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Cancer stem cell enrichment in *in vitro* models: Techniques, insights, and applications

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ABSTRACT

Due to the substantial role of cancer stem cells (CSCs) in therapeutic resistance and metastasis, the study of them is of great importance. However, their low abundance within the tumor cells makes it challenging to study their molecular pathophysiology. This limitation highlighted the critical need for reliable enrichment techniques to provide enough cells for in vitro tests. This review focuses on various methods that implemented for enrichment of cancer stem-like cells' subpopulations from the heterogeneous population of cancer cells. In this regard, various key physicochemical parameters such as hypoxia, shear stress, high glucose concentration, autophagy, and chemotherapy-induced stress have been explored for their potential to enhance CSC enrichment. The results presented in the literature confirmed that modifying these parameters can improve the enrichment of CSCs by promoting stem-like cells phenotype. However, there is still a necessity for comprehensive studies that compare the impact of these parameters on CSC enrichment, which should be addressed in future works.

1. Introduction

Tumor-initiating cells can possess the ability to self-renew, develop heterogeneous cells with different fates, and proliferate extensively, which are parallel to the features that distinguish stem cells from other cell types. These malignant cells, known as cancer stem cells (CSCs) [1,2], form a subpopulation within tumors characterized by two common properties: self-renewal and plasticity, driven by de-differentiation [3]. CSCs are known for their ability to activate tumor growth and proliferation, invasion and recurrence after treatment [4]. According to hierarchical model of cancer, CSCs are capable to evolve into various types of cancer cells while maintaining their own population with their self-renewal capacity [5]. This implies that CSCs with tumorigenicity can be the source of all malignant tumor cells. For example, studies have demonstrated that transplanting only one hundred $CD133^+$ human glioma cells (CD133 being a specific marker of CSCs for glioma) into the brains of immunodeficient mice led to tumor development. In contrast, there was no evidence of tumor initiation by transplanting 100,000 CD133-glioma cells.

Recent reports have provided an interesting connection between studies of CSCs and epithelial-mesenchymal transition (EMT), as one of the primary mechanisms involved in the metastasis process. EMT is a process in which epithelial cells lose their polarity and cell-cell adhesion, acquiring invasive and migratory properties to adopt a mesenchymal phenotype. The presence of tumor cells with such characteristics is associated with drug resistance and poor prognosis [6]. In addition to

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being invasive and migratory, cancerous cells can metastasize to far tissues under certain conditions, such as environmental stresses, low oxygen levels, and increased acidity. Recent studies showed that metastatic colonization is primarily facilitated by rare tumor-initiating cells, i.e., CSCs. These cells can circulate in the blood stream, survive hostile conditions, adapt to the new microenvironment at metastatic sites, and initiate new tumors through mesenchymal-epithelial transition (MET) [7].

CSCs are protected by special molecular mechanisms and are a major cause of tumor relapses after treatment. Despite all the advances in cancer treatment, cancer remains one of the leading causes of death worldwide, accounting for nearly 10 million deaths in 2022 [8]. According to recent data, more than 35 million new cancer cases will be increased globally by 2050, representing a significant rise of 77 % in comparison with 20 million estimated cases in 2020 [9]. Lung cancer is the leading cause of cancer death mortality, contributing to 1.8 million deaths, while colorectal and liver cancer are considered as second and third mortality rates, respectively [9]. Conventional therapies are usually able to eliminate cancer cells in the bulk of tumor masses but fail to remove CSCs. The resistance of these cells to treatment arises from their innate or acquired adaptation mechanisms, such as expression of anti-apoptotic proteins, increased expression of ATP binding cassette (ABC)-transporters, stability in hypoxic conditions, increased activity of repair enzymes, survival in quiescence state, and protection within the tumor niche (Fig. 1) [10]. This persistent resistance highlights the urgent need to develop innovative therapeutic strategies targeting CSCs to improve treatment outcomes.

Identifying distinct molecular markers that differentiate CSCs from other cancer cells is currently the primary approach to recognizing these cells. Cell surface biomarkers and functional assays are the main tools used for the detection and isolation of CSCs. Some of the most commonly studied biomarkers are listed in Table 1.

There are various methods for identifying CSCs, including biomarker-based and label-free techniques. These techniques are utilized to obtain CSCs through the detection of cell surface marker expressions and other internal cell biomarkers, such as flow cytometry [5], mass cytometry [101], immunostaining [102], molecular profiling [103], and single-cell RNA sequencing (scRNA-seq) [103,104]. However, such methods for the characterization of CSCs are not only time-consuming and error–prone but also require extensive training, highlighting the need for label-free and automated techniques [105, 106]. Imaging, a label-free technique, provides additional insights into CSCs by enabling researchers to understand the pathogenesis of different cancers, predicts treatment outcomes, and assess patient prognosis [107]. Molecular imaging (MI), a state-of-the-art technology, has the potential for improving our insight into cancer stem cell biology. For example, advanced imaging modalities such as magnetic resonance imaging (MRI) [108] and positron emission tomography (PET) [109] can enhance the identification of CSC characteristics, thereby supporting the development of effective and personalized cancer therapies. These imaging strategies enable tumor biology visualization and *in vivo* tracing of CSCs, enhances our insights into their specific role in tumor biology and initiating therapeutic resistance [108]. In addition, the combination of advanced imaging techniques with innovative computational methods could facilitate the monitoring of CSCs in tumors [110].

Recent progress in microfluidics has enabled researchers to perform high-throughput imaging and analysis of individual cells under a controlled setting [111]. To evaluate the morphological features of CSCs, such as size and shape, brightfield microscopy is employed to distinguish them from non-stem cells [112,113]. However, the lack of specificity of brightfield imaging reveals the importance of cutting-edge image analysis methods to precisely quantify various cellular phenotypes [114]. Deep learning (DL) has recently emerged as a robust

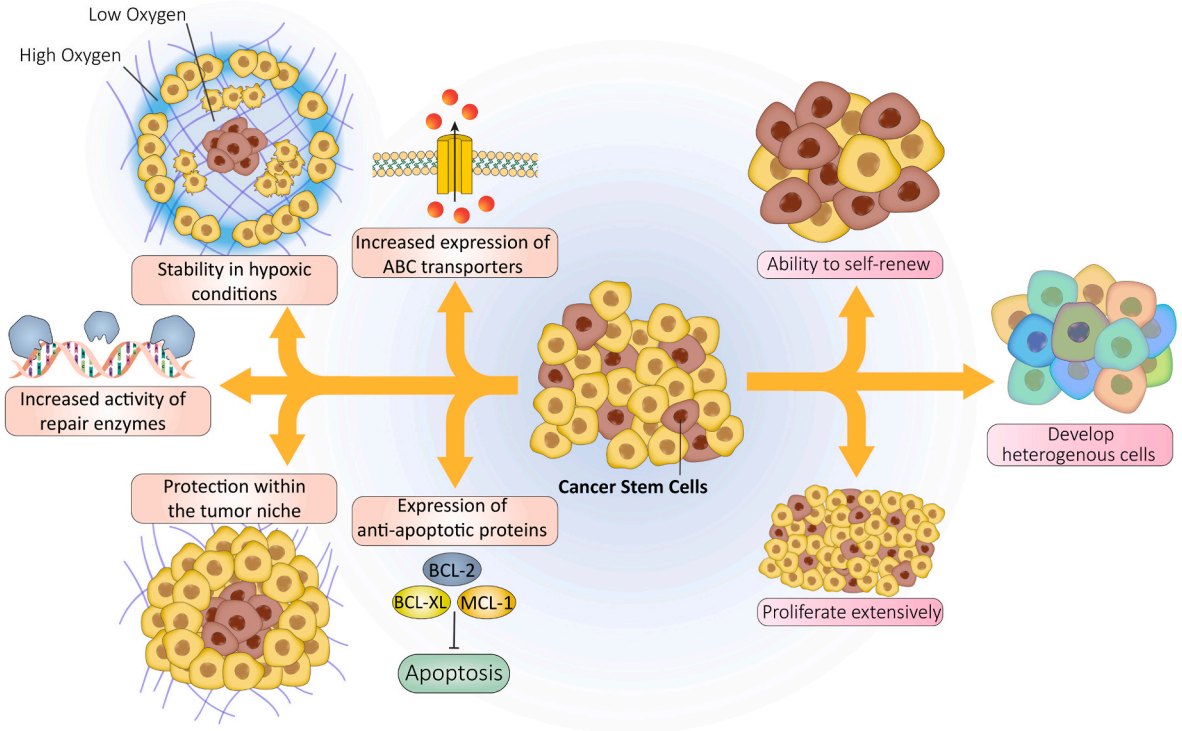

Fig. 1. CSC characteristics in tumorigenesis. CSC exhibits a significant role in tumor progression and development. Their self-renewal ability ensures their survival and preserves their population. Generating a wide variety of cancer cells from CSCs within the tumor leads to the higher heterogeneity of cell types and cell adaptability and survival [5]. Rapid tumor proliferation is caused by their extensive proliferation, and increased expression level of ATP-binding cassette (ABC) transporter plays a significant role in resistance against chemotherapy treatments [11]. Moreover, by the expressing anti-apoptotic protein [5], CSCs can evade from programmed cell death. They show resilience in hypoxic environments, enabling them to be maintained and survive [12]. The upregulation of DNA repair enzymes under genotoxic stress promotes metastasis and recurrence [5].

