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Cancer Stem Cells in Tumor Modeling: Challenges and Future Directions

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Microfluidic tumors-on-chips models have revolutionized anticancer therapeutic research by creating an ideal microenvironment for cancer cells. The tumor microenvironment (TME) includes various cell types and cancer stem cells (CSCs), which are postulated to regulate the growth, invasion, and migratory behavior of tumor cells. In this review, the biological niches of the TME and cancer cell behavior focusing on the behavior of CSCs are summarized. Conventional cancer models such as 3D cultures and organoid models are reviewed. Opportunities for the incorporation of CSCs with tumors-on-chips are then discussed for creating tumor invasion models. Such models will represent a paradigm shift in the cancer community by allowing oncologists and clinicians to predict better which cancer patients will benefit from chemotherapy treatments.

1. Introduction

Cancer stem (or stem-like) cells (CSCs)[1] show distinctive properties such as self-renewal, the capability to differentiate into multiple lineages, and extensive proliferation.[2] Malignant features have been observed in primary tumors during the conversion of tumor cells into CSCs, resulting in distant metastases. The role of CSCs in tumor progression, metastasis, and drug resistance has been postulated by several researchers.[3–5] Welch and Hurst defined the hallmarks of metastasis by four distinguished features, including motility and invasiveness, ability to modulate microenvironments, plasticity, and ability to colonize secondary sites.[6] The metastatic cascade's multistep process begins with the development of metastatic cells and uncontrolled cell proliferation, followed by angiogenesis, motility and invasion, intravasation, dissemination and transport, cellular arrest, vascular adhesion, and extravasation, and colonization in distant organs within the body.[6–8] Considering these steps at the cellular and tissue levels is critical for developing diagnostic tools and effective cancer treatments. The early CSC-based models for studying the response of cancers to systemic anticancer drugs have been 2D culture models. Engineered 3D culture models have addressed a few of the challenges in using 2D models; however, they still lack some features of the tumor microenvironment (TME)[9] and proper control over cell–cell interactions.[10] Biomimetic platforms can process and manipulate small volumes of fluid through channels to support tumor growth.[11,12] They provide spatial and temporal control at the micrometer and millimeter scales resulting in different extracellular matrices (ECMs), various types of cells, and fluids, which can be arranged to mimic the TME better and replicate the in vivo growth behavior of tumor cells.[13]

Microfluidic platforms support small sample sizes, low reagent consumption, short processing times, enhanced sensitivity, real-time analysis, and automation together in one unit.[14] The delivery of nutrients and maintenance of physiological stresses in the microreactor enables cells to transform into tissue-like structures.[10] Many microfluidic platforms use polydimethylsiloxane (PDMS) as the framework, in which the PDMS substrate provides simple fabrication, optical transparency, tunable elasticity, gas permeability, and cost effectiveness.[15] Some examples have been used for manipulating proteins and cells via biosensors, single-cell assays for disease diagnosis and

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modeling, organs-on-chips, and other applications.[16,17] Microfluidic platforms can be used to study tumor progression in unconventional, radical ways with high-throughput screening potentials.[18] However, they have been unable to capture and replicate ECM stiffness, proper conditioning of tumor cells, and ECM–cell interactions.

To tackle the challenges associated with conventional microfluidic devices in mimicking TMEs, tumors-on-chips have been introduced.[19,20] Tumors-on-chips have been used to model oxygen and nutrient gradients,[21,22] cell signaling and migration,[23,24] proliferation behavior,[25–29] protein and gene expression,[30,31] morphological and organizational changes,[28,32] and drug responses.[33,34] They are composed of microfluidic channels in which media with nutrients are supplied to the surrounding cells,[35] and the presence of multiple cell types in tumors-on-chips has been shown to mimic the key factors of the TME and the progression of tumor cells.[36] These models allow us to simulate cell–cell signaling and the physical cues in the TME, such as hypoxia and physical forces. Therefore, these models can influence cancer cell growth.

CSCs contribute to anticancer therapeutic resistance, and patient survival depends on our ability to target CSCs in the treatments.[37–40] The level of spatial and temporal control provided by microfluidics incorporating 3D cell culture has opened up new avenues for research on cancer invasion, extravasation,[41] and drug response.[42] Not only have tumors-on-chips reduced drug development time and cost, but they have also eliminated ethical issues related to animal studies[43] and are compatible with CSC research.[44] The authors anticipate that the inclusion of CSCs into tumors-on-chips will provide novel insights into the metastatic behavior of the tumor cells and other key responses. In this Review, the authors discuss the biological cues of the TME and how they are modeled by engineering technologies. Then, some approaches are proposed to tackle the challenges associated with incorporation of CSCs into tumors-on-chips.

2. Cancer Stem Cells and the Tumor Microenvironment

Tumors exhibit significant interpatient and intrapatient heterogeneity. Even if individual cells within a tumor all share a common genetic reflection of their clonal origin, single-cell analysis has shown the existence of variations in genetics and epigenetics between different cells or locations within a tumor.[45] One possible approach for explaining the heterogeneity is the clonal evolution model (i.e., stochastic model), which is based on the accumulation of mutations that leads to tumorigenic potentials in time via genetic and epigenetic changes. A second common approach includes the hierarchical conversion of stem cells to CSCs (i.e., hierarchy model) with malignant features associated with stem-cell niches. The first approach differs from the second because the mutation hypothesis in the clonal model cannot explain carcinogenesis, abnormal divisions, and epigenetic changes.[46] Genetic and cell biology studies support the hypothesis that tumors contain more than the monoclonal growth of cells, and they should have CSCs. The inclusion of CSCs may ensure the invasion of malignant cells with its TME with myeloid cells, phagocytose, immune cells, and tumor-associated fibroblasts.[1,47] Phenotypic plasticity of cancer cells denotes their capacity to interconvert between stem-like and differentiated states, which correlates with the hierarchical and evolution models.[48] In this perspective, contingent on the genotype and the TME signals, cancer cells may return to the CSC pool to redeem long-standing tumor repopulation.[49] This differentiation capacity is inherited (or the hierarchical model) or via mutations to a stem-cell-like permissive epigenome (or the stochastic model). The cell plasticity is thus considered as a third model that combines the first two models to provide the reversible transformation of cancer cells between stem-like and differentiated states.

The presence of CSCs has been a subject of controversy in the literature.[45] Recent studies in stem cell biology have supported the CSC hypothesis.[50] Wicha et al. explained the role of accumulated multiple mutations in normal stem cells for carcinogenesis. For example, the women exposed to radiation during their late adolescence in Hiroshima and Nagasaki showed the highest susceptibility of developing breast cancer.[51] This is substantial as the mammary glands have the highest number of stem cells in the late adolescents period. In the case of carcinogenesis, tumor-initiating cells are the first mutated cells in the bulk that promote cancer origin to more mutagenic properties, but CSCs have a higher self-renewing capacity.[52] Also, native stem cells can differentiate to mature cells for a specific tissue while using common signaling pathways (such as Wnt/β-catenin, Hh, Notch), similar to those of CSCs. Their self-renewability is processed by asymmetric replication and stochastic differentiation.

