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Expansion of Rare Cancer Cells into Tumoroids for Therapeutic Regimen and Cancer Therapy

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Rare cancer cells, such as circulating tumor cells (CTCs) and cancer stem cells (CSCs), are small cell population found in cancer patients. CTCs have been recognized as tumor avatars for real-time cancer monitoring, while CSCs are the most malignant tumor cells that play a dominant role in drug resistance and metastasis. Interestingly, these two types of cells share the same surface markers, such as EpCAM, CD44, and CD133. While capturing these rare cells is available, the expansion of these cells is still challenging due to the limited cell number. These cells are susceptible to the microenvironment and lose the capability to grow in vitro, especially after an intense capturing process. A technology called patient-derived tumor organoids (PDOs) or tumoroids is a rising start in cancer modeling but the applicability is still questionable. Recently, assembloids containing multiple tumor-related cells have been developed which is one step closer to the real tumor. In this review, strategies for in vitro expansion of tumoroids are summarized implying that artificial tumor niche composed of optimized biophysical and biological cues is vital in the tumoroid generation. Tumoroids containing rare cancer cells is believed to be beneficial in the diagnosis, therapeutic regimen, and drug discovery for personalized therapy.

cells.^[1] Despite advances in cancer biology, metastasis remains the primary reason that holds 90% of deaths from solid tumors.^[2] CTCs are the cells detached from both primary and metastatic lesions. The definition of CTCs and cancer stem cells (CSCs) is shown in **Scheme 1**. The number of CTCs is often between 0.1 and 10 cells per milliliter of peripheral blood, depending on the patient's status. CTCs could be an alternative to invasive tumor biopsies to detect, characterize, and monitor cancers.^[3,4] Limitations of invasive biopsy such as sampling bias, sampling difficulty for deep tumors, and harm to patients make the diagnosis confirmation of cancer complicated. Liquid biopsy has been considering much attention recently and has a huge potential in existing sampling methods. Since tumor biopsies obtained by fine-needle aspiration usually conduct together with medical imaging, evidence of the pathological progress may not be helpful in real-time diagnosis.^[5] Indeed, one of the most

significant drawbacks of tissue biopsy is routine disease monitoring. Presently, 305 CTC-related clinical trials are listed in "clinicaltrials.gov."

Many studies have confirmed that CTCs are a prognostic cancer marker; for example, breast cancers,^[6] prostate cancers,^[7,8] and colorectal cancers (CRCs)^[9] associated with low CTC

1. Introduction

1.1. Circulating Tumor Cells (CTCs)

Circulating tumor cells (CTCs) was first described in 1869 by Prof. Ashworth by comparing CTC morphology to other tumor

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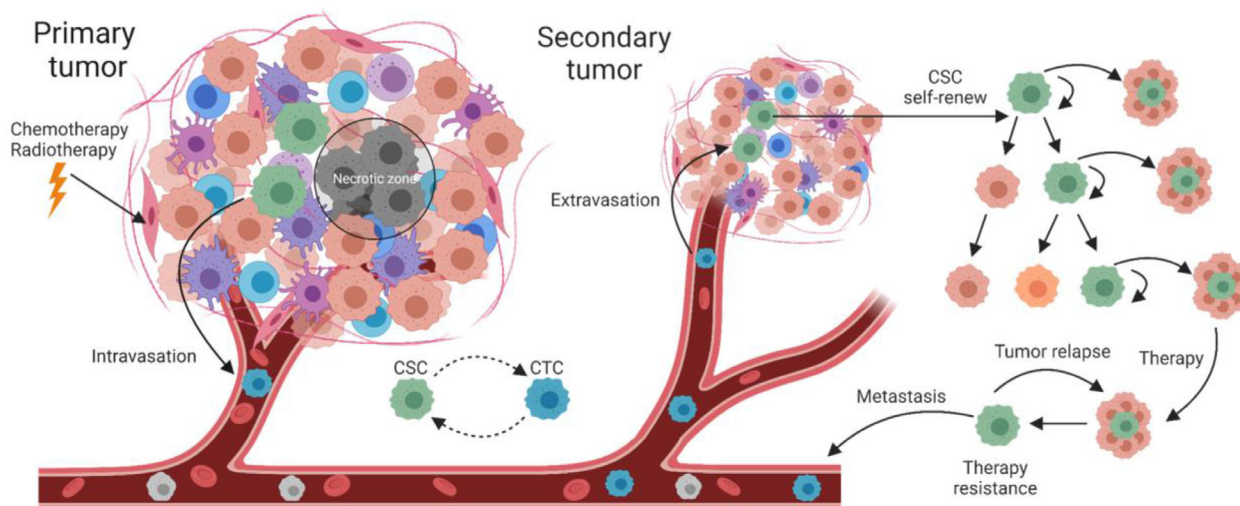
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Definition of circulating tumor cells and cancer stem cells



Scheme 1. Definition of CTCs and CSCs. Although the definitions of CSCs and CTCs are different, they share some surface markers and properties, such as metastatic capability. The number of these two cancer cells is meager. Capture and expansion of these two types of cancer cells will benefit clinical diagnosis and therapy.

concentration reflected longer survival time. Furthermore, several studies proposed that CTCs were present in cancer patients' peripheral blood when they were in the early stage or even before diagnosing the primary tumor.^[10–12] CTCs also guided therapeutic management, indicating therapeutic effectiveness or necessity even in the absence of detectable metastases, and offered insights into drug resistance mechanisms. Notably, genetic and genomic analysis of CTCs paved the way toward saving a patient from a life-threatening condition with unsuitable medications.^[13] Capturing CTCs from cancer patients' blood is a current technological challenge. Structured surfaces offer an excellent opportunity to capture CTCs from patients' blood by providing unique cell–substrate interactions. Microfluidic devices also hold great potential to enhance cell–surface interactions. Thus, combining structured surfaces and small flow channels could promote CTCs capturing^[14,15] and expansion^[16] in the near future.