Table 1
Biomarker expressions in various cancer types.

Cancer Type	Biomarkers	Ref.
Brain Tumors	CD133, CD44, CD49f, N-CAM-L1, A2B5, Nestin, SOX2, Musashi-1, CD15	[4,13–21]
Breast Cancer	CD24, CD44, CD133, Ep-CAM, ALDH-E1, LGR5, SSEA-3, CD166, ITGA6, Trop-2, ABCG2, SSEA-3, CD70, and PROCR, Mucin-1, GD2, Nectin-4.	[22–29]
Colon Cancer	CD133, CD44, LGR5, CD24, CD26, CD29, Ep-CAM, ALDH1A1, CD166, SOX2, NANOG, OCT4, CD51	[30–36]
Cervical Cancers	CD133, CD44, CD49f, CD13, CD29, CD105, ALDH1A1, SOX2 and OCT4, NANOG, MSI1 (Musashi-1)	[37–40]
Ovarian Cancers	CD133, CD44, ALDH1, CD24, CD117, CD105, CD106 (VCAM-1), Ep-CAM, Nestin, SSEA1, CD90.	[41–50]
Head and Neck cancer	CD44,CD24,CD98, CD133, CD166, ALDH1, EpCAM Bmi-1, Nanog, Oct-4, SOX2	[51–54]
Hematological cancer	CD34, CD38, CD19, CD26, CD133, CD123, IL-1RAP	[55–59]
Liver Cancer (HCC)	CD44, CD90, CD24, CD133, CD13, Ep-CAP, Ep-CAM ALDH,	[60–62]
Lung Cancer	CD44, CD133, CD166, CD90 (Thy-1), Ep-CAM, CD117 (c-KIT), ABCG2,CD87	[57, 63–68]
Esophagus cancer	CD271, CD133, CD44, CD24, CD90, CD338, CD14, CD71	[35, 69–76]
Pancreas Cancer	CD24, CD44, CD133, DCLK1, CXCR4, Ep-CAM, Oct4, ABCB1	[77–84]
Prostate Cancer	CD44, CD24, CD133, CD166, CD151, ABCG2, ALDH1	[85–91]
Skin cancer	CD20, CD271, CD133, CD44, ALDH1, Nestin	[57, 92–95]
Gastric Cancer	CD44, CD24, CD54, Ep-CAM, CD133	[35, 96–100]

Abbreviations: N-CAM-L1 (Neural Cell Adhesion Molecule L1), A2B5 (A2B5 Antigen), Nestin (Nestin), SOX2 (SRY (Sex Determining Region Y)-Box 2), Musashi-1 (Musashi RNA Binding Protein 1), Ep-CAM (Epithelial Cell Adhesion Molecule), ALDH-E1 (Aldehyde Dehydrogenase 1), LGR5 (Leucine-Rich Repeat Containing G Protein-Coupled Receptor 5), SSEA-3 (Stage-Specific Embryonic Antigen 3), ITGA6 (Integrin Alpha 6), Trop-2 (Tumor-associated calcium signal transducer 2), ABCG2 (ATP-Binding Cassette Sub-Family G Member 2), PROCR (Prothrombin Receptor), Mucin-1 (Mucin 1), GD2 (Disialoganglioside GD2), Nectin-4 (Nectin Cell Adhesion Molecule 4), ALDH1A1 (Aldehyde Dehydrogenase Family 1 Member A1), NANOG (Nanog Homeobox), OCT4 (Octamer-Binding Transcription Factor 4), DCLK1 (Doublecortin-Like Kinase 1), CXCR4 (C-X-C Chemokine Receptor Type 4), ABCB1 (ATP-Binding Cassette Sub-Family B Member 1).

method, playing a vital role in the detection of CSCs, significantly improving both efficiency and accuracy of diagnosis and prognosis [115]. Nonetheless, the broad application of DL faces several challenges, including issues with interpretability, data availability, generalization, and computational resources demands [115].

Dielectrophoresis (DEP) is an innovative microfluidic technique that separates cells based on their unique electrical features [116]. This approach is increasingly acknowledged for the isolation and label-free recognition of circulating tumor cells (CTCs) and cancer stem cells (CSCs) [117]. The process of DEP depends on the cell behavior when exposed to varying electrical fields [118]. This electric field polarizes the cells, generating a dielectrophoretic force that can effectively facilitate cell separation and preserve their viability without the need for labeling. This minimizes potential cell damage caused by technical errors [119]. The combination of DEP and microfluidic systems significantly improves high-throughput sorting of CSCs, which is crucial for early cancer diagnosis and choosing treatment modalities [120].

Targeted therapy with innovative therapeutic agents such as tyrosine kinase inhibiting agents, monoclonal antibodies, chimeric antigen receptor (CAR)-T cell and cancer vaccines aimed at selectively eradicating CSCs [113]. However, the study of drugs that specifically target CSCs has been challenging due to the low abundance of these cells within the cancer cell population and their instability in cell culture conditions [121]. Therefore, researchers have developed methods to enrich the population of these cells in test samples (CSC enrichment), enabling focused experiments to better understand their function and role in drug resistance [10,122]. This review highlights the innovative methods reported in the literature for CSC enrichment and discusses their findings in detail.

2. 3D-culture-based platforms for CSC enrichment

Since 3D models provide a more physiologically relevant microenvironment for in vitro studies, research on CSCs using these models could improve our insight into the mechanism of carcinogenesis, tumor progression, metastasis, tumor recurrence, and drug resistance (Fig. 2). Such insights are critical for advancing drug discovery strategies [123].

2.1. Suspension culture

In cancer research, suspension culture conditions facilitate the growth and expansion of cancer cells more effectively than adherent systems [127]. Suspension culture methods are increasingly acknowledged for their effectiveness in enriching CSCs, which plays a pivotal role in the process of tumor initiation, metastasis, and development of resistance to treatment [128]. The spinner culture method is the constant stirring of cell suspension in a bioreactor flask enable to facilitate spheroid generation effectively. The interplay between bioreactor dimension and stirring condition plays a significant role in size determination of spheroid [129,130]. Massai et al. designed a bioreactor for dynamic suspension culture of cancer cell spheroid under shear stress conditions. Human lung cancer cell line showed improvement in spheroid morphology, increased in size (2–4 fold) and cell cycling (1.58-fold), and decreased in DNA damage (1.58-fold) in dynamic suspension culture system [131].

By optimizing growth factor levels and culture conditions, researchers can improve the purity and yield of CSC populations, which is essential for exploring their biological activities and proposing possible therapeutic interventions [132,133].

2.2. Ultra-low attachment surface for 3D culture

Ultra-low attachment culture condition is a key strategy in cancer research and serves as a fundamental technique for isolating and enriching CSCs [134]. This approach involves using plates coated with non-reactive materials, such as poly-hydroxyethyl methacrylate (pHEMA) or agarose, which effectively disrupt the plating of cells [128, 135]. This feature is vital for CSC enrichment and isolation due to their unique characteristics such as the ability to resist anoikis and proliferation without attachment. Gao et al. used an agar-based 3D culture system to successfully isolate CSCs from different tumor cell lines, including colorectal carcinoma (HCT116), prostate cancer (LNCaP, VCaP, DU145, and 22Rv1), and hepatoma (HepG2). This method demonstrated a remarkable improvement in enrichment of CSCs compared to ECM-based 3D culture, which contain fewer CSCs [134].

