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Review Article

Current Methods to Study Cancer Stem Cells

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Abstract

Cancer stem cells are a rare population of cancer cells with the ability to self-renew and differentiate into various cell lineages within tumors. These cells play a critical role in the chemoresistance and radioresistance of cancers. Therefore, to develop new and effective cancer treatment options, it is essential to understand the specific characteristics and functions of cancer stem cells. In this review, we discuss the most successful methods for identifying cancer stem cells in tissue samples and introduce strategies for studying these cells in environments that closely resemble the tumor microenvironment.

Keywords: Cancer stem cell; Flow cytometry; Spheroid; Organoid; Tumor

Introduction

Uncontrolled growth and immortality of cancer result from multiple genetic alterations in otherwise healthy cells [1]. According to the AACR workshop (2006), Cancer Stem Cells (CSCs) are a population of cells within the tumor environment that possess self-renewal capacity and are primarily responsible for tumor initiation and maintenance [2]. CSCs contribute to the significant cellular heterogeneity observed in various tumors [3]. They have been identified in most human cancers, including lung, pancreatic, colon, liver, brain, breast, prostate, gastric, head and neck cancers, as well as in melanoma, leukemia, and multiple myeloma [4].

Historically, as early as 1858, Rudolf Virchow proposed the theory that tumors originate from immature cells, forming the foundation of the cancer stem cell model [5]. In the 1970s, the colony-forming test demonstrated that tumors could develop from rare cells with self-renewing capabilities. This technique detected self-renewing cells in several types of leukemia [6]. With advancements in flow cytometry and stem cell biology, CSCs could be isolated from tumor tissue based on specific surface markers, often the same markers used to identify adult stem cells. This approach enabled the identification of CSCs in both hematological malignancies and solid tumors [7-9].

CSCs are central to the fundamental processes of tumor growth and metastasis, with their potential for self-renewal contributing to their immortality [9]. Numerous studies have demonstrated that CSCs, like other types of stem cells, exhibit increased resistance to chemotherapy and radiotherapy [10-12]. The origins of CSCs are not yet fully understood; they may arise from oncogenic mutations and transformations in normal tissue stem cells or progenitors [13]. CSCs are capable of continuous proliferation, giving rise to all differentiated cells within a tumor. The initial genetic and epigenetic changes promote cancer cells to alter complex cellular and molecular mechanisms involved in tumor development. Cancer formation unfolds in three stages -initiation, tumor growth and angiogenesis, and progression and metastasis- all of which occur concurrently with complex and dynamics [4]. By manipulating both intrinsic and extrinsic adaptability, CSCs play an active role in cancer pathogenesis. This includes activating cell survival signals, promoting uncontrolled proliferation, resisting growth inhibitory signals, enhancing angiogenesis, evading apoptosis, and facilitating metastasis (Figure 1). Moreover, CSCs promote glycolysis, evade immune surveillance, and initiate the Epithelial-to-Mesenchymal Transition (EMT) [9,14]. A single CSC can grow and develop into a sizable tumor. As the tumor progresses, various CSC subpopulations may arise due to extensive epigenetic alterations and mutations [15,16]. These new CSC populations may develop more aggressively and contribute to the progression of malignancy. Therefore, studying CSCs is a crucial area of cancer research, and recent studies indicate that combined treatments targeting CSCs lead to improved treatment responses [12,17].

The first step in studying CSCs is to isolate and characterize these cells. CSCs possess properties similar to those of normal stem cells, including the expression of specific surface markers such as CD44, CD90, and CD133, as well as being identified as a Side Population (SP) in flow cytometry analysis [18]. However, the specificity of CSC markers may vary between different tumor types. It is essential to verify whether the isolated cell population exhibits the fundamental properties of CSCs, including the ability for self-renewal and tumorigenesis. Functional tests are employed for this purpose and can be categorized into *in vivo* and *in vitro* tests based on the methodological approach. An overview of these methods is provided in the following sections.

Methods for Isolating and Analyzing CSCs

The physical and functional characteristics of CSCs can be identified using a range of approaches described in this section.

Detection and Isolation of CSCs Through Surface Marker Analysis

Analyzing surface markers is valuable in the diagnosis, prognosis, and treatment of various diseases, including cancer. This method

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Table 1: Cancer stem cell surface markers.