Following a successful capturing of CTCs from a patient's blood, the expansion of these cells is a current technological and intellectual hurdle. Capturing rare CTCs in peripheral blood has been receiving considerable attention. Only a few approaches with limited success could expand CTCs, such as the *ex vivo* animal models.^[17–20] CTCs are heterogeneous and have inherent proliferative potential, as uncloaked by Ki67 expression differences.^[21,22] Studies showed that some normal stem cell and CSCs markers were expressed on CTCs surfaces such as Oct4^[23] and ALDH1.^[23,24] At present, CTC expansion relies on specific growth factors, hormones, tissue extracts, and/or small molecules with ultra-low attached plates.^[17,19,25] 3D cell culture has been recognized as a better way for CTC proliferation due to the senescence of CTCs in a monolayer culture system.^[17,26] Currently, 3D cultures for CTCs research have been developed, including multicellular spheroids grown in suspension and natural ECM embedded cell culture system (i.e., Matrigel)^[25] and collagen.^[16]

On the other hand, organoid cultures have not utilized structured materials in the system. Decellularized extracellular matrixes (ECMs) can be potential materials for organoid culture but have limited tunability and reproducibility. Thus, artificial cell niches, composed of optimized physical and biological supports to mimic the *in vivo* microenvironment, are beneficial in the PDO generation and then the subsequent diagnosis, drug screening, genome/transcriptome profiling, and drug discovery for personalized medicine. Strategies and applications of expansion of rare but important cancer cells are summarized in **Figure 1**.

1.2. Cancer Stem Cells (CSCs)

The traditional cancer biology theory states that all cells within a malignant tumor have equal potential to proliferate and metastasize. In the late 1930s, Furth and Kahn reported that a single cell from a mice tumor could initiate a new tumor in recipient mice.^[27] Afterward, the first evidence of the existence of CSCs was found in acute myeloid leukemia (AML), in which only 0.01–1% of the total population was able to induce leukemia after transplantation into immunodeficient mice.^[28,29] There is now a growing body of evidence illustrating that only small subpopulations of human cancer cells can initiate tumorigenesis by undergoing self-renewal and differentiation after transplantation into immunocompromised NOD/SCID mice.^[28–34] Therefore, the theory of CSCs claims that a subpopulation of tumor cells within cancers has hallmarks of self-renewal, highly metastatic capacity, proliferative ability to drive the expansion of malignant cells, therapy resistance (i.e., chemotherapy and radiotherapy),^[35] and 3D spherical formation capability.^[36]

In the last decades, CSC identification from various solid tumors was achieved, including breast cancers,^[32] brain cancers,^[33] CRCs,^[37,38] pancreatic cancers,^[39] and prostate cancers.^[40] CSCs

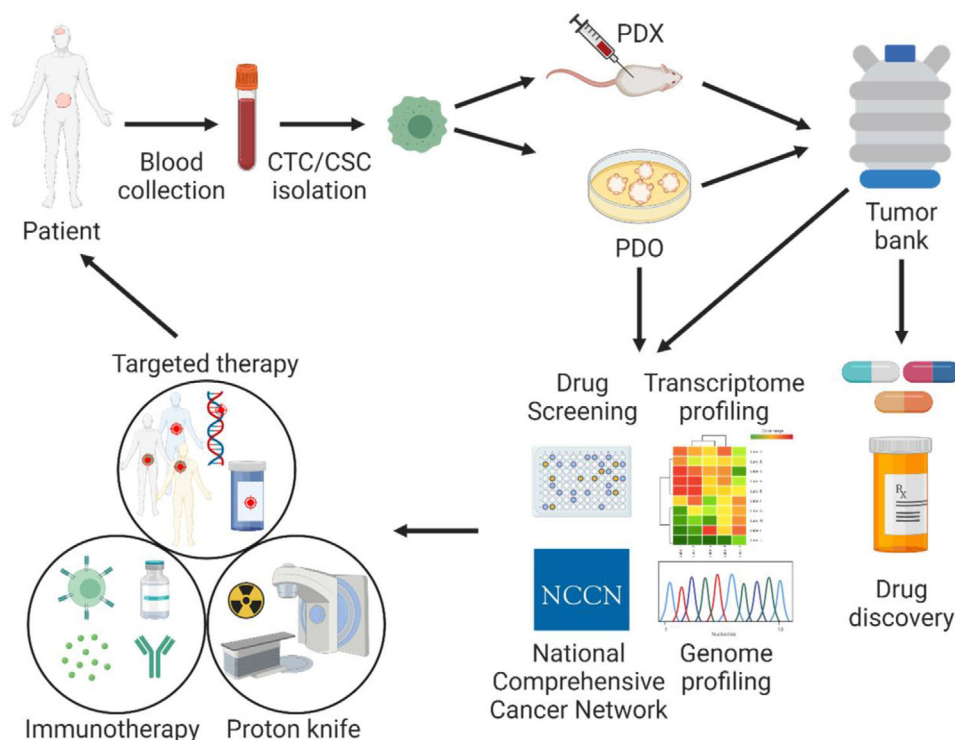


Figure 1. Schematic concept of personalized medicine using rare cancer cells-derived tumoroids. Patient-derived tumor organoid (PDO) developed from CTCs or CSCs have a huge potential for personalized drug screening, new drug discovery, and tumor molding.

Table 1. Surface markers of CTCs and CSCs.