Serum-free medium (SFM) supplemented with growth factors, including epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), plays a key role in the optimal enrichment of CSCs [136]. A study on colon cancer stem cells revealed that the yield of CSCs was notably influenced by various concentrations of EGF and bFGF when cultured for 10, 20 and 30 days. The highest enrichment was achieved with a combination of 20 ng/ml EGF and 20 ng/ml bFGF after 30 days, leading to the highest expression levels of CD44 and CD133 (stem cell markers) and a considerable number of spheroids [128]. Utilizing a serum-free strategy effectively reduces the variability in cells due to different serum components, resulting in more reliable and reproducible results in the enrichment of CSCs. Additionally, the absence of serum reduces potential contamination from unknown sources, providing

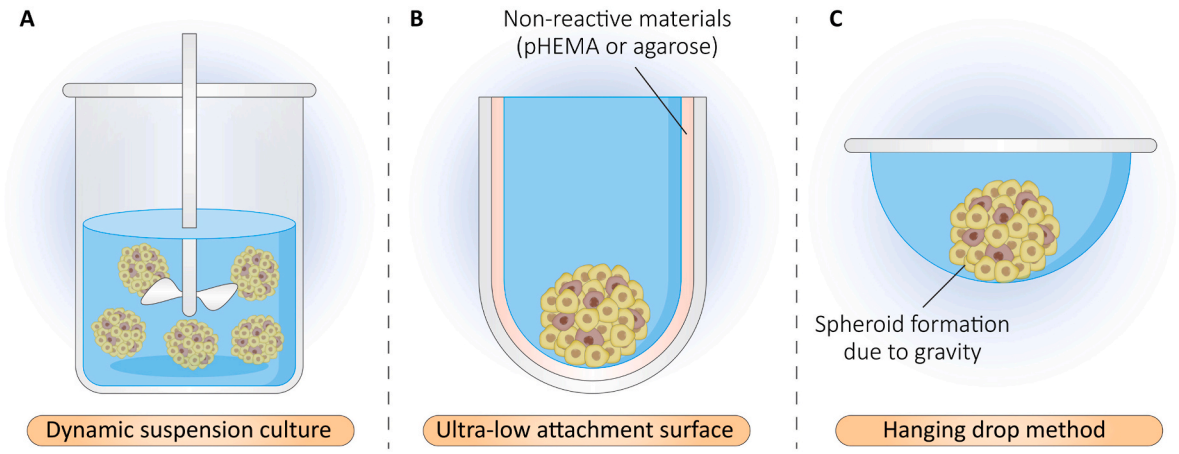

Fig. 2. Spheroid formation in dynamic and static suspension culture can be achieved through various methods; Spinner flask (dynamic), ultra-low attachment and hanging drop method (static). A. Dynamic suspension culture can be conducted by using spinner-flask approach. Continuous agitation of cell suspension in bioreactor containers or spinner flasks enhances spheroid formation [124]. B. Ultra-low-attachment and anchorage –independent culture can be optimized for 3D spheroid formation by coating poly (2-hydroxyethyl methacrylate) (pHEMA) or agarose on the surface [125]. C. The formation of spheroid in the hanging–drop technique can be achieved by inverting droplet of cell suspension and inducing aggregation through gravity [126].

controlled culture conditions and a clearer platform for studying CSC biology. This strategy facilitates the broad application of enriched CSC populations in preclinical studies, including drug development and discovery [137].

Spheroid culture systems are increasingly recognized as a practical strategy for raising the population of CSCs, either through single-cell-derived spheroids (SCDS) or multicellular spheroids (MCS) [138–140]. Research has shown that SCDS exhibit higher expression levels of CSC marker genes, suggesting that single-cell cultures may be more efficient at enriching CSC populations due to their superior self-renewal properties in a 3D environment [141]. Although spheroid cultures, which consist of multicellular tumor spheroids and tumor-derived organoids, are powerful tools for investigating CSCs, the heterogeneous nature of multicellular aggregates complicates the identification and isolation of CSCs. This variability leads to inconsistent expression of CSC markers, hindering the accurate evaluation of spheroids' actual stem cell population [133]. These three-dimensional cultures can be generated from various cancer types, including ovarian, breast, glioma, colon, and prostate cancers. They are practically useful for evaluating CSCs properties like tumor formation capacity and developing resistance to chemotherapy [142].

Establishing spheroid from patient-derived circulating tumor cells (CTCs) is a promising strategy for enriching and investigating CSCs [143]. The effective isolation and proliferation of patient-derived hepatocellular carcinoma CTCs have demonstrated that aggregates containing two or more CTCs, or cluster of CTCs, produce colonies with greater metastatic potential rather than a single CTC [144]. In addition, short-term recurrence of HCC in patients can be predicted more effectively using CTC enrichment in 3D culture condition compared to conventional methods, such as immunogenic separation via nanoparticles or physical isolation based on cell size [145]. EpCAM and Wnt/β-catenin pathways are usually expressed with CTC spheroid formation [144], notably in HCC and other cancers. These signaling pathways are essential for the tumorigenicity and spheroid formation capacity of tumor cells [144].

2.3. Hanging drop method

The hanging drop technique is a scaffold-free method for developing 3D cell culture systems [146]. This method evaluates the sphere formation capacity of cancer cells. In the hanging drop method, the suspension of cancer cells in a small droplet leads to spheroid formation due to gravity. This approach provides a low-adhesion environment, enabling cells to aggregate and simulate tumor-like conditions [147]. The formation of tumor 3D spheroids using this approach induces the expression of cancer stemness genes and elevates the levels of CSC markers, such as CD44 and CD24 [148]. By adjusting different factors in the tumor microenvironment, like oxygen levels and nutrient availability, the function and behavior of CSCs could be modulated. Recent research have shown that optimizing these parameters promotes CSC enrichment [149].

Monico et al. established a 3D melanoma spheroid model by hanging drop method embedded in the artificial dermis to enable tumor study and behavior with human skin components. In this study, multicellular aggregates derived from WM 1617 melanoma cells resulted in spheroids about 420 μm in diameter after 14 days [150]. Hepatotoxicity presents challenges in drug development, for this reason, Hurrell et al. focused on (3D) spheroid cultures by hanging drop-Hep-G2 spheroid culture to more accurately represent in vitro liver physiology [151].

2.4. Organoids

Organoid technology has significantly advanced the study of CSCs by recapitulating their microenvironment [152]. Organoids enable researchers to study CSCs and their complexities in tumor biology using a 3D model [153]. These models provide a valuable platform for mimicking the heterogenicity of primary tumor tissue, thereby improving therapeutic outcomes [154]. This is crucial for understanding of CSC biology, which is important for tumor initiation, progression, and metastasis [155]. The procedure begins with the isolation of CSCs from tumor tissues using fluorescence-activated cell sorting (FACS) [5]. The use of patient-derived organoids provides a practical platform for drug screening based on individual tumor profiles [156]. Organoid-derived CSCs can be embedded in a 3D extracellular matrix, such as Matrigel, containing specific growth factors [157]. Using CRISPR/Cas9 techniques for genetically modifying target cells and combining stromal and immune cells within organoids facilitates the study of CSC interactions with other cells and enhances CSC enrichment (Fig. 3) [158]. In addition, the investigation of CSC behaviors gives rise to uncovering the mechanism underlying tumor resistance and developing an effective approach to overcome this obstacle [159].

Although organoid culture is promising, it encounters various challenges, such as variations in cultural settings leading to differences in organoid characteristics [163]. Current organoid culture methods often lack critical components of the tumor microenvironment, such as immune cell recruitment and blood vessel network [164]. Advances in