The kinetic properties of native stem cells include slow growth rate, residing in a specific niche, and short frequency of abundance. The CSC pools carry specific surface biomarkers and have short telomeres that can initiate a rapid tumor growth.[53] When healthy stem cells transform to CSCs evading from immune surveillance and dysfunctional cell divisions can be observed. Differentiated cells can transform the other cells by their cancerous components. In a heterogeneous microenvironment, communications between native stem cells and CSCs promote malignancy.

The TME includes biochemical or signaling molecules, different cell types, the ECM, and some biophysical cues.[54] The specific features consist of fibroblasts, endothelial cells (ECs), and immune cells. The secreting factors and receptors control the signaling cascades for self-renewal and differentiation.[55] In addition to these key factors, there is hypoxia induced by a lack of oxygen, as well as elevated solid stress and interstitial fluid pressure, all of which promote cell proliferation and resistance to drug transport (as shown in **Figure 1**). Tumor-associated hypoxia promotes uncontrolled proliferation, angiogenesis, invasion, and metastasis of tumor cells. Hypoxic regions occur at the center of the tumor, and the aldehyde dehydrogenase+ (ALDH+) population of CSCs stimulates the mesenchymal-like nature of cells and gains a high proliferative rate as well as their resistance to death. In one study, hypoxic effects in the TME, including CSCs were evaluated in a study for glioma related to rapid tumor growth.[56]

Solid stress was shown to impact gene expression of tumor cells and trigger the potential invasiveness of cancer cells.[57] Among TME factors, the CSC niche contributes to the invasion, metastasis, and promotion of angiogenesis. The following factors play critical roles in cell–cell communication: cancer-associated fibroblasts, tumor-associated macrophages, tumor-associated

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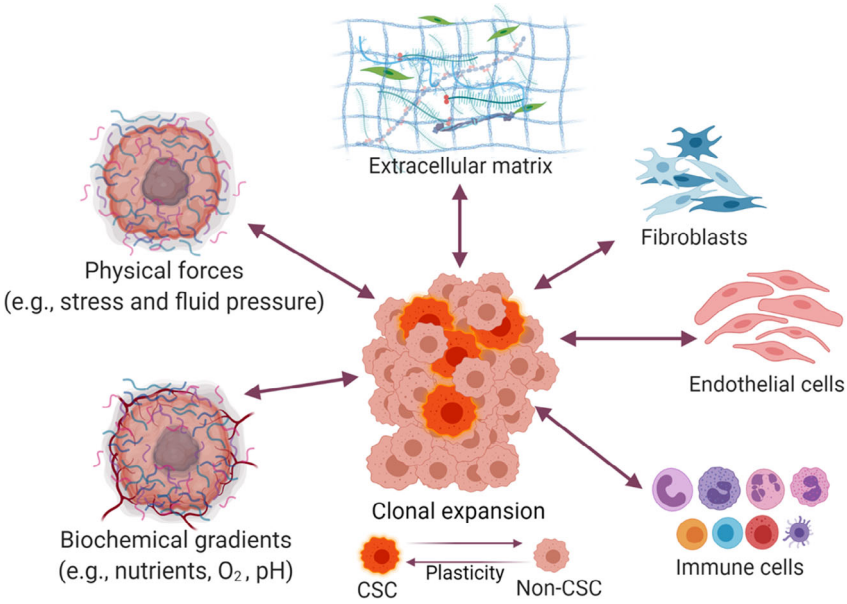

Figure 1. Schematics of TME factors.

neutrophils, mesenchymal stem cells (MSCs), cell-mediated adhesion, and soluble factors.[58] It is difficult to distinguish the TME factors associated with the biological activity of CSCs, and further research is required to study the contribution of each factor into CSCs activities.

The identification and characterization of the new CSC biomarkers are important in selecting proper treatments. They can include miRNAs (miR-34a, miR-199a, miR-181, miR-125b-2, and miR-128), cluster of differentiation (CD) biomarkers (CD44, CD34, CD38, and CD133), enzymatic activity of some markers (such as ALDH1), and other kinetics measurements of CSCs. As CD biomarkers seem to be insufficient to characterize the CSC, cancer-specific surface biomarkers seem necessary to identify the CSCs.[59] In a heterogeneous tumor bulk, each cell has different kinetics ranging from a quiescent state to aggressive growth and invasion. Poleszczuk et al. indicated that migration rate, proliferation potential, spontaneous cell death, and symmetric CSC division show behavior of cells and differences between the stem cells and CSCs. Cell proliferation rate is a good indicator of the cell behavior; for instance, some CSCs can cause rapid growth in the tumor, whereas other CSCs remain dormant for a prolonged period.[60]

CD44 is a phenotypic marker that is expressed in various cell types, and overexpressing of this marker is recognized in the tumor state. CD44 has isoforms which are named CD44v, and CD44s play roles in the pathogenesis of cancer and epithelial–mesenchymal transition (EMT). Initiation of EMT begins with the expression of CD44v to CD44s, and it was shown that the process of EMT required upregulation of CD44 in pancreatic cancer cells. The biomarker CD44 and its isoforms have multifunctional properties in the activation of cell signaling pathways and cancer pathways.[61]

A minor population of tumor cells was originally found to be clonogenic and metastatic, for in vitro and in vivo, suggesting the existence of CSCs.[62–67] Al–Hajj et al. isolated tumorigenic and nontumorigenic subsets of cancer cells from breast tumor biopsies as the first evidence of CSCs in solid tumors. They have serially passaged these tumorigenic cells and observed each in vitro model exhibited elevated $CD44^{+}CD24^{-/low}$. These biomarkers are maybe insufficient to identify CSCs because human breast CSCs and normal stem cells express these biomarkers. They suggested epithelial-specific antigen $^{+}CD44^{+}CD24^{-/low}$ $lineage^{-}$ and observed that $CD44^{+}CD24^{-/low}$ $lineage^{-}$ tumorigenic cancer cells can undergo processes analogous to the self-renewal and differentiation of normal stem cells.[68] Traditional CSC identification protocols have been based on surface biomarkers and self-renewal capacity and propagating into the tissue. Some functional biomarkers have been recently introduced to improve CSC identifications, such as adenosine triphosphate (ATP)-binding cassette (ABC) transporter and ALDH activity, the activation of some key signaling pathways, live-cell intercellular molecules, and single-cell detections.[69] For example, Ginestier et al. isolated normal and malignant breast stem cells utilizing the enzymatic activity of ALDH1.[70]

Biomimetic cancer models may include one or several TME factors. These can be introduced through a preconditioned cell culture medium.[71] For example, in one study, a group of induced pluripotent stem cells (iPSCs), regulated by epigenetic effects, was injected into a mouse model while DNA hypomethylation was postulated simultaneously to cause the conversion of iPSCs into CSCs.[72,73] External modulators can also be introduced to induce stemness in different types of cancers.[74] Some examples of external modulators are extracellular such as ATP, metabolite lactate and ketones, hypoxia, and hepatocyte growth factor (HGF). Stem cell signaling pathways provide developmental homeostasis, and their dysfunction can cause the transformation of stem cells to CSCs.[75] These pathways include Janus-activated kinase/signal transducer and activator of