CTC markers	Cancer types	CSC markers	Cancer types	Refs.
EpCAM	Breast, lung, colorectal, hepatocellular carcinoma, bladder	EpCAM	Hepatocellular carcinoma, liver	[9,43–48]
CD44	Breast	CD44	Breast, liver	[32,49–51]
ALDH1	Breast	ALDH1	Breast	[52,53]
CD133	Colorectal	CD133	Brain tumor, colorectal, liver	[33,38,50,51,54]
Vimentin	Breast	CD90	Pancreatic, liver	[55–57]
EGFR	Colorectal, breast, gastric	CD24	Breast, gastric	[32,58–61]
CEA	Colorectal, breast, pancreatic	CD49f	Brain, breast	[59,62–65]
Bmi1	Lung, breast	CD146	Rhabdoid tumor	[66–68]
HER2	Breast, gastric	CD117	Ovarian	[69–71]
CD227	Prostate, breast, colorectal, ovarian	CD26	Colorectal	[65,72–76]
EphB4	Breast, head and neck, colorectal	CD29	Breast	[10,63,77,78]
Cytokeratin 19	Breast, prostate	CD9	Leukemia	[73,79–82]
Cytokeratin 20	Pancreatic, gastric, colorectal	CD123 (IL-3 receptor)	Leukemia	[83–85]
		Notch2,3	Pancreatic, lung	[86–88]

EpCAM, epithelial cell adhesion molecule; ALDH1, aldehyde dehydrogenase-1; EGFR, epidermal growth factor receptor; CEA, carcinoembryonic antigen; HER2, human epidermal growth factor receptor 2; EphB4, ephrin receptor; Notch2,3, neurogenic locus notch homolog protein 2,3.

were not found in all solid tumors, and the frequency of CSCs varied among different types of tumors. Schatton et al. reported that the frequency of CSCs in human melanoma was lower than 1 per million cells,^[41] while Matheret et al. revealed that CSCs could be isolated from established cell lines and genetically modified cells.^[42] Significantly, the cell surface marker is crucial for identi-

fying and isolating CSCs from both tumor biopsy and peripheral blood. However, the universal surface marker that can apply to all CSCs has not been found yet. Canonical surface markers for CTCs and CSCs are summarized in **Table 1**.

The tumor microenvironment (TME) is very different from normal tissue, such as lower pH, remodeled ECM, and hypoxia.

Therefore, many cancer therapies are designed according to these differences. It has been repeatedly reported that the hypoxic regions in the TME could stimulate various stress that induces CSCs invasion^[89] and reduces drug efficiency.^[90,91] In vitro, CSCs grow in a hypoxia condition that can increase cell survival and metastases. For example, hypoxia enhances glioblastoma (GBM) cell growth, while brain CSCs are also promoted via induced hypoxia-inducible factor-1 α (HIF-1 α). One proposed mechanism is that HIF-1 α positively regulates TWIST, an E-cadherin transcriptional repressor, as a consequence of promoting epithelial–mesenchymal transition (EMT), a major metastatic phenotype.^[92–94] Likewise, under hypoxic conditions, Notch signaling enhances EMT by activating SNAIL, which represses the adhesion molecule E-cadherin transcriptional factor.^[95] Significantly, Sahlgren et al. reported that the upregulation of lysyl oxidase (LOX) stabilizes the SNAIL protein elevated by hypoxia induction due to HIF-1 α recruitment to the LOX promoter.^[95] Hence, the HIF-induced gene products are investigated as markers of CSCs detection^[96] and targeted therapy.^[97]

Organoid cultures open up an avenue for studying biology at a multicellular level, where the function and structure resemble human physiology more closely than conventional static 2D cell culture systems or non-primate animal models. Also, organoid cultures create opportunities to build huge biobanks with relevant patients, perform drug screenings, and facilitate novel drug development. Self-assembled cell aggregation or spheroid formation was reported as a physiologic tumor model or an excellent CSC collection method. Researchers have utilized different types of basement membrane components to form tumor spheroids, including collagen^[98] and Matrigel,^[99] as well as alginate, an anionic polymer typically obtained from brown seaweed.^[100] However, CSCs are challenging to maintain in vitro, severely restricting suitable treatment for patients. From a clinical perspective, CSCs could improve diagnostics and therapies to identify and target CSCs better within the tumor. Therefore, high-throughput screening requires a significant number of CSCs, wherein it is necessary to kill and prevent the metastasis of CSCs to eradicate cancer.

2. Capturing of Rare Cancer Cells

Currently, sampling is required from cancer patients to confirm a diagnosis and for further therapeutic strategies. In a solid tumor, an invasive method like a biopsy test from the primary tumor is the golden standard. Simultaneously, CTCs isolation from peripheral blood has a tremendous potential to confirm early tumor metastasis, real-time monitoring of cancer progression with an earlier evaluation of cancer recurrence, and treatment response. However, CTCs exist in exceedingly low numbers in peripheral blood, making the isolation procedure technically challenging. Once CTCs are captured, DNA mutational analysis of CTCs can be revealed via whole-genome sequencing (WGS) that can leverage targeted therapy. Besides, protein analysis of CTCs is regarded as a significant aspect of elucidating CTCs. Likewise, the detection of CSCs is a challenging task, primarily relying on the CSCs-specific surface markers. The frequency of CSCs within the tumor is \approx 1% of the majority of tumor cells. Anticancer drugs that kill these cells are considered highly effective cancer treatments if tumors are derived totally from CSCs, even metastatic

cancers. Therefore, the detection of these rare cancer cells is critical for fighting cancer.