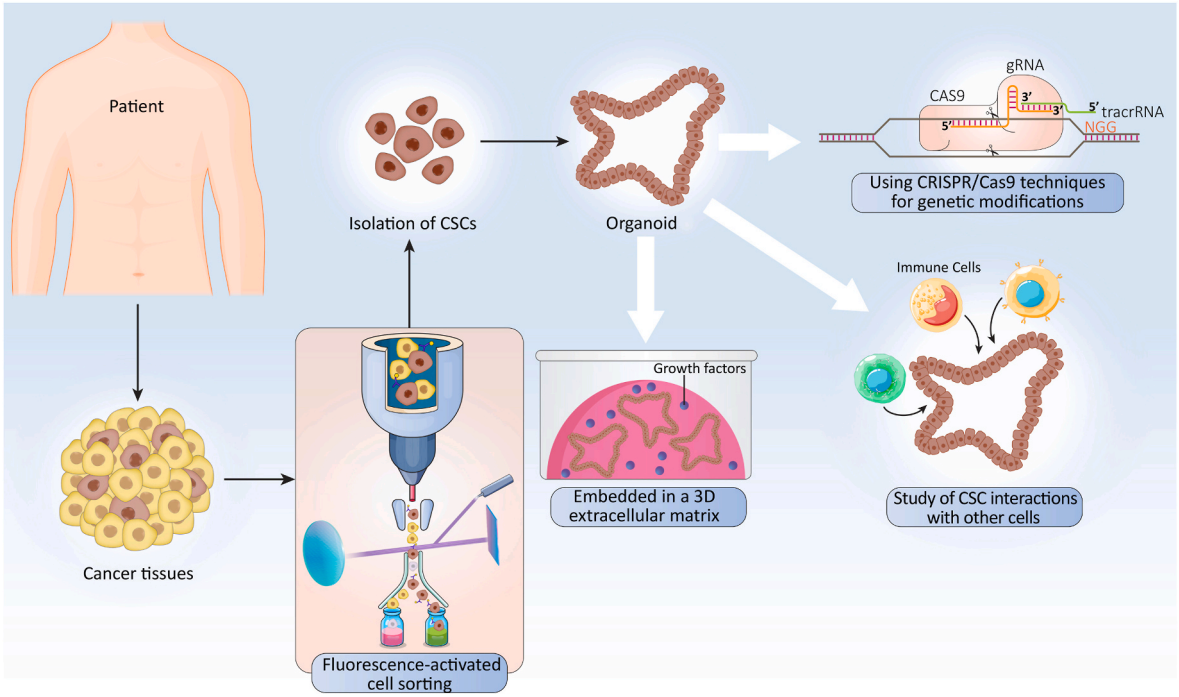

Fig. 3. This figure demonstrates the study of CSCs through the application of organoid systems. A. By isolating CSC from patient-derived tumor tissue, researchers can generate organoid [160]. B. The development and functionality of organoids can be supported by embedding in ECM. C. Employing CRISPR/Cas9 techniques facilitates the generation of genetically modified organoids for studying cancer-associated genes and cancer-associated mechanisms [161]. D. The cross-talk among organoid and other tumor microenvironment cells, especially immune cells, improving our understanding of CSC behavior [162].

microfluidic systems and imaging technologies are likely to improve the utility of organoids in cancer research [165].

2.5. Scaffolds

A supportive microenvironment is necessary for the maintenance and function of CSCs. This microenvironment comprises the extracellular matrix (ECM), cancer-associated fibroblast (CAF) [166], hypoxic condition [167], immunologic cells [168], cytokines, and growth factors [169]. Recent advancements in biomaterial-based 3D culture systems for CSC enrichment have created opportunities to better mimic tumor microenvironment interactions compared to solid tumor tissue slice, mammospheres, and traditional 2D culture systems [170]. Research findings demonstrated that a 3D cell culture system employing natural ECM-based scaffolds like collagen was developed for CSC tumorgenicity studies using breast cancer cell lines. This platform showed that the expression of pro-angiogenic growth factors and CSC markers, including OCT4A and SOX2, were significantly increased. These 3D-models effectively recapitulate the tumorigenic features of CSCs and mimic the *in vivo* microenvironment in term of physiologic activities [171].

In a study focused on osteosarcoma, hybrid scaffolds fabricated from magnesium-infused hydroxyapatite and collagen have been used. These scaffolds facilitated the formation of sarcospheres, whose aggregation enriched CSCs and increased the expression of stemness-associated genes [172]. In addition, the advancement of 3D cell culture systems using scaffolds reflects the heterogeneity inherent in the tumor microenvironment. Studies have highlighted the importance of scaffolds for improving CSC research. This approach provides an environment that closely mimics *in vivo* conditions, with polymer-based scaffolds playing a critical role in studying CSCs, particularly in terms of drug responses and interactions with the tumor microenvironments [171]. Research has shown that different scaffolds, such as polycaprolactone (PCL), collagen, and hyaluronic acid, remarkably increase the CSC populations derived from different sources [134,171,173,174].

2.6. Microfluidics

The application of microfluidics allows researchers to generate dynamic microenvironments and perform single-cell analyses. In addition, various parameters such as oxygen pressure, glucose concentration, and fluid shear stress can be controlled using microfluidics systems [149]. Adjusting specific environmental/external factors through these systems can also further promote the enrichment of CSCs (Fig. 4). Microfluidic platforms designed for spheroid cultures can optimize the similarity of the microenvironment to tumor tissues, thereby enhancing CSC populations and maintaining their viability [170]. While conventional culture methods often fail in single-cell analyses, microfluidic systems allow researchers to efficiently evaluate and analyze the heterogeneous population of CSCs [175]. One study highlighted the application of a microfluidic platform containing 18,000 wells to culture renal CSCs, facilitating drug screening for testing drug resistance-related factors while preserving CSC self-renewal capacities [176]. In addition, microfluidic technology operates with picoliter to nanoliter volumes, significantly reducing sample consumption and improving detection sensitivity [177].

Recent advancements in microfluidic platforms have enabled the efficient isolation of cancer stem cells. This system is designed to minimize reagent consumption while effectively isolating small cell populations. The isolation process is based on the distinct characteristics of cancer stem cells compared to non-stem cancer cells, such as adhesion properties, flexibility, and antibody expression profiles. In these systems, heterogeneous cancer cells samples are processed so that target cells attach to the inner surface of the microfluidic device, while non-target cells are excluded from the system.

The advantages and disadvantages of all techniques mentioned in Table 2.

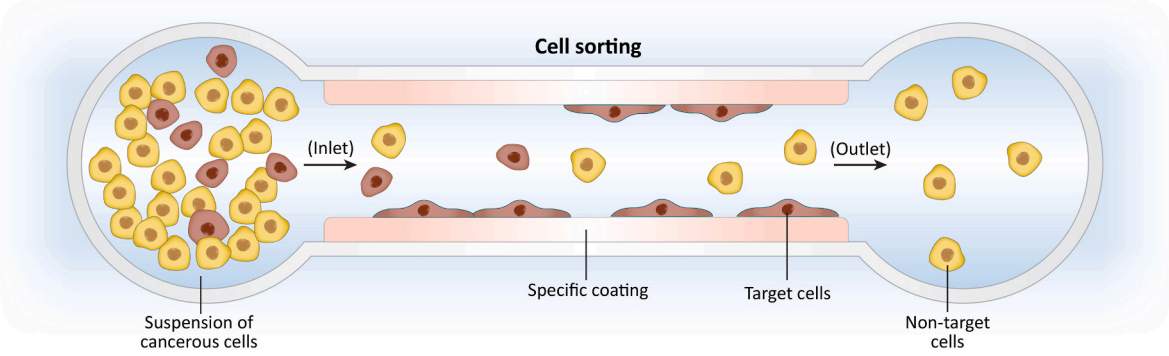

Fig. 4. Basics of CSC isolation in microfluidic devices.

Table 2
CSC enrichment techniques.

Technique	Principle	Advantages	Limitations	References
Suspension Culture	Culturing cells in agitated condition to form the sphere	Provide physiological conditions for cell-cell and cell-ECM interaction Scalability and operational efficiency	Optimizing agitation speed to conserve cell viability. Media volume and cell concentration	[178]
Ultra-low Attachment Surface	Minimizing cell attachment by inert substance-coated plate for cell culture (agarose or poly-hydroxyethyl methacrylate)	Suspension and anchorage-independent growth of cells promote spheroid formation and CSC enrichment.	Spheroid size variation, Reducing cell viability and yield because of necrotic center	[173, 179–182]
Hanging Drop Method	Gravity-driven spheroid formation		Labour-intensive Variation in spheroids' diameters	[173, 183–185]
		Uniform Spheroid formation without using scaffold	Time-consuming	
		Cost–benefit	Media evaporation and high osmolality in droplets.	
Organoids	A self-organized 3D cell culture structure, derived from primary tissue	Cellular heterogeneity of real organ Important for personalized medicine applications		[161,186]
		Improved the architecture of the tumor microenvironment	Lack of innate immune cell population.	
		Mimics the heterogeneity of the original tumor by a high level of plasticity	The technology is still in the early stages.	
ECM-based Scaffolds	Natural-based biomaterial (collagen, gelatin, etc.) and Engineered-based 3D structure for cell seeding and growth	Facilitate co-culture and the investigation of cells with intracellular matrix.	Variability and complex protein mixtures in the composition of natural ECM-based scaffolds have various effects on CSC behavior.	[187–189]
Microfluidics (Organ-on-a-chip)	Engineered microchannel network enhancing tissue-level perfusion and flow dynamic	Provide meticulous regulation of biochemical concentration and mechanical stress, which is essential for enhancing CSCs	Failing long-term maintenance of CSC-enriched culture	[190,191]

3. Mechanisms of CSC enrichment

3.1. Anoikis

Normal cells typically adhere to the surrounding extracellular matrix and upon detachment, they undergo programmed apoptosis, known as called anoikis. However, cancer cells can escape anoikis and, instead, acquire invasive and migratory traits (Fig. 5) [192]. Anoikis resistance is closely intertwined with the driven mechanism of EMT and cancer stemness marker enrichment [193]. EMT induces anoikis resistance through the repression of epithelial junction like E-cadherin and the induction of mesenchymal markers like N cadherin, Twist, Snail, and Zeb1 [194]. This molecular reprogramming facilitates tumor cells to promote anoikis resistance and acquire stem-like properties. PI3K/Akt and Wnt/β-catenin are among the key signaling pathways for anchorage-independent survival which are activated during cell detachment, inhibiting apoptosis and increasing the expression of stemness-associated markers like SOX2 and CD44 [195,196]. Activation of the integrin-mediated PI3K/Akt signaling pathway sustains cell viability, which supports anchorage-independent proliferation. E-cadherin reduction leads to CSC marker expression (e.g., ALDH1 and CD133) through lncRNAs-based epigenetic regulation (e.g., APOC1P1-3) and β-catenin nuclear accumulation [197]. As a result, the population of cancer stem-like cells could be enriched by inducing anoikis. A 3D cell culture system provides a simple and effective approach to achieving this.