Cancer stem cell	Markers	reference
Breast cancer	SSEA3, SSEA4, TRA-1-60, TRA-1-81, CD133, Cripto-1(TDGF1), PODXL-1(Podocalyxin- like protein 1), ABCG2, CD24, CD10 (Neprilysin), CXCR4(fusin/CD184), CD55 (DAF), CD29(Integrin beta1), CD44(variants)	[20.23.56]
Teratocarcinoma	SSEA3, SSEA4, TRA-1-60. TRA-1-81, SSEA1	[20]
Prostate cancer	TRA-1-60, CD133, CD117/c-kit, CD44, $\alpha_2\beta_1$ integrin, α_6 integrin, CXCR4, E-cadherin, EpCAM, Cytokeratin 5, PSA^{lo}, ABCG2, Trop2, AR variant 7, CD166/ALCAM,	[20.138]
Renal cancer	SSEA1, CD105 (Endoglin),CD105, ALDH1, OCT4, CD133, CXCR4.	[19,20]
Lung cancer	SSEA1, CD133(AC133), Cripto-1(TDGF1), PODXL-1, ABCG2, Notch, CD56(NCAM), CD166(ALCAM), CD44, uPAR/ CD87 , CD90, CD117, CD133, CD166, ALDH, BMI-1, EpCAM, FZD, PODXL-1, PTCH, SP	[20,139]
Colon cancer	CD133(AC133), CD326(EpCAM), Cripto-1(TDGF1), CD26 (DPP-4), LGR5, DLL4 (Delta-like ligand 4), CD29 (Integrin beta1), CD166 (ALCAM), CD44	[22,105]
Glioblastoma	CD133(AC133), CD49f (Integrin a6), CD44, CD15, CD70 (CD27 L), S100A4, ALDH1A3, Nanog, OCT-4, SOX-2, Nestin	[140,141]
Liver cancer	CD133 (AC133), CD90(Thy-1), CD326(EpCAM), CD13 (Alanine aminopeptidase), CD44, OV6, epithelial cell adhesion molecules (EpCAM), CD24, CD13 (ANPEP), CD34, sex determining region Y-box 9 (SOX9), ATP-binding cassette, subfamily G, member 2 (ABCG2), CD44, aldehyde dehydrogenase (ALDH), CK19 (KRT19), sex determining region Y-box 12 (SOX12), and CD47, SOX12	[142,143]
Ovary cancer	CD133(AC133), CD117(c-KIT), DLL4 (Delta-like ligand 4), CD44, ALDH1 , CD24, CD105 (endoglin), CD106 (VCAM-1), EpCAM, SOX2, Nestin, SSEA1, Thy-1 (CD90),	[23,144]
Pancreatic cancer	CD133(AC133), CD326(EpCAM), PODXL-1, CD24, Notch, CXCR4 (fusin or CD184), C-met, ALDH1	[19,145]
Brain cancer	CD90(Thy-1), ABCG2, CXCR4(fusin or CD184)	[20,23]
Squamous Cell Carcinoma	CD326 (EpCAM), ABCG2, Notch, CXCR4(fusin or CD184), CD34, CXCR1, 2, CD29(Integrin beta1), CD166 (ALCAM), CD97, Ki-67, SENP3, KIF2A, EGFR, SLC16A6, SLC2A13, Podoplanin, HMGA2, P63, P75NTR, GRP78	[21]
Leukemia	PODXL-1,CD26(DPP-4),CD34,TIM-3 (HAVCR2), CD96, CD9, CD123 (IL-3R), CD38, CD123, CD25, CD32.	[146]
Gastric cancer	CD24, CD54 (ICAM-1), CD44, EPCAM, ALDH1, CD90 (THY1), CD133 (PROM1), TFRC(CD71), OCT4, SOX2, LGR5 (GPR49), NANOG, ABCB1, ABCG2, CXCR4, CD166 (ALCAM), DCLK1, ITGA6(CD49f)	[147]
Rhabdoid Tumor	CD146(MCAM), CD133	[148]
Sarcoma	CD146 (MCAM)	[22]
Head And Neck/ HNSCC (Head And Neck Squamous Cell Carcinomas) And oral Squamous Cell Carcinomas	CD10(Neprilysin), CD271, CD44	[21,149]
Melanoma	CD271, CD20(MS4A1), ABCB5, ALDH, Sox10	[23,150]
Intestinal	LGR5, Musashi-1, BMPR1α, phospho-PTEN, DCAMKL1, EphR, integrins	[151]
Neuroblastoma	CD114(CSF3R), LGR5	[152]
Salivary Gland Cancers	HER2(HER2/neu), ALDH1	[21,153]

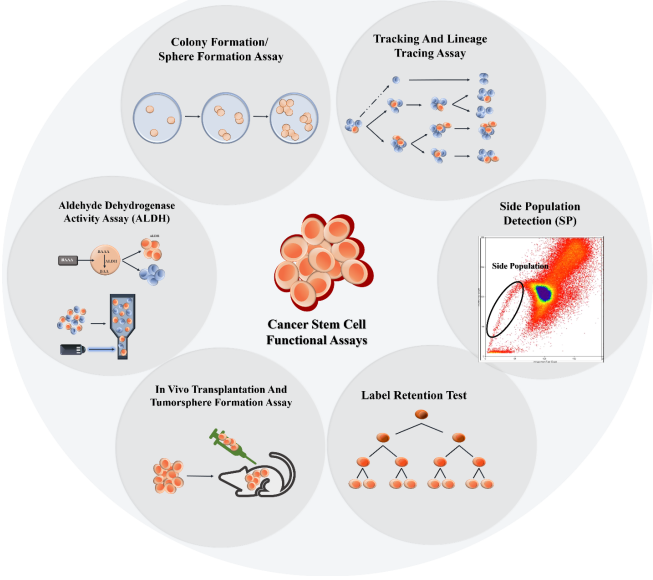

Figure 1: Role of Cancer Stem Cells in Tumor Formation. Cancer stem cells contribute to tumor growth and progression by driving fundamental processes, including cell proliferation, angiogenesis, invasion, and metastasis.