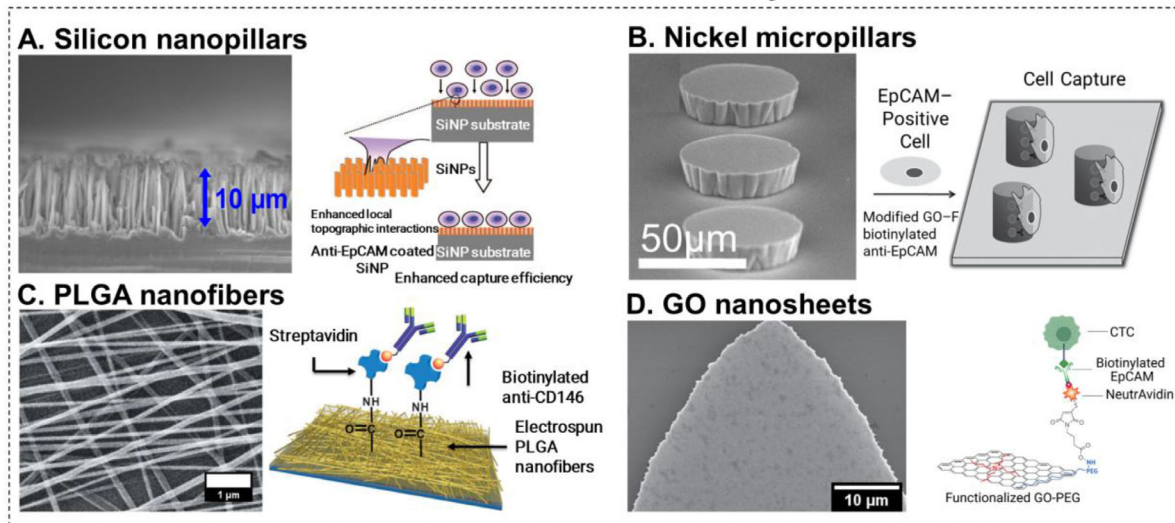
2.1. CTC Collection

Capturing CTCs from blood samples is critical in many aspects. Generally, the CTC capturing approach from patients can be classified into chemical and physical approaches. The most reliable protocol nowadays uses cell surface markers, for example, epithelial cell adhesion molecule (EpCAM).^[14,101] EpCAM is a glycoprotein that is the most selected surface marker due to its expression on the majority of tumor cells, including lung,^[102] pancreas,^[103] prostate,^[104,105] breast,^[106] esophagus,^[107,108] and liver.^[109,110] Alternatively, other surface markers can also be used for CTC capturing from specific cancer cells, such as PSMA (prostate-specific membrane antigen) for prostate cancer,^[111] HER2 (ERBB2; human epidermal growth factor receptor 2) for breast cancer,^[112,113] and platin 3 for metastatic CRC.^[114] The surface marker-based approach is not a perfect approach; for instance, EpCAM can be downregulated during EMT, a process attributed to metastasis.^[115] HER2 expression is also not consistent in all breast cancer patients,^[70] where a high concentration of HER2 was found in the patient's serum diagnosed with HER2-negative primary tumor.^[71] There are few capturing systems using surface marker approaches that are commercially available such as Adnatest, CellSearch assay, and Biofluidic, but only CellSearch was approved by the Food and Drug Administration (FDA), USA. However, CTCs captured from these systems are challenging to grow in vitro using traditional cell culture protocols. One possible reason is that capturing CTCs using cell surface markers, for example, EpCAM, can retard cell adhesion and proliferation in the subsequent culture.

With technological advances, material-based approaches including cell-size-based filtration,^[116,117] cell-density-based separation,^[118,119] hydrodynamic separation,^[120] dielectrophoretic (DEP)-based cell sorting,^[121] acoustic,^[122] and immunomagnetic separation^[123–125] have been proposed in the last decade. Nanomaterials hold great potential to improve the capturing efficacy due to their high surface-area-to-volume ratio and enhance cell–surface interactions (Table 2). Although many nanostructured surfaces have been proposed for cell culture,^[126,127] there is only CytoTrapNano by Cytolumina (also known as NanoVelcro chip) capturing systems available in the market. Altogether, we hypothesize that capturing CTCs without blocking cell surface markers could be a reliable way to expand cells in vitro and maintain the phenotype of captured CTCs. Nanotechnology provides a new set of tools that has the potential to overcome current obstacles associated with CTC capture and expansion.

One of the most exciting approaches for capturing and expanding CTCs is using one surface with nanotopography without trypsinization.^[128] Previous studies have shown that various micro- and nanostructures can enhance tumor cells' capturing efficiency using this concept.^[4] The scheme of CTC capturing using nanostructured surfaces is shown in Figure 2. The microfluidic handling system composed of a chaotic mixing channel and a patterned nanostructured substrate can be utilized for CTC capturing with increased efficiency due to