Tumor multicellular spheroids are among the most well-established models for studying cancer biology. These models serve as a bridge between conventional two-dimensional (2D) cultures and *in vivo* models, effectively recapitulating key tumor cell characteristics, including growth kinetics, cellular heterogeneity, signaling pathways activity, and gene expression profile [199,200]. Moreover, CSCs can be successfully cultured in the spheroid format. Compared to 2D culture systems, spheroid better preserve the essential properties of CSCs, including gene expression patterns, colony formation capacity, tumorigenesis, differentiation potential, cytokine secretion profile, and resistance to chemotherapy [201–208]. Therefore, some researchers utilize this approach (generating cancerous spheroid aggregates and inducing anoikis) to enrich cancer stem-like cells.

Spheroids and patient-derived organoids are two distinct types of three-dimensional (3D) cell culture models that are widely used in biomedical research. Each of these models has its own set of characteristics and applications. Spheroids are pretty basic 3D groups of cells. They are usually made when one or more types of cells, like cancer cells

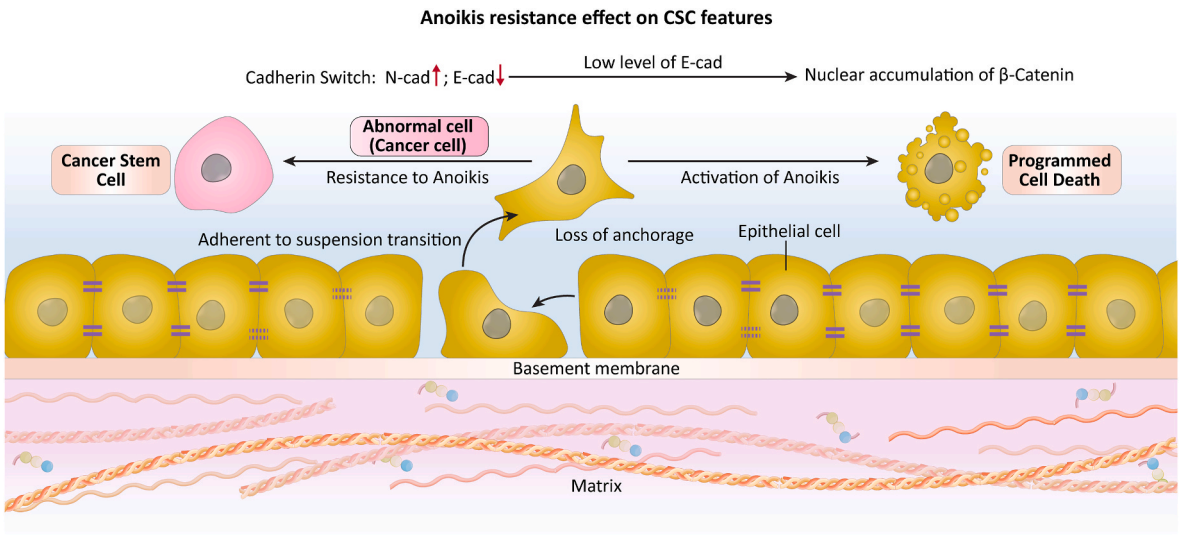

Fig. 5. Anoikis is a specific form of programmed cell death that typically occurs when normal cells lose their attachments to the extracellular matrix (ECM). This process is activated by the disruption of integrin-mediated signaling, which generally supports significant signals for adherent cell survival. In contrast, cancer cells utilize specialized strategies to escape anoikis, facilitating their survival. This mechanism is moderated by two signaling; Autocrine signaling – the survival of cancer cells can be promoted by producing growth factors for self-support; and paracrine signaling – supporting growth factors in the surrounding microenvironment of cells produced by adjacent cells that support resistance to anoikis [192]. Signaling pathway activation results in the stabilizing of β-catenin and its subsequent translocation to the nucleus, where it facilitates the transcription of genes associated with CSC characteristics, EMT and resistance to apoptosis. This process facilitates tumor progression by allowing cancer cells to avoid anoikis and improve survival during metastasis [198].

or immortalized cell lines, grow together on their own as free-floating culture. They typically lack organized tissue architecture and are made up of a single or mixed cell population with minimal differentiation. In contrast, patient-derived organoids are more advanced three-dimensional structures that are created from stem or progenitor cells that are directly isolated from patient tissues. These organoids replicate tissue architecture, various differentiated cell types, and certain organ-specific functions by self-organizing into smaller, more simplified representations of organs. The complexity of organoids enables a more accurate modeling of physiological processes, including hormone secretion and drug metabolism, compared to spheroids. Additionally, while spheroids form quickly but are less stable over long periods of time, organoids have the ability to regenerate, allowing for long-term culture and maintenance. Because of these distinctions, spheroids are frequently used to study basic cell behavior, tumor biology, and drug response in a controlled, simplified setting, whereas organoids serve as advanced models for investigating organ-specific biology, disease mechanisms, and precision medicine [209].

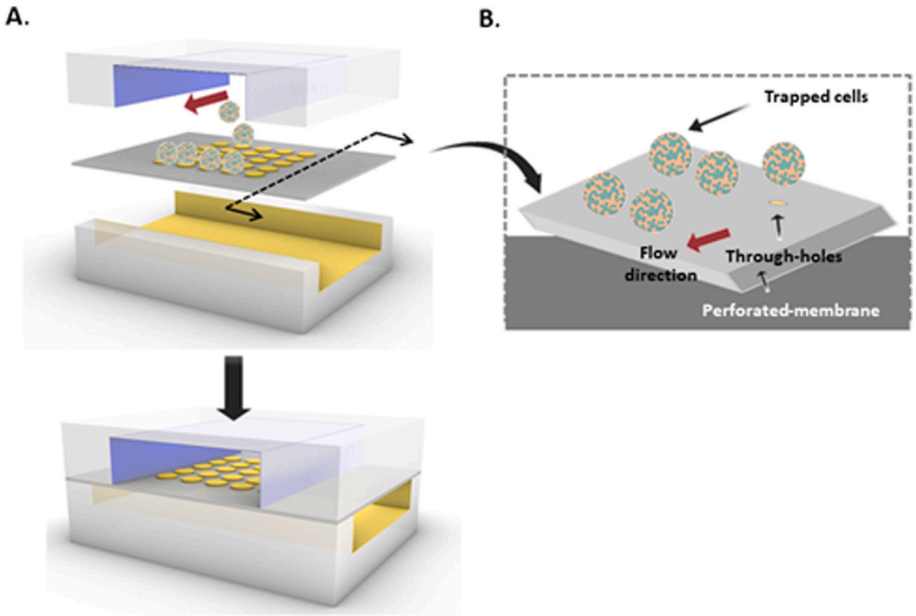

Fig. 6. A multi-layered, assembled chip is designed by Kue et al., comprised of dual superimposed microfluidic systems. Each layer is controlled separately; however, the fluid-fluid interface of two sets of reagents increases efficiently. In addition, a perforated membrane plays a significant role in increasing selective permeability and making mass transfer easier between the two channels. The vertical orientation of two channels enables better optimization of fluid flow in different layers, supporting multiple analyses and reactions simultaneously. This also prevents the risk of contamination. The membrane which contains a unique perforation pattern, facilitates the separation of specific particles with high precision [210].