allows for the identification of biomarkers for different cell lineages before and after treatment, enabling the tracking and prediction of disease recurrence or progression. Additionally, CSCs exhibit high plasticity in their phenotype and function. These changes can occur during drug treatment, radiation therapy, or as a result of cell senescence in tumors when the surrounding microenvironment is altered. In recent years, significant efforts have been made to distinguish between markers of normal and cancer stem cells. Table 1 summarizes the CSC markers reported across different cancers [19-23]. Different technologies, such as flow cytometry and magnetic-based methods can be used to analyze and isolate CSCs based on the cell surface markers. Flow cytometry remains the most widely used technique for isolating, counting, and sorting CSCs. The simultaneous use of multiple markers, enhances its applicability and specificity. Multiparametric flow cytometry, the preferred method for CSC analysis, enables the simultaneous analysis of various cellular characteristics with high performance and reliability. Additionally, fluorescence-activated cell sorting enables the separation of live cells based on marker expression or functional properties, allowing for the isolation of rare cells within the tumor bulk- one of the most significant advantages of this technique. Quantification is also achievable with volume-based flow cytometry. Markers that offer insights into stem cell-specific metabolic functions, such as side population analysis and Aldehyde Dehydrogenase (ALDH) activity are a focus of CSC research [24]. This method also enables the simultaneous analysis of different tumor cell populations. For example, Shanshan Wan et al. analyzed tissue biopsies from patients with hepatocellular carcinoma and found a correlation between $CD44^+$ CSCs and $CD14^+$ tumor-associated macrophages in hepatocellular carcinoma [25]. Advancements in cytometry techniques, such as imaging flow cytometry and mass cytometry, have enabled the analysis of cancer cells at the single-cell level. Imaging flow cytometry combines flow cytometry with single-cell imaging to generate rich data sets and can be used to detect circulating tumor cells from liquid biopsy samples in various cancers, such as hepatocellular carcinoma (HCC) [26,27]. Cytometry

Table 2: miRNAs in CSCs.

Ccancer type and miRNAs	References
Hepatocellular carcinoma miR-1, let-7a, miR-9, miR-16, miR-16a, miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, miR-21, miR-23a, miR-23b, miR-24-2, miR-25, miR-26a, miR-27a, miR-34a, miR-92a, miR-93, miR-101, miR-106b, miR-122, miR-122a, miR-124, miR-127, miR-143, miR-146a, miR-155, miR-181a-1, miR-181a-2, miR-181b-1, miR-181b-2, miR-181c, miR-195, miR-200b, miR-203, miR-221, miR-223, miR-224	[113,154]
Breast carcinoma let-7a, miR-7, miR-9-3, miR-10b, miR-17-5p, miR-20a, miR-21, miR-27a, miR-31, miR-31a, miR-96, miR-124a, miR-125a, miR-125b, miR-126, miR-127, miR-128a, miR-141, miR-146a, miR-146b, miR-155, miR-182, miR-193b, miR-196a-2, miR-199b, miR-200a, miR-200b, miR-200c, miR-204, miR-205, miR-206, miR-210, miR-221, miR-222, miR-335, miR-373, miR-429, miR-451, miR-510, miR-516-3p, miR-520c, miR-661	[17,155]
Prostate carcinoma let-7c, miR-146a, miR-15a, miR-16, miR-16-1, miR-17-3p, miR-21, miR-23b, miR-34a, miR-101, miR-125b, miR-126*, miR-127, miR-145, miR-221, miR-222, miR-330, miR-449a, miR-521	[17,106]
Colorectal cancer let-7a-1, miR-17-92, miR-18a*, miR-21, miR-34a, miR-34b, miR-34c, miR-124a, miR-126, miR-127, miR-140, miR-141, miR-143, miR-145, miR-192, miR-194, miR-196a, miR-215, miR-342, miR-451	[113]
Ovarian cancer let-7a, let-7c, let-7d, let-7g, let-7i, miR-9, miR-146a, miR-15a, miR-16, miR-34b, miR-34c, miR-125a, miR-200a, miR-200b, miR-200c, miR-214, miR-429	[113]
Glioblastoma miR-7, miR-21, miR-34a, miR-153, miR-181b, miR-221, miR-222, miR-451	[17]
Pancreatic carcinoma miR-10a, miR-21, miR-34a, miR-34b, miR-34c, miR-155, miR-221	[156]
Head and Neck Mir-21, miR-145, miR-200c, miR-218-5p	[113]
Thyroid cancer Mir-21, Mir-148a	[113]

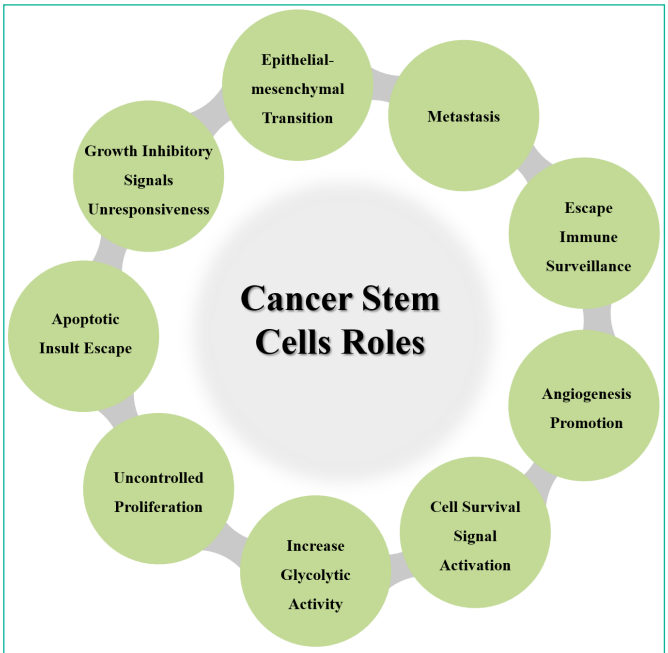

Figure 2: Functional assays for identification of cancer stem cells.

by time-of-flight, or mass cytometry, is another powerful technique that employs metal isotopes, overcoming the fluorophore limitations of conventional flow cytometry. Mass cytometry can detect over 100 biomarkers at both cellular and subcellular levels and has been used to assess tumor heterogeneity and identify subpopulations of cancerous cells in human breast cancer samples [28].