Microfluidic device-based flow system



Static system

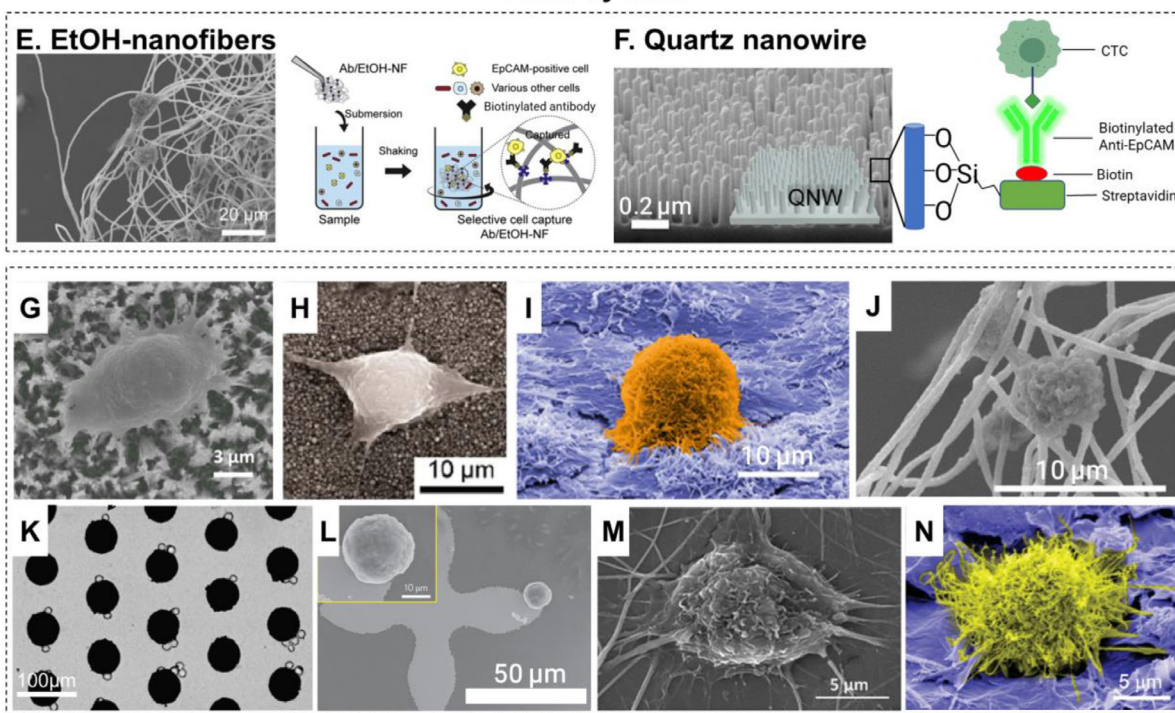


Figure 2. Nanostructured substrates for cancer cell capturing and characterization. A) Silicon nanopillar (SiNP) array on a silicon wafer, obtained by the wet etching process. Adapted with permission.^[136] Copyright 2009, Wiley-VCH. B) Nickel micropillars were obtained by the electroplating process. Adapted with permission.^[135] Copyright 2013, Wiley-VCH. C) PLGA nanofibers on a substrate by depositing onto commercial laser microdissection (LMD) slide. Adapted with permission.^[130] Copyright 2013, Wiley-VCH. D) Graphene oxide (GO) nanosheets fabricated using etching technique on gold patterns. Adapted with permission.^[134] Copyright 2013, Springer Nature. E) PS-PSMA nanofibers were prepared by the electrospinning technique. Adapted with permission.^[132] Copyright 2017, Elsevier. F) Quartz nanowire (QNW) arrays fabricated by the wet etching process. Adapted with permission.^[131] Copyright 2012, American Chemical Society. G) MCF-7 cell was captured on a silicon nanowire substrate (NanoVelcro chip). Reproduced with permission.^[4] Copyright 2014, American Chemical Society. H) MDA-MB-231 cell captured on nanorough surface. Reproduced with permission.^[3] Copyright 2012, American Chemical Society. I) MCF-7 cells captured on the surface that replicated MCF-7 cell. Adapted with permission.^[137] Copyright 2017, American Chemical Society. J) MCF-7 EpCAM⁺ cell captured on ethanol-dispersed polymer nanofibers. Adapted with permission.^[132] Copyright 2017, Elsevier. K) HCT116 EpCAM⁺ cell captured on nickel micropillar device decorated with graphite oxide-coated Fe₃O₄ magnetic nanoparticles. Adapted with permission.^[138] Copyright 2013, Wiley-VCH. L) MCF-7 cell captured on the gold pattern. Inset: magnified SEM image of the captured MCF-7 cell. Adapted with permission.^[134] Copyright 2013, Springer Nature. M) HCT116 cell captured with fully outspread pseudopodia and attached to TiO₂ nanofibers. Reproduced with permission.^[139] Copyright 2012, Wiley-VCH. N) MCF-7 cell captured on reduced graphene oxide films. Reproduced with permission.^[140] Copyright 2015, Wiley-VCH.

Table 2. Nanostructured surfaces developed for CTC capturing.

Structure	Dimensions ^{a)}	Materials ^{b)}	Markers	Cell type ^{c)}	Yields	Ref
Vertical Nanopillar	L = 12–15 μm	Silicon	Anti-EpCAM	MCF-7, PC3, T24	95%	[136]
Nanorough	Rq = 150 nm	Glass	–	MCF-7, MDA-MB-231, HeLa, PC3, SUM-149	80%	[3]
Nanofibers	–	PS PSMA	Anti-EpCAM	MCF-7	59–67%	[132]
Nanogratings	W = 500 nm S = 500 nm H = 150 nm; 560 nm	PDMS	–	MDA-MB-231	14–36%	[141]
			–	MCF-7 & A549	41.0 \pm 5.0% & 51.7 \pm 4.9%	
Nanopillars	D = 500 nm S = 450 nm H = 150 nm; 560 nm	–	–	–	47.5 \pm 2.2%	–
Vertical Nanowires	L = 12–15 μm	Silicon	Aptamers	A549	80%	[142]
Vertical Nanowires	D = 50–160 nm L = 5–10 μm	Silicon	AuSi droplets (T = 2-3 nm)	MCF-7	88%	[143]
Vertical Nanowires	D = 80–100 nm L = 250–350 nm	Quartz	Anti-EpCAM	A549	65.1 \pm 25.2%	[131]
Nanopillars	D = 500 nm H = 130 nm S = 300 nm	Quartz	Lipid bilayer-Anti-EpCAM	MCF7	92%	[144]
Vertically aligned Nanowires	D = 200 nm L = 2 μm	PPy	Disulfide-biotin-Anti-EpCAM	HCT-116, MDA-MB-468, MIA-PaCa-2,	93%	[145]
Micro-posts	D = 100 μm	PDMS	Anti-EpCAM	H1650 & A549	87% & 60%	[16]