Researchers have used this technique to enrich CSCs [210,211]. Their microfluidic system consisted of two superimposed channels separated by a perforated membrane (Fig. 6A). The upper channel was designated for cell culture and loaded with culture medium to maintain cell survival, while the lower channel generated suction through the porous membrane to trap the cells (Fig. 6B). Their microchip was separately employed for 2D and 3D cell culture systems. Collagen type 1 and Pluronic copolymers were used to coat the membrane for 2D and 3D culture systems, respectively. They compared SKOV3 cells (the ovarian carcinoma continuous cell line) cultured in spheroid format within the microchip to those cultured in a conventional 2D system. Their observations revealed a decrease in expression of some epithelial markers (CD326) and upregulation of mesenchymal genes (*CDH2*, *VIM* and ***FN1***) in the spheroid culture in comparison to the 2D model. Fundamental to these alterations are essential survival signaling pathways, including PI3K/Akt/mTOR and MAPK/ERK, which enable cells to avoid anoikis by suppressing apoptotic signals and facilitating cell cycle arrest [212,213]. Additionally, these pathways involve in TGF-β and Wnt/β-catenin signaling cascades to maintain epithelial-mesenchymal transition (EMT) and improve stemness [214,215]. Additionally, wound healing analysis indicated an increased migration capacity of spheroid cells compared to 2D-cultured cells. By evaluating drug resistance to cisplatin and paclitaxel, they demonstrated that, across all tested doses, cellular resistance to therapy was greater in the spheroid model than in the 2D model.

Researchers studied ovarian epithelial cancer cells by culturing serum-free spheroids and compared the results with attached cells [216–218]. The spheroids exhibited more CSC-related properties than the adherent cells. Also, these cells were more tumorigenic and demonstrated resistance to cisplatin, paclitaxel, Adriamycin, and methotrexate. The results showed an upregulation of *NANOG, SOX2, OCT4,* ***NES****, ABCG2,* ***PROM1*** *(CD133)*, and ***KIT*** (CD117). In addition, the number of CD117+ and CD133+ cells increased significantly. They also found that EMT plays an important role in the enrichment of ovarian CSCs. They blocked EMT using P13K/AKT pathway inhibitors, and showed that stemness markers, mesenchymal markers, and resistance to paclitaxel were decreased.

In a serum-free spheroid culture of lung cancer cell lines A549 and H446, Yan et al. [219] showed that tumorigenicity and the expression of stemness genes (*SOX2* and *OCT4*) were significantly increased compared to 2D cultures. They also proposed CD90 as a specific biomarker for lung CSCs. The stimulation of the PI3K/AKT pathway can induce anoikis resistance in lung cancer cells. A wide range of proteins modulate anoikis resistance via the PI3K/AKT pathway. CEA, TrkB, FAK/Src, and βIII-tubulin facilitate the phosphorylation of AKT to provide resistance to anoikis, as noted. The activation of PI3K/AKT facilitates epithelial-mesenchymal transition (EMT) and suppresses caspases, enabling cells to evade anoikis [220].

Pandit et al. [221] established a serum-free spheroid culture of Hepa1-6 cells (Hepatocellular carcinoma cell line). Their findings indicated that tumor cells cultured in the spheroids form showed greater tumor formation capacity and growth in a mouse model compared to adherent cells. In addition, their results demonstrated that both CD90 and CD44 surface markers (known as HCC-CSC markers) were upregulated under 3D culture conditions. An Upregulation of the Wnt/β-catenin signaling pathway was also observed in spheroid cultures.

Gheytanchi et al. [222] cultured HT-29 and Caco-2 cells (human colon adenocarcinoma cells) using hanging drops (3D) and forced floating techniques under serum-free and non-attachment conditions. When comparing spheroids with parental adherent cells, they found that all examined CSC surface markers (CD44, CD133, and CD166) were upregulated in both HT-29 and Caco-2 cells in spheroids. Their results further indicated an upregulation of stemness-related genes, including *SOX2*, *C-MYC*, *OCT4*, and *NANOG* in HT-29 spheroids, and *SOX2*, *MYC*, and *KLF4* for Caco-2 cells in Caco-2 spheroids. The activation of survival signaling pathways, specifically PI3K/AKT, MAPK, along with the increased expression of ABC-transporters, specifically *ABCB1* and *ABCG2,*was observed in HT-29 spheroids [223]. In addition, the expression of EMT genes was upregulated, including *TWIST1, SNAI1, ZEB1*, and *CDH1* in HT-29 spheroids, and *SNAI1* and *VIM* in Caco-2 spheroids. However, the expression of *CDH2* was downregulated in spheroids of both cell lines.

Calvet et al. [224] assessed four cell lines, B16-F10 (murine melanoma), HT-29, MCF-7, and MDA-MB (human breast adenocarcinoma) by spheroid formation on an ultra-low adherence surface. They concluded that spheroid-based CSC enrichment is cell-line dependent. For example, they discovered that B16-F10 spheroid cells exhibited lower tumorigenic, clonogenic, and proliferative capacities than adherent cells. Moreover, *VIM* and *CDH1* were downregulated in B16-F10 spheroids, and the percentage of cells expressing the CSC markers (CD133, CD44 and CD24) remained low, similar to adherent cells. The mechanism of anoikis resistance in B16-F10 melanoma cell is primarily through the activation of FAK/RhoA/Akt signaling pathways, consistent with the low expression of stemness markers [225].

3.2. Hypoxia

Based on the distribution of oxygen, nutrients, and waste products, the tumor mass is divided into three regions: the necrotic core, the quiescent zone, and proliferative rim, as shown in Fig. 7. In the proliferative region, cells receive an adequate supply of oxygen and nutrients, allowing them to divide and grow rapidly. In contrast, the necrotic zone experiences low concentrations of nutrients and oxygen due to lack of blood capillaries and the accumulation of waste products, ultimately leading to cell death. The quiescent zone contains cells that are temporarily inactive; if they do not receive adequate nutrients or oxygen, they may undergo apoptosis [226,227].

Hypoxia occurs when the oxygen level within the tissue falls below normal physiological levels due to an imbalance between vascular supply and tissue demand [229,228]. Since the 1950s, researchers have observed that hypoxic tissues exhibit greater resistance to radiation compared to normal tissues and require higher levels of radiation exposure [230]. Hypoxia-induced factors (HIFs) are transcription factors that regulate cellular responses to hypoxia [231]. These factors prevent cell differentiation, promote angiogenesis, and regulate apoptosis. HIFs also contribute to resistance against therapeutic agents targeting DNA integrity by activating dsDNA repair enzymes [228,232]. Hence, hypoxia has a significant effect on cancer treatment, and researchers have developed analytical and numerical models to predict hypoxia inside tumor aggregates [233–239].

Hypoxia maintains stemness of CSCs through six different mechanisms: 1) Induction of EMT, 2) Activation of reactive oxygen species in response to stressors, 3) Activation of stemness-related signaling pathways, such as Wnt/β-catenin [240], TGF-β/SMAD [169], Notch [241], SIRT1 [242], STAT3 [243], 4) Upregulation of stemness-related genes, including *OCT4, NANOG, SOX2* [244], *BMI1* [245], *MYC and KLF4* [246,247], 5) Suppression of differentiation-associated genes, and 6) cross-talk between endothelial cells and CSCs [248].

To recapitulate, hypoxia actively contributes to poor clinical outcomes by increasing the risk of metastasis, promoting angiogenesis, inducing treatment resistance, and enhancing CSC enrichment [229,232,248–250].

Heddleston et al. [251] isolated CSCs and non-stem cancer cells (CD133+ and CD133-cells) from glioblastoma tumor masses and cultured them in two-dimensional at oxygen concentrations of 2 % and 21 %. Their neurosphere formation assays revealed that hypoxia promotes the self-renewal ability of both cell types. Furthermore, their findings demonstrated that hypoxia induces cancer stem-like cell phenotype in the non-stem cancer cell population by upregulation of *OCT4*, *NANOG*, and *MYC*.

Lan et al. [252] established a 2D culture model of human breast cancer cells under oxygen concentrations of 1 % and 20 %. The results demonstrated that hypoxia upregulated adenosine receptor 2B (AR2BR)

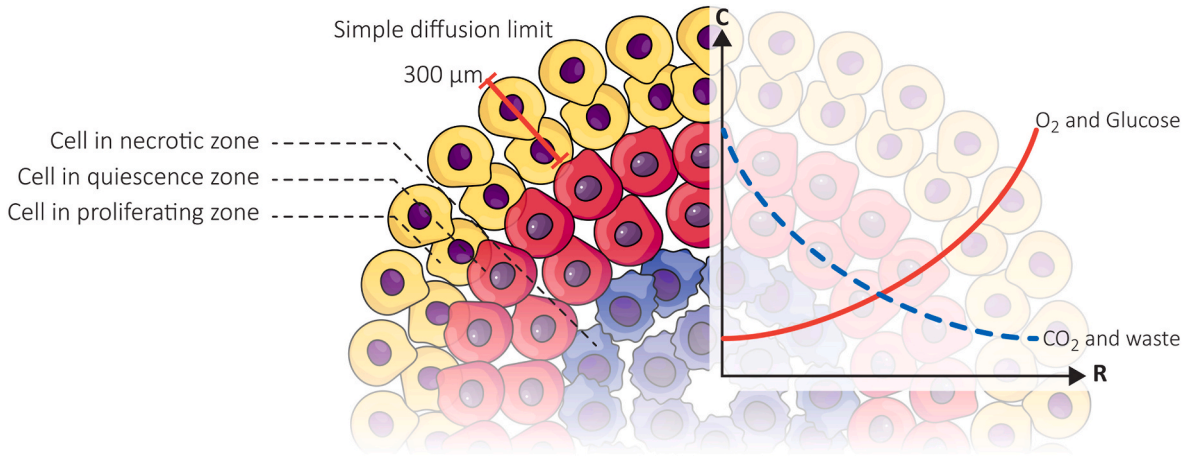

Fig. 7. Microfluidic-3D-based studies are suitable for CSC enrichment and the regulation of O2 and glucose levels instead of using conventional-2D cell culture. A simple diffusion limit within cancer spheroid results in the hyperproliferative zone in the outer layer, hypoxic zone in the intermediate, and necrotic zone in the inner layer. Utilizing microfluidic systems significantly regulates the O2 and Glucose levels which are essential for CSCs enrichment [228].