"In Magnetic-Activated Cell Sorting (MACS), magnetic beads are attached to highly specific monoclonal antibodies that recognize CSC markers on the surface of target cells. A heterogeneous cell suspension is passed through a separation column within a magnetic field, which retains cells labeled with the magnetic beads and antibodies. When the magnetic field is turned off, the target cells elute. MACS is a quick and straightforward cell separation method, particularly useful for isolating rare cell populations within tumor bulk, such as CSCs [29]. Magnetic cell sorting can be performed using High-Gradient Magnetic Separation (HGMS) or Low-Gradient Magnetic Separation (LGMS). In HGMS, low-volume columns containing stainless steel beads or wool that respond to magnetic fields are used to create a uniform magnetic field for separating labeled cells. Once cells bind to the wool or beads, the magnetic field is turned off to isolate the bound cells [30]. In LGMS, permanent magnets generate the magnetic gradient to accommodate larger sample volumes, and larger beads are used to counteract opposing forces, such as sedimentation. Both methods enable positive and negative selection. During positive selection, the supernatant is removed, retaining the particles of interest.

CELLSEARCH® CTC (Janssen Diagnostics Inc., formerly Veridex LLC) is the first FDA-approved method for isolating circulating tumor cells from the blood of patients with metastatic breast, colorectal, and prostate cancers. In this technique, non-specific particles are retained during negative selection, while particles of interest are collected in a separate fraction [31-33].

A combination of techniques, such as flow cytometry followed by MACS, can be used to increase the purity of isolated cell populations. For example, CD133+ Lung Cancer Stem Cells (LCSCs) sorted by flow cytometry were further enriched for cancer stem cells with an additional step using MACS. These isolated cells showed a greater capacity to form tumors in NOD/SCID mice [34]. Similarly, CD24-/CD44+ cells were isolated as an enriched population for squamous cell carcinoma [35]. However, like most technologies, MACS has limitations. The cell collection and processing procedure can be time-consuming, and the number of sorted cells is limited by the volume of the sorting column. Typically, only one surface marker can be used at a time, as combining antibodies for positive selection is not feasible. As a result, multiple rounds of MACS may be required to isolate and enrich CSCs, which can impact the viability of the final cells. Additionally, the equipment and magnetic beads used in MACS are costly [36].

Detection and Isolation of CSCs Based on their Functions

A well-designed *in vitro* functional assay should offer sufficient

specificity and sensitivity for detecting low-abundance populations of CSCs while allowing for quantitative evaluation. The most commonly used *in vitro* functional tests include the sphere formation assay, Colony-Forming Unit (CFU) assay, Side Population (SP) analysis, label-retention assay, and aldehyde dehydrogenase activity test [1,11,36,37]. *In vivo* assays, such as the tumorigenesis assay and lineage tracing assay, are conducted to further validate CSC characterization. A schematic of various functional assays used to identify CSCs is presented in Figure 2.

Side population as a stem cell-rich population: Side Population (SP) analysis identifies CSCs based not on cell surface markers, but on the activity of the ABCG2 transporter protein, which is highly expressed in CSCs. This protein actively exports the Hoechst 33342 dye, leaving cells unstained. These cells then appear as a distinct side population when analyzed by flow cytometry [38]. The SP phenotype has been reported in several cancers, including human gastrointestinal cancers and hepatoma cell lines [39]. SP fractions are commonly studied for cancer stem cell isolation [39,40]. In CSCs, ABCG2 expression is elevated, contributing to their resistance to chemotherapy [22,39]. A limitation of using Hoechst 33342 to detect SP cells is the need for a UV excitation laser (355 nm), which is often not included in standard flow cytometer configurations. As an alternative, rhodamine 123 (Rho123) can be used [41]. Studies show that dye cycle violet (DCV) offers advantage over Hoechst 33342, with lower toxicity and easier cell permeability. DCV shares a similar chemical structure with Hoechst 33342 but has excitation/emission maxima of 405/440 nm [36].

Label retention assay: Another *in vitro* method for identifying CSCs isolated from solid tumors is the label-retention assay [42-44]. For cell labeling, various fluorochromes can be used, such as PKH26 and DiI, which bind to the plasma membrane, or Carboxyfluorescein Diacetate Succinimidyl Ester (CFDA-SE), which freely passes through the plasma membrane and covalently binds to intracellular proteins. During cell division, these fluorochromes are evenly distributed between daughter cells, resulting in a reduction of fluorescence intensity by half for each division. After a certain number of divisions (usually 8–10), the fluorescence intensity decreases to levels comparable to those of unlabeled cells. By evaluating the fluorescence of individual cells, the proliferation rate of a given cell population can be determined. Cancer cells that retain the fluorescent signal due to an extended cell cycle exhibit other characteristics typical of CSCs, such as colony formation, *in vivo* tumorigenesis, and the expression of stem cell marker [45]. For instance, this assay has been employed to detect CSCs in glioblastoma spheres [46]. In prostate cancer spheres, it was found that label-retaining cells represent a mixed population of quiescent and active cancer stem cells [47].