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transcription (JAK/STAT), Hedgehog, Wnt, Notch, and others. These pathways each play a role in many biological processes and also support CSC function.[76] For example, JAK/STAT-related genes were found to be overexpressed in stem-like cells isolated from prostate cancer cells.[77]

The EMT is a key step in metastatic progression that triggers the transformation of the normal to cancer cells in the TME. A large number of transcription factors (e.g., Snail, Slug, deltaEF1, Zeb1, and Bmi-1) induce and regulate the EMT.[78] Following the EMT process, the tumor cells are disseminated from primary regions, thus migrating into the circulatory system. Known signaling pathways of EMT regulation are HGF, epidermal growth factor (EGF), transforming growth factor β (TGF-β), Wnt/β-catenin, notch, and hedgehog.[40,79] Human healthy mammary epithelial cells were found to express stem cell markers associated with the induction of the EMT process. Induction of EMT increased overexpression of transcription factors like Slug and Sox9 cause stem-like state. Low expression of E–Cadherin, high levels of vimentin, and quiescent state can be observed in the CD44+/CD24− population, and the ALDH+ population shows a high expression of E–Cadherin and low expression of vimentin. The TMEs are regulated by CSCs population and states throughout transcriptional switch from mesenchymal-to-epithelial transition (MET) to EMT or vice versa.[80]

In addition, the expression of E-cadherin (as an epithelial biomarker) was increased while MMP2 expression was decreased in the stem cells of glioblastomas.[81] In another case, the role of CSCs in EMT—MET was observed in breast cancer patients whose bone marrow samples had a large population of CSC biomarkers.[82] TME's acidic environment around the tumor cells further inhibits nutrients and oxygen. It results in further tumor progression and increased drug resistance due to proteases breaking down in tumor adaptation. Another difficulty involving TME, which has been associated with impaired vascularization and triggered stem cell dysregulation, is hypoxia.[83] In addition, in the mesenchymal-like CSCs and epithelial-like CSCs show proliferative properties, whereas ALDH+ cells create more hypoxic conditions at the center. The presence of ALDH+ cells can lead to ineffective treatment of antiangiogenic agents (making hypoxic and acidic conditions).[84] The acidic condition induces stem cell transformation to CSCs, which can change the TME by transforming neighbor fibroblasts into cancer-associated fibroblasts (CAFs).[85] TME's hypoxic-affected cells reduce the uptake of chemotherapeutic agents that eventually leads to multidrug resistance. All these factors can control the behavior of tumor cells, in particular for CSCs, and researchers need to consider them in their solutions (**Table 1**).

Table 1. Selected TME models based on various ECM composition for cancer modeling.

Cancer type	Model	ECM and cellular composition	Refs
Brain	Spheroid culture	GBM, tMVECs, bFGF and EGF, collagen, HA, matrigel	[104]
–	Tumors-on-chip	GB3, HUVECs, VEGF, Matrigel, Fibrin	[145]
–	Hydrogel-based organoid	SU3, U87, bFGF, EGF, gelatin, alginate, fibrinogen mixed hydrogel	[144]
Breast	Spheroid culture	MDA—MB-231, MCF7, collagen, Matrigel, FGF, EGF	[106]
–	2D Microfluidics	MCF-7, MDA-MB-231, T47D	[133]
Liver	Spheroid culture	HePG2, HCSC, PLC/PRF/5, Matrigel	[107]
–	2D Microfluidics	HePG2, HeP3B, PLC/PRF/5,	[135]
Lung	Spheroid culture	A549, NCI—H1395, NCI—H1650, NCI—H1975, NCI—H1993, NCI—H2228, NCI—H23, NCI—H358, NCI—H460, HCC827, PC9, and SW900, EGF, bFGF, Matrigel	[109]
–	2D Microfluidics	LCSC, dLCSC, EGF, bFGF	[134]
Ovarian	Spheroid culture	OVCAR3, U937, CSC/M2-macrophage, PBMCs, Cytokines	[105]
Bone	Spheroid culture	MNNG/HOS, bFGF, EGF,	[108]

3. Culture Models for the Tumor Microenvironment

3.1. 3D Culture Models

Traditional 3D culture models have been established to mimic tissue-specific TME, where tumor cells can proliferate and differentiate.[86] The culture models can be categorized into nonscaffold, anchorage-independent, and scaffold-based systems in which formed spheroids are integrated into a biomaterial scaffold.[87] These methods may differ in terms of cell sources, protocols for cell preparation, and the time periods necessary for the creation of 3D culture models. Some detailed summaries of 3D culture methods can be found in the literature.[88–90] One well-known approach for the creation of 3D culture models includes causing floating conditions for tumor cells.

The floating culture method works based on nonadherent surfaces of plates. There are three main floating models, named as 1) hanging drop method, 2) the forced floating method, and 3) the agitation-based method (as shown in **Figure 2**A).[34,91] In the hanging drop method, medium-based cell suspension droplets are first created in well plates. The well plates are then inverted; thus, the droplets hang due to surface tension. The forced floating method prevents the adhesion of cells to any substrate using a nonadhering coating; therefore, cells are able to float. In the agitation method, cells in the suspension are stirred gently to prevent the attaching of the cells to the substrates. Recently, a low-cost and efficient method was developed for CSC prostate cancer cells enrichment culturing using a hydrophilic filter paper. Hydrophilic filter paper allows the spontaneous formation of tumor spheroids while the expression of CSC biomarkers is elevated. This spontaneous formation was found to be associated with increased hydrophilicity of cellulose fibers. Cell aggregation is promoted through limited space and niche between the fibers.[92]

Stem cell-based spheroids provide cell–cell and cell–matrix interactions, tissue-specific conditions, biological functions, a controlled TME, and in vivo-like physiology.[93] An initial spheroid tissue model was created using a soft hydrogel.[94] The differentiated tumor cells and stromal cells were not able to grow into a spheroid formation. Their material model was used

