Cancer cells can be reprogrammed into induced pluripotent cancer (iPC) cells by specific transcription factors or microRNAs, similar to the manner in which normal fibroblasts can be reprogrammed into induced pluripotent stem cells. The iPC cells have an increased sensitivity to conventional chemotherapy.

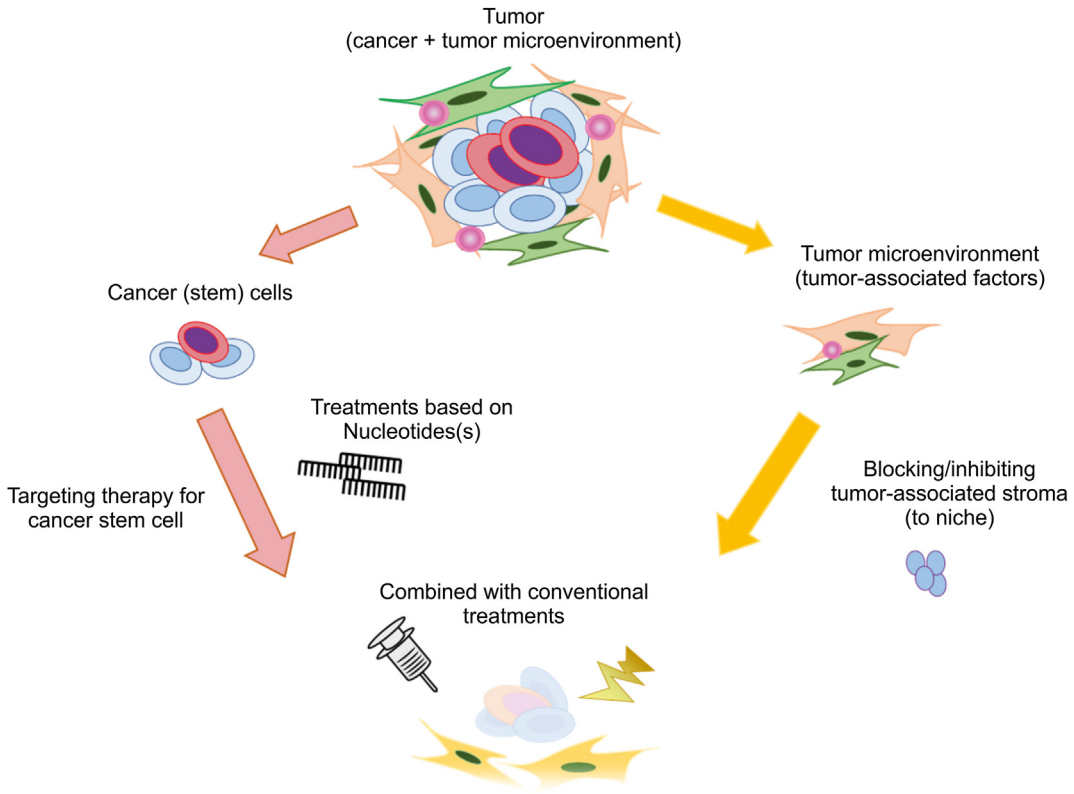

Fig. 5. Treatment strategy for cancer focusing on the stem cells and the tumor microenvironment. The heterogenous tumor and its microenvironment protect the tumor from anticancer therapies. Stem cell-specific markers or a visualizing system can be used to detect the cancer stem cells. Then, targeted therapy and reprogramming-based treatments can improve the efficacy of conventional treatments. Furthermore, targeting the tumor microenvironment can enhance the treatment strategy, leading to the reduction of recurrence and eradication of the cancer.

increases the induction efficiency of iPS cells and maintains their immortalized state [49–51].

Although these reprogrammed cells have not been fully characterized, because they are distinct from their parental cells, it is possible that the generation of induced pluripotent cancer (iPC) cells will allow for so-called differentiation therapy *via* the induction of drug susceptibility in cancer cells. This reprogramming approach in cancer cells supports that transduction will cause cell differentiation into unique lineages. The initial goal is to exploit drug discoveries with the aim of producing therapeutic and diagnostic reagents. We also demonstrated that iPC cells have the capacity for multipotent differentiation. Originally, we hypothesized that cells differentiated from iPC cells would revert to their original phenotypes. However, we found that the differentiated iPC cells lost the ability to form tumors after transplantation into mice. Furthermore, the differentiated iPC cells became more sensitive to chemotherapy. These findings suggest that reprogramming and epigenetic modifications are promising strategies to understanding CSCs and identifying novel cancer treatments, regardless of any genetic mutations in the parental cells.

A recent report showed that G9a activity is essential for the maintenance of the embryonic-like transcriptional signature that promotes self-renewal, tumorigenicity, and the undifferentiated state. Therefore, this might be useful when transforming pluripotent cells as a surrogate model for CSCs [52].