Aldehyde dehydrogenase activity assay: CSCs, like non-cancerous stem cells, exhibit high aldehyde dehydrogenase (ALDH) activity, which can be utilized to define and isolate them [33,48]. (Alison et al., 2008; Storms et al., 1999). There is a commercially available system for the detection of ALDH activity under the name AldefluorTM. The general method is that a substance called BAAA (BODIPY aminoacetaldehyde) enters the cells as a substrate. It is converted into another substance called BAA (BODIPY amino acetate) by ALDH. BAA has fluorescence properties and can be measured with a flow cytometry device. As a result, more increased the activity of ALDH, the more BAA is produced, and the fluorescent light is brighter [49-51]. Increased expression of ALDH has been reported in CSCs and is associated with chemoresistance through the detoxification of anticancer drugs [52]. Various studies have employed ALDH activity to isolate CSCs from tumor tissues, including breast and colorectal cancers [53,54]. However, non-cancerous stem cells, such as hematopoietic stem cells, also exhibit high ALDH enzyme activity; thus, this property alone is not a definitive marker of CSCs (Hilton, 1984). Furthermore, using additional markers alongside ALDH has shown more reliable results in detecting CSCs, highlighting the heterogeneity within the CSC population [55,56]. This consideration is crucial when searching for CSCs in tissues containing non-cancerous cells, such as when detecting circulating tumor cells in peripheral blood.

Colony formation assay & Tumorsphere formation assay: Both the colony formation assay and tumorsphere assay are based on the ability of CSCs to self-renew and generate clones of identical cells. These assays allow for the quantification of the self-renewal capacity of CSCs. CSCs can form Colony-Forming Units (CFUs) or spheres. In non-adherent conditions, CSCs can grow and form floating aggregates called spheres, while non-stem cells undergo anoikis [57]. These techniques can isolate CSCs from a mixed population of cancer cells. The tests are performed in a medium with a defined concentration of growth factors to minimize the influence of external cell signals and under non-adherent conditions (spheres) to confirm their independence from substrate adherence, or in a semi-solid culture environment, such as Matrigel (CFUs). In the sphere assay, it is necessary to passage the spheres repeatedly to verify the self-renewal of CSCs. Spheres are enzymatically converted into a suspension of individual cells and cultured again under defined conditions [58,59]. The number of spheres formed in subsequent generations can be used to determine the self-renewal and clonogenicity of the individual cells.

These assays can be semi-automated with the help of flow cytometry. By analyzing the expression of surface markers, cells can be sorted using the Fluorescence-Activated Cell Sorting (FACS) method and seeded into 96-well or 384-well microtiter plates. This approach significantly reduces the time and manual complexity of the tests and enables rapid quantitative evaluation, such as with a fluorescence scanning cytometer [60,61]. Spheres have been derived from various tumors and are often named according to their tissue origin or the specific tumor type, including: breast carcinoma (mammospheres) [62], neuronal tumors (neurospheres) [63,64], rhabdomyosarcoma (rhabdospheres) [65], colon carcinoma (colonosphere) [66], prostate carcinoma (prostaspheres) [67], osteosarcoma (sarco spheres) [68], and hepatoma (hepato spheres) [60]. These assays are also valuable for drug discovery purposes. For instance, the colony formation assay demonstrated that chelerythrine inhibited the self-renewal potential of Cancer Stem Cells (CSCs) in osteosarcoma [69].

***In vivo* Tumorigenicity and transplantation assay:** The tumorigenicity test is currently the most effective functional assay for characterizing Cancer Stem Cells (CSCs). This *in vivo* test simultaneously verifies the ability of CSCs to self-renew and form tumors that replicate the cellular heterogeneity of the original tumor. In this assay, fractionated tumor cell populations are xenografted

into immunocompromised mice. A limiting dilution assay, along with repeated tumor transplantations, is employed to determine the frequency of CSCs and the multi-lineage potential of specific cell populations. These assays have demonstrated the presence of CSCs in various human cancers, including breast cancer, glioblastoma, colorectal cancer, Acute Myeloid Leukemia (AML), and Chronic Myeloid Leukemia (CML) [70-72]. Depending on the origin and homogeneity of the tested cell population, anywhere from 100 to several million cells are typically required for transplantation. The injection is generally performed subcutaneously, though cells can also be implanted directly into specific organs such as the brain, muscle, or mammary glands. Mice are then closely monitored for tumor development. The tumorigenic ability is assessed based on the ratio of mice that develop tumors to the total number of injected mice. Other evaluation criteria include tumor size, the time until tumors are detected, and the number of injected cells. However, the self-renewal capacity must be further verified by isolating CSCs from the xenograft tumors and transplanting them into additional animals [2,37,62].

The time-consuming nature of the tumorigenicity test is one of its significant drawbacks. Research has shown that using more highly immunocompromised mouse models, such as NOD/SCID IL2Rγ null mice, instead of NOD/SCID mice can enhance the tumorigenic potential of CSCs [73]. Another limitation of this assay is the microenvironment at the transplantation site and the method of cell injection. Stem cells are known to be largely dependent on signals produced by the surrounding stroma [74]. The preparation of single-cell suspensions may alter the characteristics of the cells due to changes in their metabolism or microenvironment. Additionally, a major flaw in this method is the lack of an immune-competent microenvironment [70]. However, patient-derived cancer stem cell xenograft models of lung, brain, colon, and pancreatic cancer have provided compelling evidence of the capacity of CSCs to initiate tumors [1].

Tracking and lineage tracing of CSCs: The proper isolation of Cancer Stem Cells (CSCs) from tumors or cell lines has provided an opportunity to study their functions and develop a deeper understanding of the molecular mechanisms underlying self-renewal, metastasis, and drug resistance. To effectively identify and target CSCs *in vivo*, it is essential to understand their behavior within their niche. Fluorescence imaging and bioluminescence imaging are commonly employed to identify and track CSCs *in vivo*. Fluorescent tracking at the single-cell level offers high-resolution images and is widely used in studies of CSC plasticity and differentiation. This method allows for the use of various fluorescent proteins to track multiple targets simultaneously [75].