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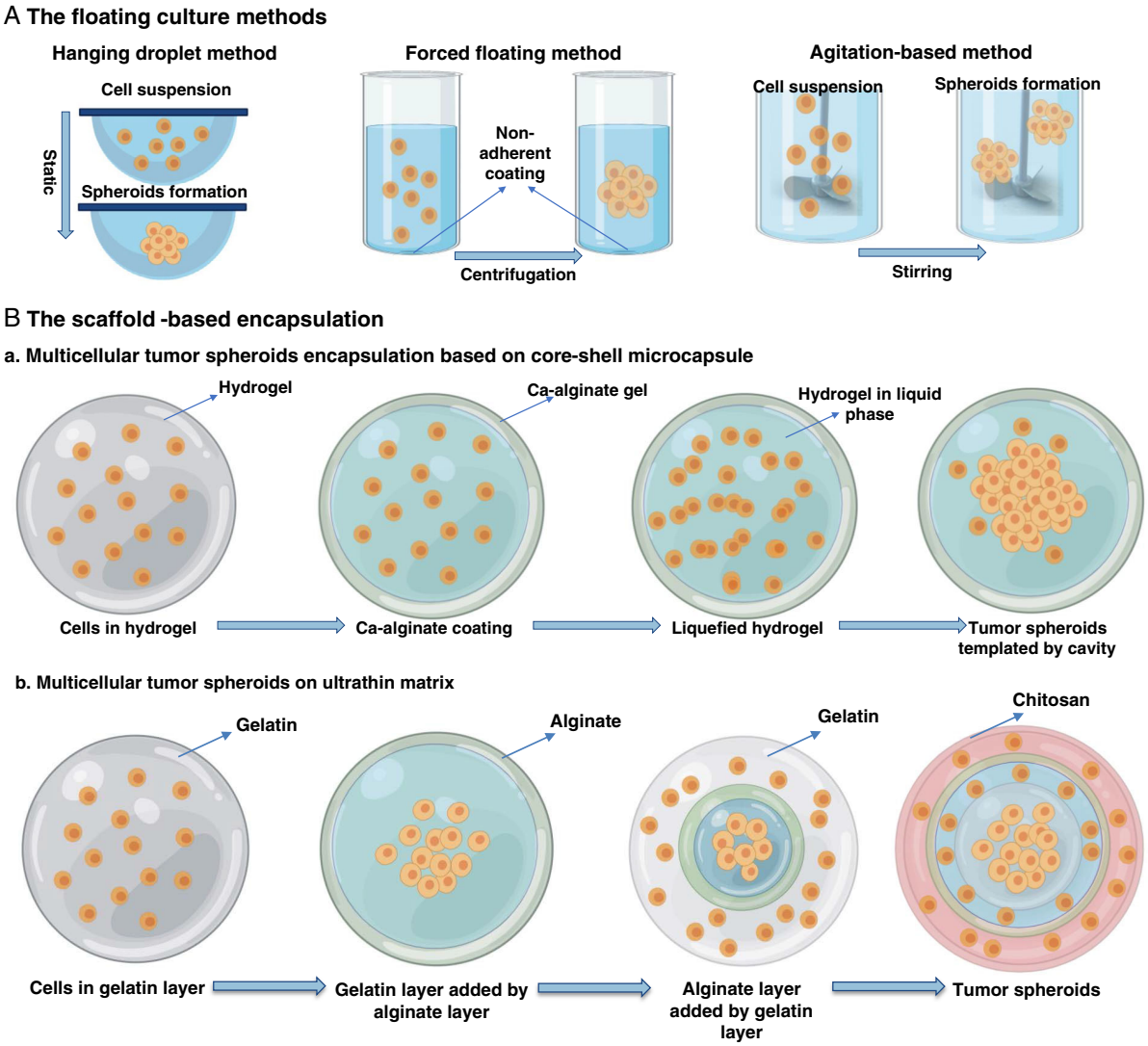

Figure 2. A) An illustration of floating sphere culture systems; these systems provide cell–cell interaction in the absence of matrix using the suspension in container walls; B) The scaffold-based encapsulation a) Multicellular tumor spheroids based on various matrix milieu; b) Multicellular tumor spheroids are based on the layer-by-layer ultrathin matrix. These structures allow the formation of the niche-like matrix for the generation of tumor spheroid microenvironment. Adapted under the terms and conditions of the Creative Commons Attribution license 3.0.[154] Copyright 2018, The Authors, published by Impact Journals, LLC.

to form new colonies in the absence of cell adhesion peptides.[95] The limited throughput ability of CSCs (representing only ≈0.4% of the tumor cells) has hampered their use for further processing.[96]

Multicellular spheroid tumor models have also been developed to investigate the interactions between cells and microenvironments.[97] A cell spheroid can be defined as compact and well-rounded (Figure 2B**)**. These aggregated cells can interact with each other and behave similarly to analogous in vivo tissues.[98,99] The spheroid model has many advantages compared with 2D cultures, such as recreating hypoxia-induced angiogenesis normally observed in tumor spheroids with diameters more than 500 μm,[22,100] as well as reduced gas,[101] nutrient,[102] and drug exchange.[103] Different ECM mimicking hydrogels were used to investigate the effect of ECM properties on the migratory response of glioma stem cells (GSCs) in a spheroid culture model. Results indicated that GSCs exhibited different single and collective migratory responses related to hydrogel porosity and stiffness values.[104] In another study, the interactions between ovarian CSCs and macrophages of hanging-drop-made spheroids were investigated. The results showed that CSCs upregulated the key macrophage biomarkers significantly compared with other ovarian cancer cells.[105]

The effect of pluripotency properties of 3D culture models on drug response was explored by comparing 2D monolayers with dispersed 3D cultures. It was found that the 2D monolayer culture and dispersed 3D culture models were not adequate to evaluate drug response and pluripotency of tumors.[106] They showed that better recreations of in vivo tumor conditions, such as chemoresistance, metastasis, and recurrence, were successfully

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represented in a 3D spheroid model by including the role of CSCs. Tumor spheroids of a hepatocellular carcinoma (HCC) cell line (HepG2),[107] human osteosarcoma cell line (MNNG/HOS),[108] and primary cultures from early-stage lung carcinoma patients[109] with enriched CSC potential were established through spheroid formation assays. Generated spheroid subclones displayed aggressive characteristics of tumor-initiating cells such as exponential growth (the passage number >30), drug resistance, and high invasion capacity, which is related to tumor progression and metastasis-association of aggressive phenotypes of CSCs. More recent culture models are adaptable with microscale technologies that are compatible with automated high-throughput screening.[86]

3.2. Organoids

Cancer tissue samples can be obtained from patients' primary tumors, patient-derived xenografts, or genetically and chemically induced animal tumors. Such samples can be used to generate organoids and provide a better understanding of the tumor bulk behavior and characteristics in their natural microenvironment. Better tumor models are created through morphology, cell–cell interactions, and gene and protein expression. These models result in a tumor bulk which more closely resembles the original tumor.[110] In addition to patient samples, adult stem cells, embryonic stem cells, or induced pluripotent stem cells can also be used by reprogramming them into an embryonic-like pluripotent state. The use of stem cell-based organoids assists in the development of unique characteristics of organs, the modeling of diseases at different stages, regenerative therapy, and the testing of many parameters at a developmental level.[111]

Stem cell properties are required to supply both intrinsic and extrinsic signals in cell autonomy and self-organization. Bioengineering approaches are used to manage the physiological control of organoids.[112] Improving the bioengineering approaches and creating complex niches are significant for downstream applications, elucidating the mechanisms of diseases and progressing toward regenerative medicine. The stem cell niche requires niche-related signals to support physiochemical conditions for differentiation; therefore, a specialized microenvironment should be created with required components for targeted tissues or organs. Growth factors or other components are used to create tissue-specific signaling pathways that play crucial roles in cell survival, self-renewal, and differentiation. Cell–cell communication is the main process for stem cell niche, and ECM components take part in this signaling cascade with laminin, fibronectin, and collagen, where they integrate with integrins.[113] The most commonly used ECM model is matrigel, which provides a scaffold structure and allows important signaling cues to activate cell–cell communication.[111] Matrigel can also be used to modulate neural stem cell and hematopoietic stem cell behavior. Synthetic polymers (such as polyacrylamide and polyethylene glycol) or natural macromolecules (for instance, agarose or collagen) have also been used. Here, the hydrogel was combined with collagen to generate the epidermal niche ECM to induce vascularization and healing for MSCs' function.[114]