With a view toward using reprogramming to eradicate CSCs clinically, we developed a method of reprogramming differentiated fibroblasts into pluripotent stem cells *via* a specific combination of miRNAs [53]. miRNAs are small noncoding RNAs that regulate gene expression to regulate development and differentiation. Specific miRNAs have been characterized in relation to pluripotency [54–56]; therefore, we searched for miRNAs that could reprogram differentiated cells into pluripotent stem cells. Candidate miRNAs that were highly expressed in iPS and embryonic stem cells were transfected into somatic cells derived from transgenic mice with green fluorescent protein driven by the *Nanog* promoter [57]. By examining the fluorescence (a surrogate for *Nanog* activation), we identified a combination of miRNAs that could reprogram mammalian cells into pluripotent stem cells and demonstrated the ability of the miRNA-induced reprogrammed cells to differentiate into cells of different lineages. As with iPS cells created using the four transcription factors, cancer cell lines reprogrammed with miRNAs also demonstrated decreased tumor-initiating capacity and became sensitive to chemotherapy [58]. In an *in vivo* model examining the feasibility, safety, and effectiveness of the miRNAs, the miRNA combination suppressed tumorigenesis, suggesting that this therapy could be useful for both preventing and treating cancer (Fig. 4). In glioblastoma, reprogramming transcription factors and miRNAs drive a stem-like phenotype [59].

However, one limitation of miRNA-based treatment is the presence of RNases in the blood, which makes miRNA-based treatments unsuitable for systemic therapy. To prevent RNA degradation, a drug delivery system was designed using super carbonate apatite nanoparticles. Because of their preference for the low pH conditions found in tumor microenvironments, these nanoparticles deliver RNAs selectively to tumors [60]. We are

currently exploring the use of synthetic miRNAs to increase the stability and efficacy of this miRNA-based therapy.

4. Targeting the tumor microenvironment

Although the refractory nature of the tumor itself is born of tumor heterogeneity, the tumor microenvironment also plays an important role by providing various self–protection properties. These include mechanisms that enable dynamic interactions with surrounding epithelial cells, infiltrating immune cells, cytokines, and chemokines, which maintain refractory cancers regulated by CSC proliferation and self-renewal. The tumor microenvironment maintains CSCs by the induction of specific features [61]. The microenvironment plays a key role in regulating the CSC population by direct cell–cell contacts in which various paracrine factors are secreted. The stemness of CSCs is maintained by microenvironmental factors through pathways that promote self-renewal, such as the Wnt/β-catenin, Notch, and Hedgehog pathways. For example, Wnt activity regulates the self-renewal of CSCs and drives transit amplifying cell proliferation and differentiation [62]. Hedgehog signaling is important for embryonic development, patterning, and differentiation, which are required for the regulation of the self-renewal of normal mammary stem cells and CSCs [63]. Additionally, during development, Notch signaling controls cell fate, and aberrant activation contributes to tumorigenesis [64]. Niclosamide, a tineacide of the anthelmintic family, is an inhibitor of Wnt/β-catenin and Notch signaling [65], and it might be useful for inhibiting CSCs by blocking these signaling pathways. Mesenchymal stem cells in the tumor-associated stroma have been shown to affect cancer cell behavior. Mesenchymal stem cells influence the phenotypes of cancer cells, and prostaglandin E2 (PGE2) secreted by mesenchymal stem cells enables tumor progression *via* creating a CSC niche [66]. Targeting the tumor microenvironment may stimulate host antitumor responses by blocking tumor-promoting inflammation with PGE2 receptor antagonists, which is a promising treatment strategy. However, to effectively target CSCs, combination therapy may be needed.

The two major superfamilies of efflux transporters in CSCs are the ATP-binding cassette transporters and the solute carrier transporters. Interestingly, because of the ability of CSCs to extrude dye, targeting these efflux transporters as well as CSC-related surface markers in combination with conventional treatments can improve cancer treatment.

5. Conclusions

Refractory cancer, which is associated with a long cellular life span, relative quiescence, and resistance to drugs, is derived from cellular heterogeneity produced by small populations of CSCs. Through their self-renewal and drug-resistant capacities, CSCs allow for proliferation and differentiation, even after treatment with anticancer therapy. No specific marker to identify all CSCs has been defined. Further, cancer cells gain plasticity during metastasis in colorectal cancer [38]. It is important to further understand the characteristics of CSCs and normal stem cells. If specific markers or tracing methods for these stem cells can be identified, we will be able to isolate, identify, and analyze these populations within heterogenous tumors. Examining these characteristics will allow gene expression profiling, which provides crucial information that will allow prediction of patient prognosis [67]. Considering the tumor microenvironment and molecular subtypes, the elucidation of CSC properties could lead to the development of effective cancer treatments (Fig. 5).

Cancer stem cells are the cause of chemo- and radioresistance, which results in tumor recurrence. Cancer stem cells have the capacity for self-renewal and multipotent differentiation, which is thought to lead to resistance to conventional treatments.

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