Bioluminescence is another imaging method that employs bioluminescent compounds to study and examine Cancer Stem Cells (CSCs) *in vivo*. In this technique, cells are tagged with luciferase and tracked using injected luciferin (76). Bioluminescent signal detection offers advantages over fluorescent detection due to reduced background noise. However, it also has limitations, as bioluminescent analysis requires at least 2,500 cells to detect a signal and generally provides lower resolution than fluorescent signals [77,78]. Quantum Dots (QDs) are semiconductor nanocrystals that emit stable fluorescence at specific wavelengths. Many studies have utilized QDs to track cancer cell populations and stem cells within the body [79-81]. QDs are particularly valuable in near-infrared Fluorescence Imaging (NIRF) due to their superior penetration compared to other optical methods, resulting in minimal damage to cells. Additionally, QD-conjugated monoclonal antibodies can be employed for *in vivo* targeting and stem cell detection with high specificity [79].

Lineage tracing requires stable labeling of Cancer Stem Cells (CSCs) and has been employed to study stem cells [82]. Gene editing technologies enable the introduction of heritable labels into the genomes of specific cells, allowing researchers to trace their fate *in vivo*. For instance, the LGR5 gene cassette, which marks mature intestinal stem cells, can be integrated into the cell genome using CRISPR/Cas9 gene editing technology to investigate cellular behavior and plasticity *in vivo* [79,80]. Using the lineage tracing approach, the plasticity of CSCs in breast cancer has been demonstrated [81]. Cellular barcoding, as the name implies, utilizes unique nucleotide sequences called "barcodes" to label target cells. These barcodes are introduced into cells via plasmids or viral vectors, followed by PCR techniques for extraction and identification [1]. In a study on glioblastoma, cellular barcoding helped identify the proliferative hierarchy of stem cells within cancer tissue, leading to the classification of two populations: slow-cycling cells and highly proliferative progenitor cells, along with a non-proliferative population of drug-resistant cells during chemotherapy [83]. One of the main challenges in lineage tracing of CSCs is the presence of common markers shared between stem cells and surrounding cell lineages, which increases the potential for experimental error. Additionally, the method is sensitive and can lead to misinterpretation during enzymatic digestion. This complexity is further heightened when studying CSCs in heterogeneous tumors, such as breast cancer, or in tumors exhibiting diverse structures and stages [84].

Superparamagnetic Iron Oxide Nanoparticles (SPION) and radiolabels are additional classes of sensitive tags used in lineage tracing and tracking of CSCs within the body. Magnetic Resonance Imaging (MRI) is employed to detect SPION-labeled cells, providing high-resolution images through probes that offer strong positive or negative contrast [78]. Research utilizing MRI has successfully identified single exogenous stem cells in small animal tumors [85,86]. Additionally, the extracellular domain B of fibronectin (EDB-FN) has been recognized as a biomarker for breast cancer stem cells. SPION-conjugated peptides targeting EDB-FN have been developed for *in vivo* detection and targeted drug delivery to breast CSCs [85,87]. A review by Monnica Carril discusses the diverse applications of smart probes in MRI [88].

Positron Emission Tomography (PET) measures high-energy rays emitted from the injection of positron-emitting isotopes or isotope-labeled molecular probes into a patient. PET is highly sensitive, noninvasive, and unaffected by the depth of signal emission, allowing for real-time tracking *in vivo*. In a specific application of PET, a radiolabeled tracer was developed to detect CD133-positive CSCs in gliomas with high resolution, with the ability to detect signals from tumors as small as 2-3 mm [89].

Each of these *in vivo* imaging techniques has its own benefits and drawbacks. High resolution is essential for tracking single cells *in vivo*. Additionally, the absence of specific markers for Cancer Stem

Cells (CSCs) limits the reliability of tracing methods. Technologies that enable simultaneous detection of multiple probes may facilitate the identification of CSCs. For example, the use of nanoprobes in multimodal imaging allows for the combination of MRI and fluorescence imaging, enhancing the accurate detection of rare CSCs within tissue [77,90].

Microfluidics

Microfluidic systems analyze the behavior of fluids manipulated through microchannels. Microfluidic technology offers a reliable, efficient, and cost-effective approach for single-cell selection and navigation [91,92]. Because CSCs and blood cells differ in size, microfluidics can effectively separate them. In a microfluidic system, fluids and particles are transported on a microscale, with microporous membranes enabling the separation of cells based on size and deformability [91]. Three commonly used separation techniques are: 1) Deterministic Lateral Displacement (DLD): Fluids pass through micro-posts, allowing particles smaller than a critical hydrodynamic diameter to flow along with the stream, while larger particles, unable to streamline, are displaced laterally upon colliding with the micro-posts [93]. 2) Inertial Flow-Based Techniques: This method leverages inertial forces generated within microfluidic devices to separate cells based on size, as the lift force acting on a particle is influenced by its diameter [94,95]. 3) Filtration: In this technique, only cells of a specific size can pass through membranes with pores of defined diameters, effectively filtering out cells based on size [96].