^{a)} D = diameter; H = height; W = width; L = length; S = spacing; Rq = root-mean-square roughness; ^{b)} PS: polystyrene; PDMS: poly(dimethylsiloxane); PPy: polypyrrole; PSMA: poly(styrene-co-maleic anhydride); ^{c)} MCF-7: EpCAM-positive breast-cancer cells; PC3: EpCAM-positive human prostate adenocarcinoma cells; T24: EpCAM-positive bladder cancer cells; MDA-MB-231: EpCAM-negative breast cancer cells; HeLa: EpCAM-negative cervical cancer cells; PC3: EpCAM-positive prostate cancer cells; SUM-149: EpCAM-positive inflammatory breast cancer cells; A549: EpCAM-positive lung cancer cells; HCT-116: EpCAM-positive colon cancer cells; MDA-MB-468: EpCAM-positive breast cancer cells; MIA-PaCa-2: EpCAM-negative pancreatic cancer cells; H1650: EpCAM-positive lung cancer cells.

the enhancement of cell–substrate interactions (Figure 2A–D). Figure 2G–N shows captured CTCs by different nanostructured surfaces, such as nanowire, nanorough, nanofibers, graphene oxide films, graphene oxide nanosheets, and micropillars.

Wang et al. reported the first-gen device, in which the spiked cancer cells were captured on silicon nanopillars (SiNP) (Figure 2A).^[129] The efficiency was >95% (maximum) with a mixer, which was significantly higher than those without a mixer (\approx 60%) and those on a flat surface with a mixer (\approx 25%). The side-by-side comparison showed that the integrated microfluidic SiNP platform outperformed the CellSearch system using clinical blood samples. It indicates that both nanostructure and high frequency of cell–substrate contact are critical for the device’s efficiency, including efficiency in immunomagnetic separation. Subsequently, a third-gen “NanoVelcro,” an anti-EpCAM-coated silicon nanowire substrate (diameters of 100–200 nm and lengths of 15–20 μm) described by Lin et al., was used to capture CTCs and enrich cells by combining silicon nanowire substrate (SiNS) with thermoresponsive polymer brushes. Third-gen devices are created by grafting thermoresponsive polymer brushes (i.e., poly (N-isopropyl acrylamide), PIPAAm) onto SiNS. Thermoresponsive “NanoVelcro” chips are able to capture CTCs by EpCAM and release CTCs at 37 and 4 $^{\circ}\text{C}$, respectively.^[4] “NanoVelcro” CTC chips detected, isolated, and purified CTCs from artificial blood

samples with an 85% success rate and 17 out of 26 clinical blood samples. Hou et al. revealed that \approx 87% of M229 cells were captured on PLGA nanofiber embedded (Figure 2C) “NanoVelcro” chip,^[130] which previously showed the ability to capture circulating melanoma cells (CMC) using EpCAM, as well as to detect, isolate, and genotype a single CMC. Besides, 43 and 36 CMCs were captured and identified from the blood samples of patients 1 and 2.

Another nanostructured surface called quartz nanowires (QNWs) (Figure 2F) was fabricated with sizes in the range of 80–100 nm for CTC capture.^[131] QNWs were functionalized with a monoclonal antihuman EpCAM antibody, and the average capture efficiency for A549 human lung cancer cells was 89.2 \pm 12.3% on QNWs compared to 22.7 \pm 3.0% on flat glass surfaces. An average CTC-capture yield of this device from human blood samples was 67.5 \pm 15.0%.

Anti-EpCAM-functionalized electrospun nanofibers (NFs) (Figure 2E) were also used for CTC capturing due to a large surface-area-to-volume ratio.^[132] The antibody-functionalized NF matrix can be used as a filter to integrate both selective capture and 3D culture of tumor cells. Antibody-grafted NF matrix has shown a reasonable capture efficiency of EpCAM⁺ MCF-7 cells (i.e., 59–67%) and the capability to expand high numbers on 3D NFs matrix compared to 2D cell culture within 6 days.

Besides, graphene oxide (GO) has a couple of advantages for biological applications, such as biocompatibility and conductivity. It is easy to functionalize using, for example, polyethylene glycol (PEG).^[133] GO nanosheets functionalized with EpCAM antibody (Figure 2D) increase the capture efficiency of both MCF-7 cells and the patient's blood sample.^[134] CTCs isolated from blood samples of metastatic pancreatic, metastatic breast, and early-stage lung cancer patients were up to 23 CTCs per mL and all patients ≥ 2 CTCs per mL captured due to functionalized graphene oxide nanosheets on a patterned gold surface. In another study described by Yu et al.,^[135] anti-EpCAM GO-modified magnetic nanoparticles were immobilized onto a micropillar device (Figure 2B). This study showed high capture efficiency of HCT116 CRC cell line ($>70\%$) and a blood sample ($>40\%$). Also, a high release efficiency of the HCT116 cell line ($>90\%$) was achieved.

Interestingly, CTCs captured from artificial blood were the majority, while CTCs captured from the patient's blood were the minority in recent publications. CTCs in cancer patients vary in different sizes, phenotypes, and concentrations per milliliter. Antibody-based CTC capturing methods have trouble maintaining the viability of CTCs due to inevitable antibody binding. On the other hand, physical approaches rely on CTC properties proposed for viable CTC isolation without antibody-antigen binding. This approach makes use of the physical properties of CTCs, such as density, electrical properties, and size.