through HIF1 transcriptional activity in the cell population. They concluded that the binding of adenosine to A2BR promotes CSC enrichment in breast cancer cells by activating protein kinase C-δ (*PRKCD*)

Instead of using a low oxygen incubator, Mahkamova et al. [253] induced hypoxia in thyroid cancer cell lines by treating 2D cultures with cobalt chloride ($CoCl_2$), which activates the HIF pathway. They illustrated an increase in the side population of thyroid CSCs following $COCl_2$ treatment, confirmed through side-population analysis and the evaluation of stemness gene expression profiles.

3.3. Shear stress

Researchers have demonstrated that mechanical stimuli, such as pressure, stretching, and shear stress, can affect the morphology, biomarker expression patterns, and aggressiveness of cancer cells [254–257]. In addition to advancements in microfluidic technologies for cancer research [258], controlled shear stress conditions make this platform practical for the simultaneous enrichment and analysis of CSCs. In a study [254], a microfluidic system was employed to culture spheroids of SKOV3 cancer cells under dynamic condition with fluid shear stress to investigate its impact on the enrichment of CSCs. They found that SKOV3 cells significantly expressed EMT and CSC markers under fluid shear stress. Moreover, resistance to paclitaxel and cisplatin under dynamic conditions (with shear stress from microfluidic media flow) was significantly higher compared to static conditions. Furthermore, sphere formation and tumor growth in nude mice were remarkably enhanced under dynamic conditions.

Triantafillu et al. [259] investigated the effect of high shear stress (equivalent to the bloodstream shear stress experienced by circulating cancer cells) on breast cancer cells. For this purpose, they utilized a syringe attached to a tube to establish a constant flow rate, thereby applying shear stress to the cells. Their findings indicated that although fluid shear stress did not significantly affect the EMT expression pattern, it remarkably increased the CSC population by increasing the clonogenicity index and the upregulation of *OCT4* and *NANOG* genes.

3.4. Glucose metabolism

In normal cells, oxidative phosphorylation (OXPHOS) in the mitochondria is the primary mechanism by which ATP is produced. This process is responsible for providing approximately 70 percent of the energy requirements of the cell [260]. Under normoxic conditions, glycolysis, which takes place in the cytoplasm, is responsible for contributing the remaining 30 % of ATP production [261]. However, under hypoxic conditions, its contribution significantly increases in order to compensate for the decreased efficiency of electron transport chain (OXPHOS) [260]. Nevertheless, cancer cells demonstrate metabolic reprogramming that is primarily characterized by aerobic glycolysis (the Warburg effect), which results in the preferential conversion of glucose to lactate even in normoxia. Metastasis and immune evasion are both made easier by the acidification of the microenvironment caused by the subsequent excretion of lactate [262]. Due to the complex range of microenvironmental conditions in which they survive, CSCs probably get their energy from multiple sources. The evidence suggests that both glycolysis and oxidative-based metabolisms play critical roles in CSC energy production. In addition, amino acids such as glutamine and lysine act as alternative fuel sources for CSCs. Glucose catabolism *via* glycolysis mediates the biosynthesis of amino acids and nucleotides, supporting rapid cell growth and division. The metabolic shift from oxidative phosphorylation to glycolysis effectively enables cancer cells to survive, proliferate, migrate to distant locations, and invade secondary tissues, even in harsh oxygen-deficient environments [263,264]. CSCs produce ATP via mitochondrial oxidative phosphorylation (OXPHOS), unlike bulk cancer cells that prefer glycolysis. Glycolysis facilitates CSC proliferation in hypoxic environments; it serves as a secondary function to OXPHOS. Therefore, the survival and stemness characteristics of CSCs can be diminished by inhibiting glycolysis [262]. The maintenance of CSCs also depends on fatty acid oxidation (FAO), which supplies necessary substrates for OXPHOS and redox balance, including acetyl-CoA and NADPH [265].The roles of NANOG and PPAR-δ in controlling lipid metabolism in CSCs are crucial, especially in enhancing CSC stemness and tumorigenicity through fatty acid oxidation (FAO). NANOG is an important transcription factor for maintaining CSCs [266]. It changes the metabolism by blocking oxidative phosphorylation (OXPHOS) and increasing mitochondrial FAO. This metabolic change promotes chemoresistance, tumor initiation, and self-renewal, particularly in CSCs from hepatocellular carcinoma (HCC) [267]. The nuclear receptor known as PPAR-δ, which is strongly associated with the expression of NANOG, further enhances the activity of FAO and the metabolic pathways involved in lipid metabolism in CSCs [268]. Overexpression of either NANOG or PPAR-δ leads to an increase in FAO, which, in turn, promotes redox balance and energy production, both of which are essential for the survival and stemness of CSCs [5] (Fig. 8). Many studies have demonstrated the importance of glucose metabolism for the maintenance and propagation of CSCs across various cancer types [263,269–273].

Lin et al. [274] cultured hepatocellular carcinoma cells (PLC8024, Huh7, and Hep3B cell lines) under 2D conditions with two different glucose concentrations (2.5 and 25 mM). They observed an increase in

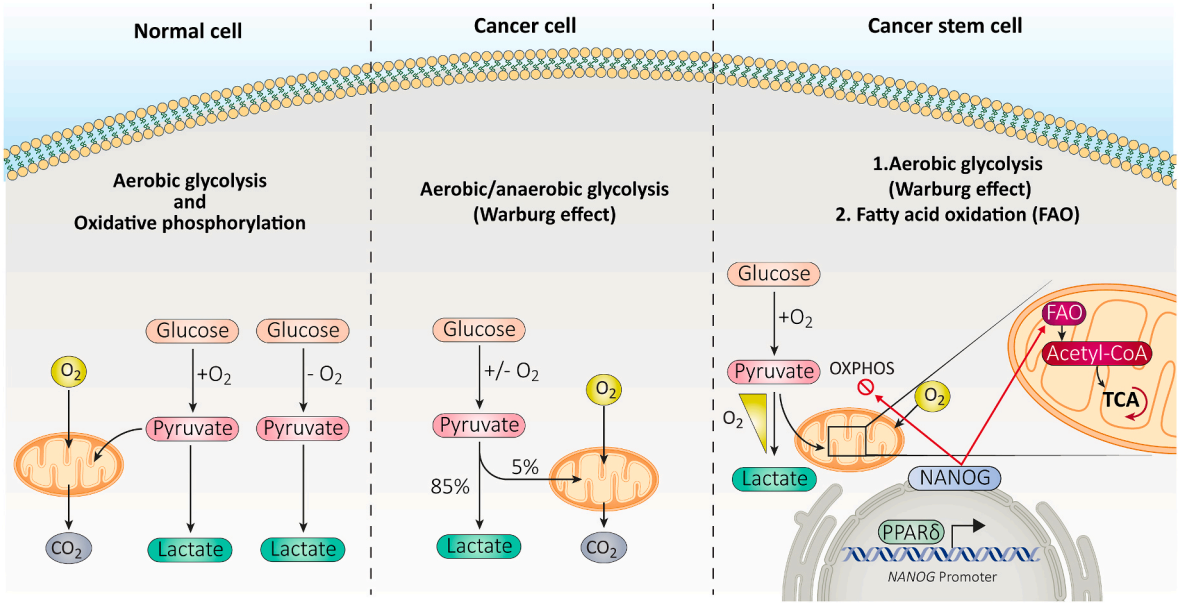

Fig. 8. Comparison of the predominant ATP production pathways, glycolysis and the metabolic functions of fatty acid oxidation (FAO) as energy metabolism in normal, cancer and CSCs. The primary source of energy production for normal cells is oxidative phosphorylation, while cancer cells prioritize aerobic glycolysis. Cancer stem cells primarily rely on glycolysis and oxidative phosphorylation depend on FAO, to maintain their energy and stemness features.