Furthermore, microfluidic devices can be functionalized with specific antibodies or other probes, such as aptamers, to selectively separate CSCs based on their surface markers. For instance, CD44-positive circulating tumor cells in the blood of patients with hepatocellular carcinoma have been successfully detected and isolated using microfluidic devices [97]. Similarly, a microfluidic device functionalized against CD133 and epithelial cell adhesion molecule (EpCAM) was used to isolate CSCs in patients with pancreatic ductal adenocarcinoma [98]. In addition, label-free detection of CSCs is possible through a tandem mechanical sorting system, which identifies CSCs based on their flexibility, deformability, and low adherence properties [99]. A droplet-based microfluidic system can further isolate CSCs and encapsulate them in a controlled extracellular matrix environment, facilitating the study of CSC functions such as their Colony-Forming Unit (CFU) potential [100].

Laser Capture Microdissection

Laser Capture Microdissection (LCM) is a histology-based technique that isolates specific cell populations at the single-cell level from a tissue slide. The isolated cells can then be analyzed for various contents, including genomic and proteomic profiles, which greatly enhances cancer research. Tumors are inherently heterogeneous, containing various cell types, so whole-tumor tissue analysis often fails to provide information on specific cell populations. LCM allows researchers to detect and isolate rare Cancer Stem Cells (CSCs) at the single-cell level. While other cell isolation methods can extract CSCs, they often disrupt the cells' microenvironment during sample processing. With LCM, target cells are identified and selected through optical identification, staining, immunohistochemistry, or immunofluorescence staining [101]. In this technique, Formalin-Fixed, Paraffin-Embedded (FFPE) tissue samples are commonly used. A laser is precisely applied to excise the selected area containing the cell of interest, after which the sample is ejected into a collection tube [102,103]. LCM has proven valuable in distinguishing cell populations within xenograft models of head and neck cancer to study Cancer Stem Cell (CSC) signaling pathways [104]. For example, this technique was employed to isolate ALDH-positive (CSC-enriched) and ALDH-negative cells from stage III colon cancer patient samples, allowing for comparative protein expression analysis between the two groups [105]. LCM differs from previous cell isolation techniques by enabling the anatomical identification of cells. Technological advancements have addressed some limitations, such as improved imaging and the ability to isolate fixed cells. However, challenges remain, including the labor-intensive sample preparation process and potential sample integrity loss, which can affect subsequent transcriptomic or proteomic analyses.

miRNAs as Emerging Biomarkers for CSCs

miRNAs are a class of regulatory RNAs that modulate gene expression, and their dysregulation has been implicated in various cancers [106-108]. For instance, miR-145 is frequently downregulated in cancers such as ovarian, cervical, colorectal, and breast carcinoma [106,109. ver recent decades, miRNAs have been explored for their therapeutic potential as biopharmaceuticals in cancer treatment [107,110]. In CSCs, as in other cell types, miRNAs play crucial roles in regulating functions such as self-renewal, differentiation, and drug resistance. Differential miRNA expression profiles between CSCs and non-stem cancer cells offer a promising avenue for CSC detection. Certain miRNAs are unique to CSCs, including miRNA-34, miRNA-200b-3p, miRNA-7-5p, miRNA-92a, and miRNA-21, which are associated with the maintenance of CSC "stemness" [111]. Additionally, some miRNAs, such as miRNA-210, miRNA-10b, miRNA-93, miRNA-21, and miRNA-142, have been linked to CSC radio resistance, indicating that changes in their expression during radiotherapy could serve as biomarkers for treatment monitoring [112]. Table 2 summarizes various miRNAs identified in different cancer stem cell types, highlighting their potential as biomarkers [17,106,113].

Single-Cell Analysis and Multi-Omics Approaches to Analyze Cscs

Recent advances in single-cell isolation and barcoding technologies have enabled the quantification of DNA, mRNA, and protein profiles at the single-cell level. These experimental methods have been applied to diverse biological systems, highlighting the effectiveness of single-cell investigations. Computational approaches are also employed to identify clusters, lineages, and networks within this data. New techniques have emerged for single-cell research that have significantly enhanced the throughput and scope of these analyses. One of the primary challenges in single-cell analysis is amplifying the small amounts of nucleic acid present to reach the threshold required for detection. To address this, substantial technical developments in Whole-Genome Amplification (WGA) have been made, specifically to support single-cell genome research [114].

In Whole-Genome Amplification (WGA), various methods can be used to amplify the DNA of individual cells. One common

approach is Multiple Displacement Amplification (MDA), which offers linear amplification of DNA using random primers and Phi 29 DNA polymerase. Another technique, known as Multiple Annealing and Looping-Based Amplification Cycles (MALBAC), enables comprehensive genome analysis with consistent amplification. MALBAC is particularly useful for detecting Single Nucleotide Polymorphisms (SNPs) and Copy Number Variations (CNVs) within single cells [114]. Similarly, transcriptomic analysis at the single-cell level faces challenges due to the wide variety of RNA species within cells. However, several technological advancements have enabled researchers to overcome these limitations, allowing for the study of tumor heterogeneity and the discovery of novel biomarkers for cancer detection and prognosis. For instance, CEL-Seq enables RNA amplification in individual cells, while Drop-seq, a microfluidics-based single-cell sequencing technology, allows for the analysis of mRNA transcripts from single cells within droplets [114,115]. In proteomics analysis, advances in technology, such as mass cytometry, have made it possible to analyze more than 40 parameters at the single-cell level [114]. Most single-cell multi-omics approaches are designed for use with freshly isolated, intact cells. However, the scONE-seq technique has emerged as a method to amplify DNA or RNA from single cells in both frozen and fresh tissue samples, eliminating the need for single-cell suspension preparation [116]. Through multi-omics approaches, several biomarkers with high specificity and sensitivity have been identified for various cancers. In ovarian cancer, for instance, specific biomarkers include promoter methylation of genes like c17ORF64, IRX2, and TUBB6, as well as an expression profile of 10 miRNAs such as miR-320a, miR-665, miR-3184-5p, miR-6717-5p, miR-4459, miR-6076, miR-3195, miR-1275, miR-3185, and miR-4640-5p [117-119]. Similarly, multi-omics analyses have led to the identification of biomarkers for screening and diagnosing colorectal cancer [120], breast cancer [121] and sarcomas [122].