Kim et al. fabricated circular tapered-slit filters (TSF) of SU8 material for capturing CTCs.^[116] SU8 TSF surface was immobilized with PEG by modifying a covalent coupling technique. They reported that the PEG-modified filter captured and released 19.4 CTCs per mL from different colorectal and lung cancer patients' blood, including EpCAM⁻ CTCs, which antibody-based methods cannot capture. The microfluidic device was equipped with a size-selective microcavity array established by Hosokawa et al. to detect CTCs from whole blood.^[117] The microcavity array specifically separated tumor cells from whole blood based on size and deformability differences between tumor cells and hematologic cells. The device exhibited $\approx 97\%$ detection of lung carcinoma NCI-H358 cells in 1 mL of whole blood spiked with 10–100 of NCI-H358 cells, while gastric, breast, and colon tumor cell lines that include EpCAM⁻ tumor cells were recovered from the microcavity array with high efficiency ($>80\%$).

Density gradient centrifugation is a straightforward approach to separate blood into its components: erythrocytes, platelets, polymorph nuclear cells in the pellet, and mononuclear cells, including tumor cells, the so-called interphase. A comparison study by Balic et al. reported that at least one CTC was detected from 14 out of 61 patients (23%) by OncoQuick and from 33 out of 61 patients (54%) by CellSearch.^[119] Besides, the detected CTCs number was more significant for CellSearch (mean 20 CTCs per 7.5 mL of blood) than for OncoQuick (3 CTCs per 7.5 mL). Therefore, the density gradient centrifugation method is less accurate and sensitive than the immunomagnetic separation method.

Size-dependent hydrodynamic force is a potential candidate device for CTCs isolation and has been exploited to separate and enrich cancer cells from peripheral blood.^[120] A microfluidic chip equipped with a deterministic lateral displacement (DLD) array (circular and triangular) is used to isolate cancer cells. As a result,

cancer cells were isolated from a spiked blood sample with high isolation efficiency (99% for MCF-7 and 80% for MDA-MB-231), and 2 mL min⁻¹ throughput was achieved.

DEP isolation of CTCs from blood in a microfluidic chip was also reported.^[121] Cancer cell sorting was achieved with a sustained 3D lateral DEP (LDEP) particle force normal to the continuous through-flow (maximum flow rate: ≈ 2.4 mL h⁻¹, linear velocity: ≈ 4 mm s⁻¹). Their experimental data showed that isolation purities of 81.6%, 91.3%, and 87% were achieved at flow rates of 10, 20, and 30 mL min⁻¹, respectively, when the original cancer cell purity was 0.01%.

The optical detector of photoacoustic waves in melanoma cells was used to detect CMCs in a flow system.^[122] Gutierrez-Juarez et al. used a continuous helium-neon laser (HeNe laser) to provide a probe beam reflected off of a glass–water interface close to the microcuvette. A high-speed photodiode detected the beam when a photoacoustic wave was generated in microcuvette, and the index of refraction changes in the water indicated the beam's reflectance. This principle was used to detect the presence of melanoma cells. Their results demonstrated a detection threshold of one individual melanoma cell without pyroelectric noise was indicated in the signals.

2.2. CSC Collection

The characteristics of CSCs are similar to embryonic stem cells. One of the astonishing approaches to identify CSCs is their expression of markers associated with stem cells. However, no universal surface markers for CSC identification were found yet. CD133 and ALDH1 have been identified as candidate markers in many tumors, including non-small cell lung cancer (NSCLC),^[35] brain tumor,^[33] CRCs,^[50,54] and breast cancer.^[53] Approximately 0.03–42% of tumor cells in solid tumors express CSC markers, depending on tumor type.^[146] Despite the rarity of CSCs, researchers use specific proteins on the cell surface to isolate and identify CSCs from a tumor biopsy with flow cytometry (Table 1). An immunomagnetic separation is also a promising tool that binds to cell surface markers via a specific antibody–antigen reaction, where an oriented displacement separates them in an external magnetic field. Particularly, magnetic-activated cell sorting (MACS) was described in many CSCs studies. Many studies used MACS to isolate or identify CSCs from human cancer cell lines;^[147,148] however, a small number of studies were conducted with clinical samples.^[33,40,148,149] Thus, there is an urgent demand for more studies to use clinical samples. Currently, surface marker-based approaches present tremendous progress for capturing rare cancer cells (Figure 3A), while other methods have also been developed, including sphere formation assay, side population assay by Hoechst 33 342 exclusion, and aldehyde dehydrogenase (ALDH) activity assay.

A subpopulation of SK-RC-42 renal cell carcinoma cells grew as tumorspheres in a serum-free medium supplemented with epidermal growth factor (EGF) and fibroblast growth factor (FGF).^[150] Sphere-forming cells (SFCs) expressed high levels of MHC class I but low MHC class II, CD80, and CD86 compared to monolayer adherent cells (MACs). However, the expression of CSCs markers (CD44, CD24, and CD133) was not found in SFCs. The subcutaneous injection of 2×10^5 SFCs generated