Modeling the heterogeneous cell colonies in TME creates an in vivo-like environment. Thus, reprogramming the different types of cancer cells seems crucial for modeling the disease. Reprogramming cancer cells using induced pluripotent stem cells from the somatic cell method is important to mimic the dynamic structure and to understand the tumorigenesis. In the past 10 years, many studies showed that the characterization of tumorigenic properties was assessed using induced pluripotent cancer cells (iPCCs).[115] For instance, Chao et al. demonstrated that myeloid monocytic leukemia cells were reprogrammed and became acute myeloid leukemia (AML)-induced pluripotent cancer cells, which stimulate epigenetic and gene expression alterations, thus approaching an in vivo-like environment. Genetic alterations in the AML-iPCCs were used to predict the response of clinical chemotherapeutic drugs.[116]

Myeloid malignancy was reprogrammed using iPSCs and iPCCs at different stages of the disease (low risk, high risk, and AML) and correlated with the observed prognosis.[117] This provided observation of the prognosis and facilitated the administration of the drug at the right time. In contrast, reprogramming of the melanoma using iPCCs caused drug resistance.[118] In addition, imatinib resistance was developed in imatinib-sensitive chronic myeloid leukemia (CML) due to iPCCs reprogramming[119] while reprogrammed cells from colorectal cancer develop sensitivity to chemotherapeutic drugs.[120] All these results show that iPCCs reprogramming is dependent on the cancer cell type and its origin. In CSC-based platforms, the prognosis is better controlled in every stage of the disease and epigenetic alteration.

Microcapsules are defined as a consistent microenvironment that provides autoimmune protection and long-term stability for enclosed cells. The microcapsule-based 3D structure is created using alginate or other hydrogels, and this structure allows the interaction of the biological cues between cells.[121] Because stem cells secrete various trophic factors to avoid immune rejection, a controlled microenvironment should be created with biocompatible material. Encapsulation of stem cells prevents immunological rejection and enhances the permeability of essential nutrients, oxygen, and most cellular secreted factors. It also restricts the passage of larger molecules, such as antibodies and immune cells.[122] For example, human MSCs were modified to express hemopexin-like proteins to inhibit angiogenesis in glioblastoma. Alginate microcapsules were designed next to these microcapsules and were transplanted subcutaneously into nude mice. Both the tumor volume and the weight of the mice reduced significantly compared with the control group.[123]

3.3. Microfluidic Models

Conventional 3D culture and organoid models have some limitations, such as geometrical and visual inspection throughout the migration process and quantifying the invasion of 3D spheroid models.[124] These limitations have led researchers to focus on microfluidic devices that allow controlled delivery of reagents and placement of tumor cells in desired patterns.[125] One key development of microfluidic devices is their integration with organs-on-chips (**Figure 3**); this has opened new horizons in the field of cancer research.[126]

Organs-on-chip is a top emerging technology with the potential to reduce drug development time and cost and to alleviate ethical considerations related to animal studies.[43] When

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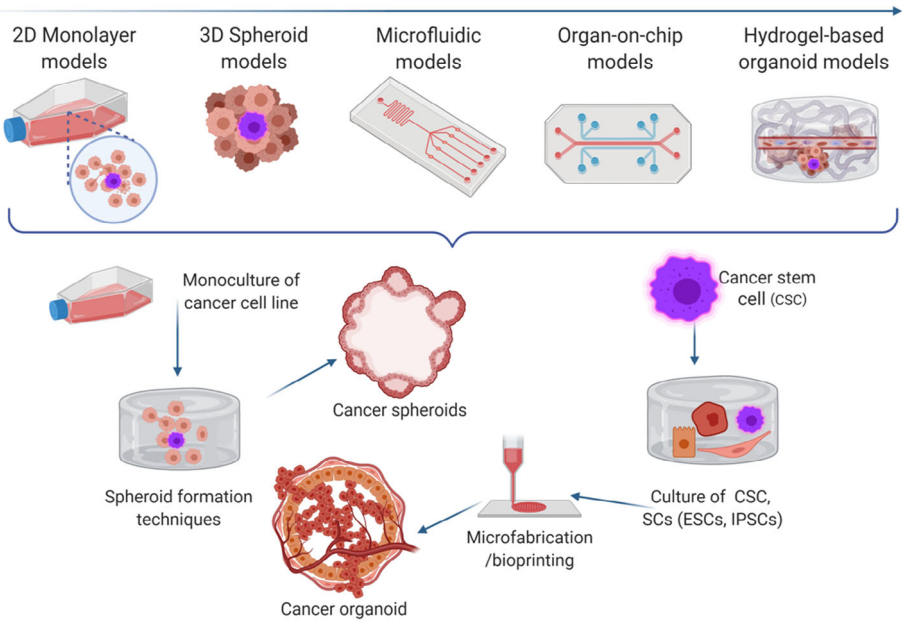

Figure 3. Evolution of cancer modeling from simple 2D cultures to cancer-on-chip and organoid.

compared with the conventional in vitro 2D cell cultures and in vivo animal models, 3D models have a better capacity to mimic and replicate human pathophysiology.[127] Organs-on-chips can be defined as 3D organotypic devices; they can accommodate 3D cultures of multiple cell types and provide flow and mechanical input.[10] Tumors-on-chips are thus microfluidic devices developed to replicate tumors through physiological mimicry that allows continuous perfusion of nutrients gasses and testing pharmaceutical agents.[128] As an experimental approach, cancer modeling through microfluidics has great potential to improve our understanding of cancer behavior and effective drug development.[129] Cancer cell motility is more sensitive to surrounding stimulations due to ECM components compared with noncancerous cells.[130] Many studies have hypothesized that the CSC phenomenon might be the key reason for anticancer therapeutic resistance, and patient survival may ultimately depend on the elimination of CSCs.[37–40] Therefore, recently emerged microfluidics models, tumors-on-chips, could have revolutionary and novel contributions to CSC research.[44]

Conventional microfluidics involves the use of lithography-based molding and casting processes. One of the most critical requirements of microfluidic devices is optical transparency; this restricts the available materials and manufacturing techniques that can be used. The processes such as replica molding, injection molding, and embossing assist in the fabrication of conventional organs-on-chips. Silicon, glass, and plastic materials are mostly used during these fabrication methods.[12] Microfluidics technology has advanced, other functionalities, such as gas permeability and biocompatibility, researchers working especially in pharmaceutical, biochemistry, and biomedical fields have asked for this technology. As a result, much of the microfluidics in this field use crosslinked polydimethylsiloxane (PDMS), an elastomeric material that provides optical transparency, biocompatibility, flexibility, and gas permeability, all of which are required for microfluidic devices used in successful cell culture processes.[131] An early example of microfluidic devices was developed for real-time tracking of the migration of hundreds of cancer cells inside mechanically constrained microchannels.[132] They used time-lapse images for quantifying the cell migration through microchannels, observing continuous and persistent motility in one direction for several hours in the absence of an external gradient. This work also examined the migration behavior of the cancer cells in the presence of drugs. The results showed that the increased concentration of nocodazole caused a reduction in the speed of cancer cell migration.