the percentage of CD133+ cells with higher glucose concentrations across all three cell lines. In addition, analysis in PLC8024 cells revealed that key glycolysis-related genes, including *GLUT2*, *PFKL*, *PGAM1*, *PKM2*, and *LDHA*, were significantly upregulated under high-glucose conditions. Their findings also indicated that the hexosamine biosynthetic pathway (HBP) plays an important role in maintaining the

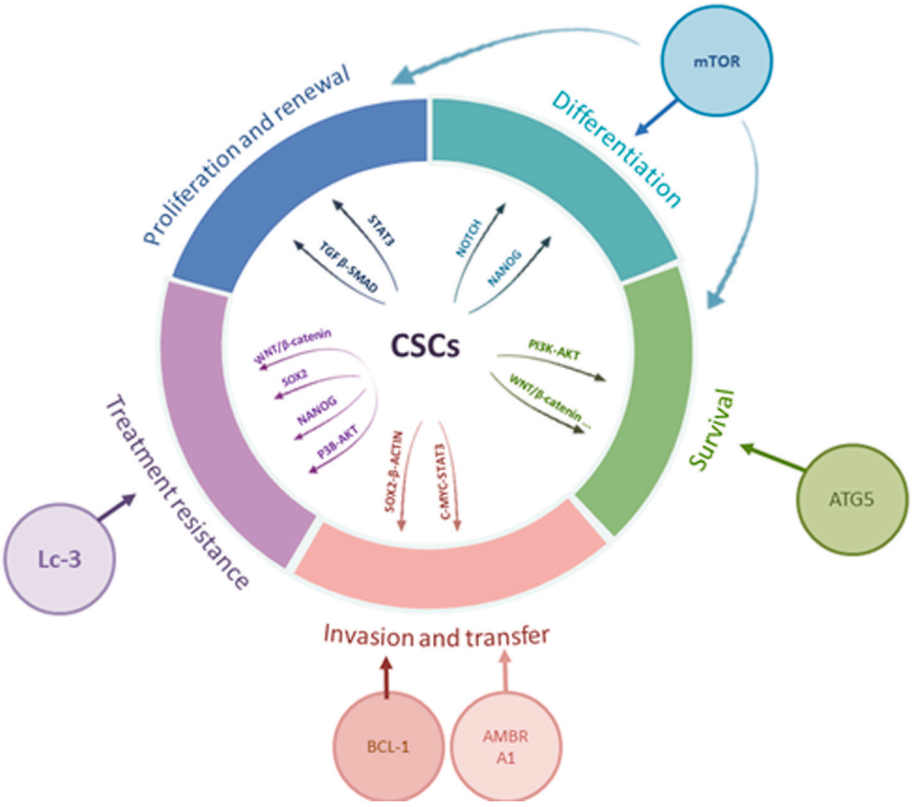

Fig. 9. Autophagy regulatory pathways in CSCs. This figure highlights the various functions of autophagy in CSCs, including differentiation, survival, invasion, treatment resistance, proliferation, renewal, and key signaling pathways. Autophagy regulates the fate of cancer stem cells by impacting proliferation through the TGF-β and STAT3 signaling pathways, and survival via the PI3K-AKT and WNT-β-catenin pathways. Autophagy contributes to therapy resistance in CSCs by recycling damaged cellular components and activating key transcription factors, such as NANOG and SOX2. The upregulation of autophagy increases the migration and invasion capabilities of CSCs and facilitates the overexpression of mesenchymal marker expression. Furthermore, autophagy facilitates tumor vascularization by promoting CSC differentiation into endothelial cells, thereby supporting angiogenesis.

CSC-like phenotype in hepatocellular carcinoma cells.

3.5. Autophagy

Autophagy is a catabolic process essential for eliminating dysfunctional organelles and damaged proteins, thereby maintaining cellular homeostasis [275,276]. The autophagic process is recognized by three main stages: initiation (forming autophagosomal structure), extension (enlargement of the autophagosome), and maturation (lysosomal fusion and degradation) [277]. Autophagy plays a dual role in cancer biology, acting as a double-edged sword. In the early stage of cancer, autophagy inhibits cancer progression by reducing the accumulation of mutagenic factors. However, during the later stages of carcinogenesis, autophagy supports cancer cell survival under stressful conditions and inhibits apoptosis, thus promoting tumor progression [278,279].

Autophagy is vital for maintaining the plasticity of CSCs (Fig. 9). Generally, CSCs exhibit higher levels of autophagy, with elevated expression of key autophagy-related genes, such as *BECN1* and *ATG5*, which are crucial for CSCs self-renewal and resistance to treatment modalities. [280,281]. In contrast, applying autophagy inhibitors has resulted in a decline in CSC population, accompanied by a reduction in autophagy marker expression.

Similar findings have been observed with mitophagy, a selective form of autophagy that targets damaged mitochondria. In CSCs, the expression levels of mitophagy-related genes, such as *PINK1* and *PRKN*, are significantly elevated [279]. These findings propose a strong association between autophagy activation and enrichment of CSCs.

3.6. Other parameters

Researchers showed that in addition to the above-mentioned points, some culture-related factors are effective in enriching CSCs within the cancer cell population. Rao et al. [282] showed that utilizing ultra-low adhesion plates (ULAP) for CSCs enrichment requires approximately ten days to achieve optimal levels. However, employing core and shell microcapsules (CSMCs) to form spheroids significantly reduced this time to just two days. Using a coaxial needle, they produced a core containing culture medium and prostate cancer cells (PC-3) surrounded by an alginate hydrogel shell. The proportion of $CD44^{+}$ CD133+ cells, which were maximal in the ULAP model on the tenth day, reached its maximum in the CSMC model by the second day, with significantly higher levels compared to the ULAP model. Additionally, the expression of pluripotency genes (*OCT4*, *NANOG*, and *KLF4*) and *in vivo* tumorigenicity of the spheroids cultured in CSMC model on day 2 were remarkably higher than those cultured in the ULAP model on day 10.

Hu et al. [283] treated the cells with carboplatin to investigate the effect of chemotherapy on the enrichment of CSCs in hepatocellular carcinoma (HCC) cells. Their findings indicated that chemotherapy treatment significantly increased spheroid formation and upregulated the expression of *SOX2* and *OCT3/4* in HCC cells.

Using two different protocols for spheroid formation in ULA plates, MCTS (multi-cellular tumor spheroid model) and SCESM (stem cell-enriched spheroid model), Goričan et al. [284] cultured Fa Du HNSCC (head and neck squamous cell carcinoma) spheroids to identify which model enriches CSCs more efficiently. The two models differed in their culture medium compositions: MCTS used DMEM/F12 medium supplemented with 10 % FBS and 1 % P/S, while SCESM included DMEM/F12 medium supplemented with 10 ng/mL of epidermal growth factor, 10 ng/mL of basic fibroblast growth factor, B-27TM (50 ×) serum-free supplement, and 1 % P/S. Their results demonstrated that cell proliferation, protein expression of CSC markers (CD44, CD73, CD90, CD133, and OCT4), and gene expression of *NANOG*, *SOX2*, and *ALDH1A1* were higher in the SCESM model compared to MCTS model.

4. Conclusions and perspectives

Understanding unknown features of CSCs is necessary for establishment of effective treatment strategies. This review highlighted various laboratory methods used for CSC enrichment and briefly discussed their advantages. Researchers could valuably increase CSC-like phenotype in cancer cell populations through factors such as anoikis, hypoxia, and high glucose concentration. Moreover, mechanical forces like shear stress, the use of chemotherapy agents, hydrogel platforms, and stem culture media have also been shown to facilitate CSC enrichment in *vitro* models. Their results have included comprehensive analyses of tumorigenicity, sphere formation, stemness-related gene expression profile, and CSC surface marker expression. However, there is still a lack of comprehensive comparative studies that assess all these parameters to determine the most effective approach for CSC enrichment.

In conclusion, future research should focus on developing innovative platforms to study and enrich CSCs more effectively, with an emphasis on understanding their drug resistance mechanism and molecular pathogenesis across different cancers.

CRediT authorship contribution statement

Fazeleh Ranjbar-Niavol: Writing – original draft, Visualization, Investigation. **Maryam Barisam:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Arezoo Khosravi:** Writing – review & editing, Writing – original draft, Visualization. **Navid Kashaninejad:** Writing – review & editing, Conceptualization. **Ali Zarrabi:** Writing – review & editing, Conceptualization. **Massoud Vosough:** Writing – review & editing, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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