In Vitro Modeling of CSCs

2-D Culture Methods

Since the early 1900s, cells have been cultivated using two-dimensional (2D) techniques. However, this approach has significant limitations, as 2D models do not effectively replicate the behavior of tissue cells *in vitro* [123]. The drawbacks of 2D cell culture include restricted x-y plane adhesion, enforced apical-basal polarity, unrestricted spreading, and lack of soluble gradients, all of which can impact gene and protein expression, cell proliferation, morphology, and viability—factors critical for CSC studies [124]. In 2D cultures, cell morphology tends to be flat, while in 3D models, metastatic CSCs are observed forming aggregates, spheres, and colonies at the tumor periphery, better reflecting *in vivo* behavior [125]. Additionally, 2D culture techniques face challenges when used to study *in vitro* interactions involving tumor stroma, cell invasion, hypoxia, and migration. A promising alternative is the development of 3D cell culture methods that employ cell scaffolds, which more accurately simulate the complexity of *in vivo* tumors [126]. Jensen et al. provided a review comparing features of 2D and 3D cell culture techniques [123].

3D Culture Models of CSCs

Three-dimensional (3D) culture of Cancer Stem Cells (CSCs) can be achieved through scaffold-based or scaffold-free techniques. In scaffold-based models, a semi-solid matrix, such as hydrogels, provides the extracellular matrix necessary for CSC growth. The porous structure of these scaffolds creates a unique microenvironment that supports CSC adhesion, migration, and invasion, closely mimicking the tumor environment *in vivo*. This model allows researchers to study CSC cellular functions and analyze drug sensitivities [127]. An example of a scaffold used in CSC culture is the poly (lactic acid) (PLA) scaffold, which has been shown to promote the proliferation of breast CSCs [128,129]. Another effective scaffold, the chitosan-alginate composite, is capable of supporting the proliferation of a variety of CSC types, including those from breast, prostate, hepatocellular carcinoma, and glioblastoma [128,130].

Scaffold-free 3D CSC culture models: The primary techniques used in scaffold-free 3D CSC culture models focus on methods that prevent cell attachment, promoting the aggregation of cells into spheroids. These methods include suspension culture, hanging drop cultures, and the use of ultra-low attachment plates [128]. Suspension culture, often conducted in bioreactors, is typically applied for large-scale cell cultures. In the hanging drop technique, surface tension and gravity allow a droplet of growth media containing cells to remain suspended on the lid of a Petri dish, encouraging spheroid formation. This method has been successfully applied to produce prostate cancer organoids [128,131]. Materials like poly-hydroxyethyl methacrylate (pHEMA) are also utilized to reduce cell attachment probability, thereby enhancing aggregate and spheroid formation [132]. These scaffold-free techniques facilitate the development of 3D spheroids, offering a more realistic model for studying CSC behavior.

Organoids and Spheroids as Promising Models to Study CSCs

Tumoroids, spheres, and organoids can be cultivated from human tumors, preserving similar genotypic and phenotypic traits to the original tumors. These models are valuable not only for studying tumor heterogeneity but also for drug screening. For instance, patient-derived glioblastoma organoids have been used to reflect key features of parental tumors, such as cellular diversity, gene expression profiles, and mutational patterns [133]. Furthermore, coculturing tumor cells with tumor-associated immune cells enables the creation of organoid models that simulate the tumor immune environment. This approach demonstrated that tumor-specific T cell receptors remain highly conserved between tumors and their organoids [134]. These models are also promising for personalized drug screening, especially for patients with tumors resistant to conventional therapies. For example, colorectal cancer spheroids were utilized to assess patient-specific chemosensitivity, retaining differences in drug responses across individual patients [135]. Additionally, the efficacy of treatments was tested using Her2-negative, patient-derived spheroids, revealing that these models, unlike standard cancer cell lines, offer a platform to identify the most effective therapies [136]. Spheres and organoids also enable research into the role of the microbiome in tumor development. For example, gastric organoids have been used to study how H. pylori contributes to gastric cancer initiation [137].

Conclusion and Future Remarks

Cancer Stem Cells (CSCs) play a crucial role in the initiation,

maintenance, and progression of primary cancers, including invasion and metastasis. This central role has led cancer research to prioritize studying CSCs. In this review, we explored the most commonly used approaches to identify and isolate CSCs and discussed advanced technologies designed to replicate tumor environments, allowing for an in-depth understanding of CSC responses to environmental changes. These advancements pave the way for personalized therapy, tailored to a patient's physiological profile, which is especially beneficial for those who do not respond well to standard treatments. Additionally, recognizing the unique properties of CSCs can help minimize side effects associated with tumor-targeted chemotherapy and radiotherapy. However, challenges persist in the identification of CSCs, even in well-studied cancers like breast and colon cancer. CSC markers often lack specificity, necessitating the use of multiple markers for detection. The discovery of a specific CSC marker would enhance targeted drug delivery, improving specificity and reducing the cytotoxic impact on healthy tissues.

Author Statements

Author Contributions

EM proposed the review conception, FS prepared the first draft of the manuscript, EM critically revised the manuscript and all authors approved the final version of the manuscript.

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