To screen CSC-specific biomarkers in a single-cell assay, Lin et al.[133] fabricated a high-throughput PDMS chip containing single-cell capturing units, using the advantage of the single-cell clone-forming capability of CSCs, to investigate specific therapeutic agents on breast CSCs (**Figure 4**B). The chip allows cell capturing, CSC identification, and clone-forming inhibition assays to be conducted on the same device using cell retentions within the upper outlet channel. After perfusing cell suspensions through the channel, single cells are trapped by the top layer intersections. This is associated with open-outlet and size retention effects. Trapped cells would lead to increased flow resistance, thus preventing other cells from passing the channel. After flushing out the residuals, the chip is rotated to place the trapped cells inside the culture chambers. The results showed that only a few cells in single-cell arrays survived and were able to form tumor spheroid. Tumor sphere formation rates of microfluidic assays were slightly lower than the conventional multiwell plate method, allowing for both cell–cell interactions and cell aggregation.

Zou et al. developed a V-shaped microfluidic network to study gradient-induced chemotaxis of lung cancer stem cells (LCSCs) and differentiated LCSTs (dLCSCs) in real time. They trapped the cells by a gap between main and connecting channels after loading cell suspension droplets with different densities

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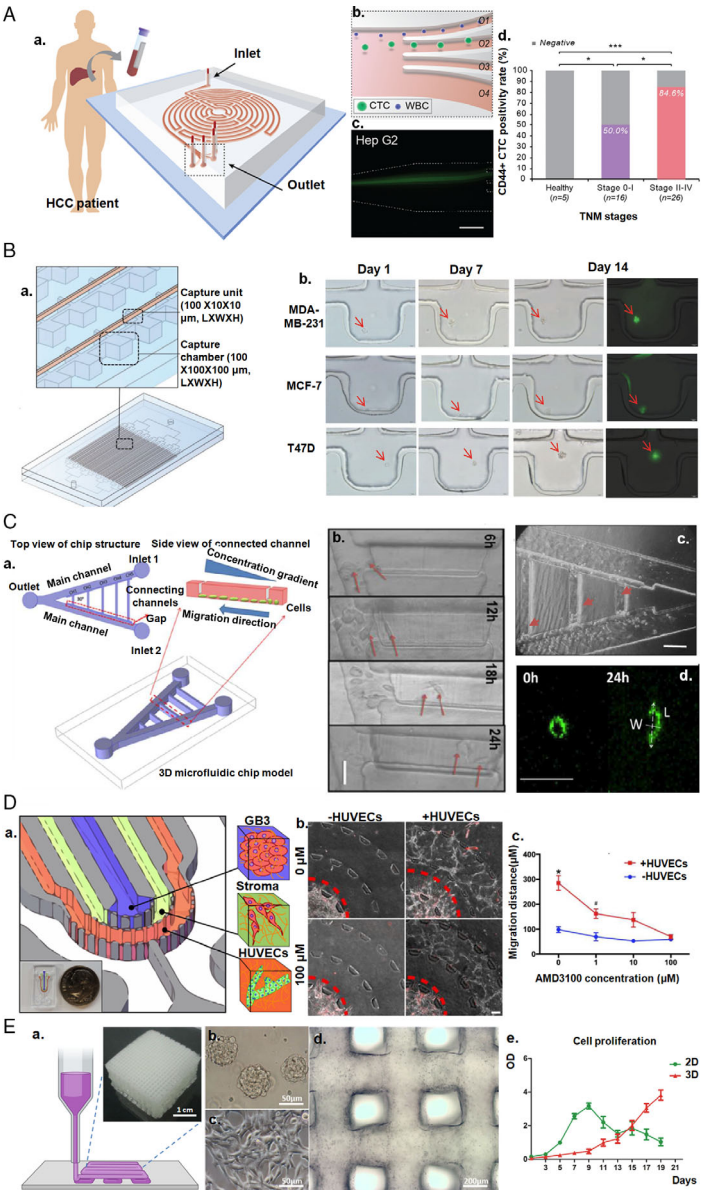

Figure 4. A) Characterization of the labyrinth device with HCC: a. CTC isolation workflow using the labyrinth device, b. CTC separation from WBCs by differential inertial focusing and collection, c. Fluorescent microscope image of differentially focused cell streaks by inertial focusing and migration for cell separation and CD44 biomarker analyses (scale bar is 100 μm). Reproduced with permission.[135] Copyright 2019, Springer Nature. B) Schematic of the microfluidic chip and continuous cell survival of the single-cell array (MCF7 cells), a. Schematic of the single-cell array microfluidic chip and the cell-capturing unit, b. Single-cell-derived clone formation rate of MDA—MB-231, MCF-7, and T47D cells ($n = 3$, $p < 0.0001$), c. Consecutive microscopic pictures showing the formation of single-cell-derived tumor spheres on-chip (scale bar is 50 μm). Reproduced with permission.[133] Copyright 2019, John Wiley and Sons. C) 3D schematic illustration of microfluidic chip design for multiple gradients generation: a. The microfluidic chip consists of two main channels forming a 30° V-shaped structure and five parallel connecting channels with different lengths, b. Image of dLCSC migration in the CH1 in normal condition (scale bar is 50 μm). c. The LCSC migration locations in channels at time point 18 h (scale bar is 100 μm). d. Graphical description of aspect ratio (L/W) of the LCSC in CH1 during chemotactic migration (0 and 24 h; scale bar is 50 μm). Reproduced with permission.[134] Copyright 2015, American Chemical Society. D) Schematic of GSC—EC interaction. a. Schematic of the vascular niche within the GBM TME. b. CXCL12—CXCR4 signaling in GSC—EC interaction. Phase-contrast image of GSC (red) invading in the presence of HUVECs in different concentrations of AMD3100. The red dashed line delineates the average migration boundary (scale bar is 100 μm). c. Quantification of invasion distance for each condition. Reproduced with permission.[145] Copyright 2019, Elsevier. E) a. The schematic of the printing process of in vitro brain tumor model and 3D-bioprinted GAF hydrogels mixed with GSCs SU3. b. SU3 grown in spheres in stem cell medium. c. SU3 grown on a 2D substrate in complete medium. d. 3D-bioprinted SU3 at day 1 of culturing. **e.** Cell proliferation of SU3 in a 2D environment and GAF hydrogels (3D). Reproduced with permission.[144] Copyright 2009, IOP Publishing.

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(Figure 4C). They showed that the β-catenin-dependent Wnt signaling pathway regulates the chemotaxis behavior of both LCSCs and dLCSCs. This study also showed the importance of cell heterogeneity, observing both cell types behaved differently to the same external stimuli. They further observed the acceleration of dLCSC to be more sensitive for gradient stimulation in comparison to LCSCs, and the application of XAV-939 inhibited the β-catenin signaling, thus leading to the suppression of chemotactic migration rates.[134] Another PDMS microfluidic device was developed for the detection of CD44, a potential CSC biomarker. Labyrinth microfluidic devices can isolate circulating tumor cells (CTCs) in blood samples obtained from HCC patients. HCC cells were collected and labeled with a fluorescent dye. Then labeled cells were diluted into non–HCC subject blood samples or a buffer solution before loading the suspension into labyrinth microfluidic device. Labyrinth device separates CTCs from white blood cells (WBCs) through differential inertial sorting and collecting principle **(**Figure 4A**)**. A correlation between CTC rates and different tumor stages in patients was shown. They also revealed that the majority of the HCC patients tested positive for CD44, a pluripotency biomarker, in CTCs that could indicate the relation between pluripotency properties and dissemination.[135] While microfluidics-based single-cell assays are generally used for CSC studies, microfluidic assays accommodating 3D cell cultures are rare.

More recent microfluidic chip designs have comprised cell-embedded 3D ECM hydrogel organs-on-chips. Hydrogel microfluidic systems have numerous advantages in replicating an organotypic model due to their high permeability and biocompatibility.[136] For example, hydrogels enable the diffusion of solute molecules (e.g., nutrient, oxygen, growth factors); they are optically transparent, thus providing an observable microenvironment for cells, and most hydrogels have comparable stiffness and tunable mechanical properties for better replicating the ECM.[137,138] Some of these models have used direct bioprinting through cell-laden hydrogels or using sacrificial hydrogels for post cell seeding processes, whereas others have used hybrid techniques, including casting cell-laden hydrogels into a lithography-based PDMS mold.[139–141] For example, Baker et al. showed that locations of the strongest gradients define positions of angiogenic sprouting within a 3D ECM model.[139] They proposed a lithography-based hybrid technique, using sacrificial microchannels patterned and molded-in sacrificial gelatin, to generate temporally and spatially defined soluble gradients. In another study, Meng et al.[142] used a hybrid bioprinting technique to recreate a TME while modeling the metastatic cancer steps such as invasion, intravasation, and angiogenesis to explore the molecular mechanism of tumor progression. They used a custom-built extrusion-based bioprinter to place tumor, stromal, and vascular cells precisely according to their physiological functions in a hydrogel-based chip that can serve as a high-throughput anticancer drug screening tool. They also printed stimuli–responsive core/shell capsules carrying growth factors that can be manipulated through programmable laser-triggered sources. These capsules are released using a GelMA/gelatin combination as the core and use an AuNR-functionalized poly-(lactic-*co*-glycolic) acid (PLGA) film as shell ink. To prepare the metastatic model, designed culture chambers were printed in the glass bottoms of the Petri dishes using room-temperature-vulcanized silicon. To create vascular cavities, they first used a pin-molding technique, then they bioprinted a droplet of A549-laden fibrin for simulating a primary tumor, placed 1 mm away from the vessel, and printed microcapsule arrays while fibroblasts were integrated into the surrounding hydrogel as supporting stromal cells. This combination promoted the remodeling of the ECM. Zhang et al.[143] combined inkjet printing with a lithography-based PDMS chip to investigate drug metabolism and the diffusion of an anticancer drug, Tegafur, on cocultured human hepatoma (HepG2) and glioma cell lines (U251). They encapsulated cells into 0.5% alginate sodium hydrogel with 10^6 cells mL^{-1} cell density to bioprint cell-laden hydrogel precisely onto a glass substrate via inkjet printer and then integrated the hydrogel with an oxygen plasma-treated PDMS layer with microchannels. After chip integration, they injected the $CaCl_2$ solution into the chip at the flow rate of 20 mL min^{-1} to crosslink the cell-laden alginate.

Dai et al. bioprinted a 3D GSC model using a modified porous gelatin/alginate/ fibrinogen (GAF) hydrogel to mimic the ECM. They crosslinked the GAF hydrogel system by adding transglutaminase as a crosslinker, targeting the gelatin component of the hydrogel (the main component of the GAF hydrogel). They then bioprinted GSC-laden GAF to observe the growth characteristics, stemness, and differentiation potential of the cells **(**Figure 4E**)**. Finally, they evaluated the results comparing the cells in the 3D-printed GAF with the 2D culture condition using a chemotherapeutic drug.[144] Truong et al. developed a hydrogel-based organotypic microfluidic model to investigate the interaction of GSC vascular niche and ECs and to identify the signaling cues which play crucial roles in invasiveness and phenotype **(**Figure 4D**)**. The organotypic platform consisted of three concentric semicircles of tumor, stromal, and vascular regions embedded in a micropatterned PDMS chip. Trapezoidal microposts were placed at the boundaries of the semicircles to allow mass transfer throughout the layers. They coated 3D cell culture regions on the chip by injecting poly-D-lysine to enhance the surface attachment of cell-laden hydrogels. HUVECs were encapsulated in a fibrin solution with a cell density of 20×10^6 cells mL^{-1} to model vasculogenesis, and then the solution was injected into the vascular cavity of the chip. GB3—RFP cells (15×10^6 cells mL^{-1}) were encapsulated in matrigel and injected in the tumor region, then matrigel was directly injected in the stroma region and incubated to allow hydrogel polymerization. They showed that CXCL12—CXCR4 signaling was involved in promoting GSC invasion in a 3D vascular microenvironment.[145]

4. Key Lessons and Future Directions

4.1. Governing Factors

CSC research is still in the early stage of development. The key challenge is to isolate CSCs to increase our understanding of their new pathways and specific roles in the well-known drug resistance.[146] The isolation strategies should be based on some vital rules, such as the isolated cells should not contain any nontumorigenic cells,[147] because CSCs and normal stem cells can express the same biomarkers.[68] Microfluidic devices can be used to overcome the limitations of biomarkers with strategies to isolate cancer cells from nontumorigenic cells. For example, labyrinth microfluidic device can separate CTCs from

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Table 2. Different considerations in selecting microfluidic tumors-on-chips.

Parameter	Options	Selection criterion
Geometrical features	Length, height, and width of channels, patterns	Biological questions 2D or 3D modeling Long-term cell culture, intact geometry, cell aggregation in spherical form Perfusable for biochemical gradient, tailorable stiffness for migratory response
Encasement materials	Silicon, glass, and polymer	Cost, biocompatibility, chemical inert, imaging, robustness, surface coating, optical transparency, stability, and permeability
Manufacturing and assembly	Cast molding, scale of fabrication, UV lithography, and 3D printers	Microinjection, cost of fabrication, prototype, and mass production
ECM material	Matrigel, fibrin, collagen, hyaluronan, decellularized ECM, and alginate	Sample type, duration of cultivation

WBCs through differential inertial sorting and collecting principle.[135] Microfluidics has been a radical solution for resolving limitations of culture methods through the development of well-controlled fluid delivery to cells. The key parameters in the fabrication of proper microfluidic tumors-on-chips are shown in **Table 2**. The range of parameters shows the flexibility in our preparation of tumors-on-chips with potential tuning toward CSCs. The feasibility of culturing CSCs in microfluidic devices has been tested and validated by some recent studies, such as improving the diagnosis of CSC-related biomarkers. In addition, microfluidics is well supported by computer-based modeling, in which simulations can be applied in such devices for in-depth analysis in contrast to most conventional methods. Despite these advancements in microfluidics, CSCs are used most commonly in drug screening platforms but are rarely used in cancer models. The challenges associated with isolating CSCs and the need for special culture conditions for both proliferation and differentiation might have slowed down their incorporation in 3D and microfluidic models. The presence and usage of TME attributes are likely to remove such limitations.

Clinical applications of microfluidic platforms depend on several practical considerations, particularly for precision medicine (**Figure 5**). The complexity in patient samples may require a high level of customization in the model. The small number of CSCs that can be isolated and kept functional may limit high-throughput screening in tumors-on-chips. Further additions, such as an increased number of cell types and proper vasculature networks that mimic the TME, should be added to engineered models.[148] The CSC niche can be introduced into new technologies, including tumors-on-chips and 3D bioprinting. Recent advancements in improving the versatility of these techniques have made them attractive choices for tumor modeling and therapeutic developments. Well-controlled studies and analysis of cell–cell interactions, CSC behavior, and CSC-specific biomarkers are now possible through microfluidics. The response of CSCs to cancer drugs is also crucial because of its pivotal role in resistance to cancer therapy as well as being the prime source of tumor recurrence. The inclusion of CSC into tumors-on-chips is needed for future cancer modeling.

4.2. Clinical Outlooks

One of the current limitations in the field of oncology is predicting which patients will respond to which therapy. For example, in patients with incurable lung cancer, the response rates to chemotherapy vary, but the typical response rate for first-line chemotherapy is between 7 and 40%. This means that up to 93% of patients who receive the therapy will suffer the toxicities of therapy without any benefit.[149] Other types of cancer can have even lower median response rates: sarcomas, for example, have a response rate of less than 20%.[150] In addition, some patients who have no response at all, even the patients whose cancer does respond to treatment, cancer may respond better to one type of chemotherapy than another. In both situations, there is a limited ability to predict which patient will respond and which chemotherapy will produce the best response. Chemotherapy can be very toxic and also expensive to both the patient and the healthcare system. To remedy this, CSC-based models seem promising in analyzing the tumor resistance to chemotherapy agents. This could lead to the ability of clinicians to develop personalized medicine and give chemotherapy primarily to patients who will benefit from such. If tumor samples or primary cells that were collected at the time of diagnosis could be placed into a chip and tested in a lab, clinicians could test which chemotherapy agents would give the best result and avoid those treatments that would not benefit the patients. The successful development of such models will represent a paradigm shift in the cancer community by improving patient's quality of life, potentially prolonging survival, and opening up new clinical trials to test various new drug formulations.

4.3. Drug Screening Platforms

A potential CSC-specific therapeutic targets the CSC and bulk tumor cells at the same time due to plasticity ability of the CSCs.[146] The use of organotypic microfluidic models allows mimicking the cellular composition in the TME, and the dynamic flow provides biomimetic body fluid;[151] they maintain the phenotypes of tumor cells and the tumor grade for applications in personalized therapeutics.[152] Advancements in organ-on-a-chip platforms can be combined with the high-throughput capacity and reproducible drug screening by adding parallel cell encasements and chemical gradients of circulating drugs in a single platform.[152] In addition, the creation of microtissue models at high structural complexity[153] and control over composition will lead to a very functional drug screening platform.[148] The authors believe that by harvesting patient tumor cells and placing them into the microtissue, it will be possible to precisely reproduce an organotypic model while creating a vascular system using advanced

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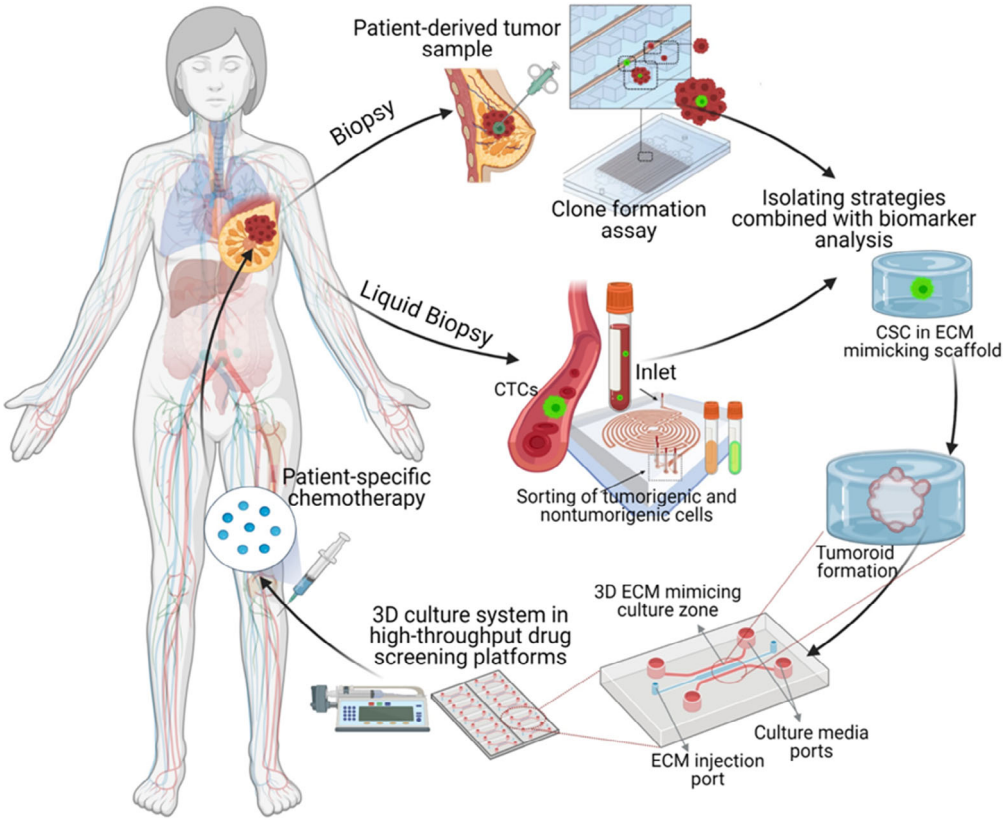

Figure 5. Proposed strategy in personalized medicine for CSC-based drug resistance.

manufacturing,[148] clinicians will be able to monitor the behavior of embedded tumor cells and CSCs. This will allow further studies on the role of CSCs and their interactions with drug candidates; thus, it will reveal a new standard in the cancer society.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

cancer stem cells, microfluidics, tumor microenvironments, tumors-on-chips

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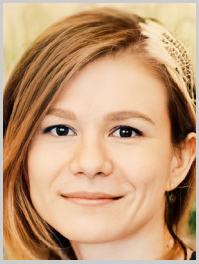

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