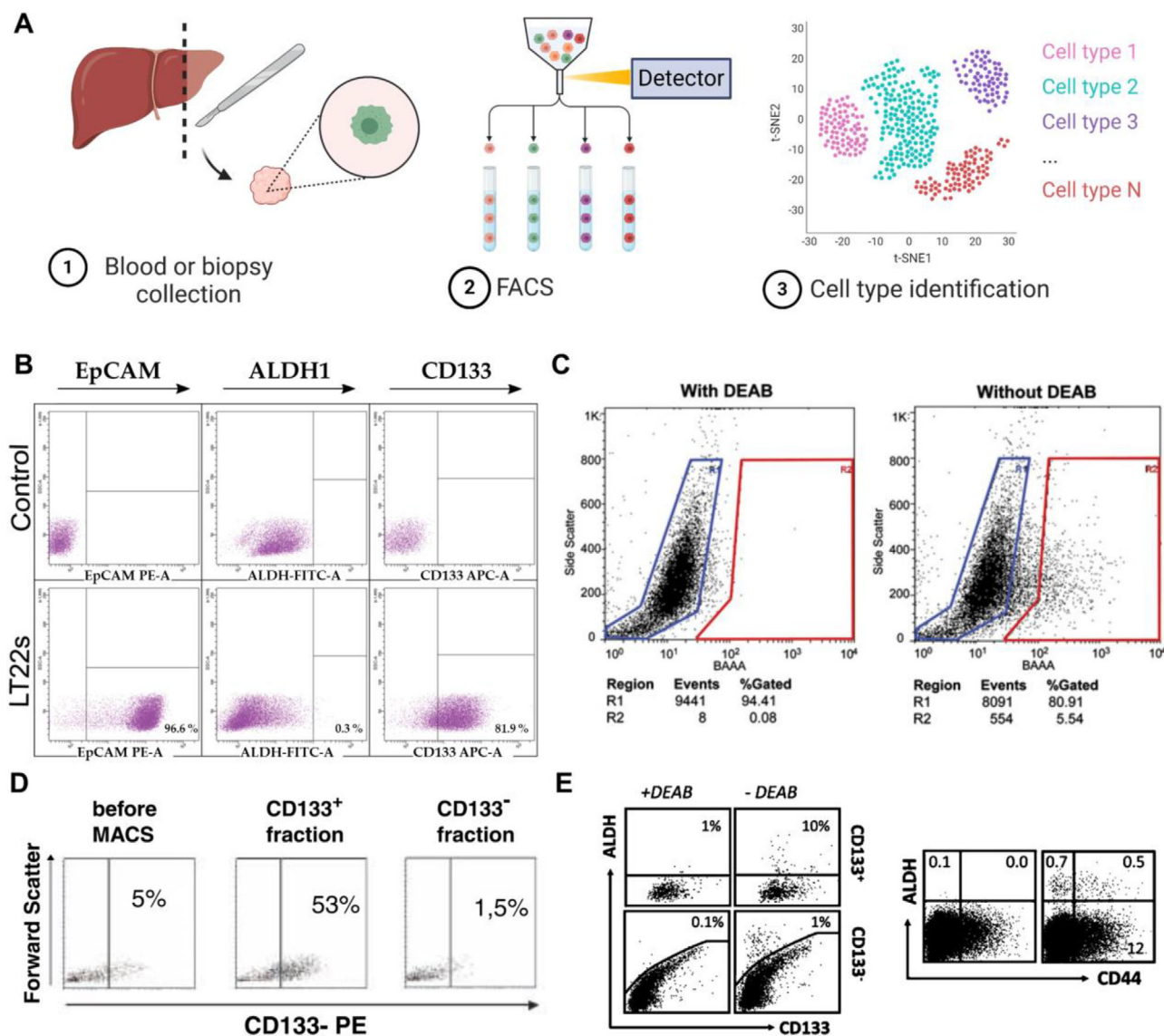


Figure 3. CSCs isolation from cancer patients with most common cancer types using FACS and activity of ALDH1. A) Schematic representation of FACS isolation of the subpopulation of CSCs. B) Isolation and identification of subpopulation cells in primary lung adenocarcinoma patients. Flow cytometry-based identification of CSC markers in LT22s cells, showing EpCAM, ALDH1, and CD133 expressions. Controls were stained with isotypes or treated with DEAB. Adapted with permission.^[35] Copyright 2017, Springer Nature. C) Flow cytometry analysis of ALDH activity in cells derived from a human breast tumor orthotopically xenotransplanted in NOD/SCID mice. Reproduced with permission.^[53] Copyright 2007, Elsevier. D) CD133⁺ CSCs in primary glioblastomas. CD133⁺ tumor cells were separated using magnetic-activated cell sorting (MACS), and purity was assessed by flow cytometry. Reproduced with permission.^[151] Copyright 2007, American Association for Cancer Research. E) Flow cytometric analysis of multiple cancer stem cell markers in fresh ovarian cancer. ALDEFLUOR assay was done on human ovarian cancer cells. As a control for ALDH activity, the DEAB inhibitor was used (left). The phenotypic characterization of CD44⁺ tumor cells. Plot is shown as the percentage of CD44⁺ cells expressing ALDH marker (right). Reproduced with permission.^[162] Copyright 2011, John Wiley & Sons.

tumors in mice after 30 days, while the same number of MACs did not produce tumors. Importantly, SFCs had many CSCs features such as self-renewing ability in vitro and in vivo and high mRNA levels of “stemness” genes, including Oct3/4, BMI, NANOG, and β -catenin, as well as chemotherapeutic agents and irradiation resistance compared to the MACs.

Primary lung cancer cells isolated from patients consisted of two biologically distinct adenocarcinoma cell subpopulations, which differed phenotypically and genotypically.^[35] The first

subpopulation was initiated and sustained as spheroids (LT22s), whereas the other subpopulation was only capable of growth and proliferation under adherent conditions. The presence of CSCs markers was investigated with FACS using CD133 and ALDH1. In particular, LT22s expressed CD133 markers with serial passages, ranging from 14% to 80% after 19 passages. EpCAM expression was frequently more than 90%, while ALDH1 expression was not found in LT22s (Figure 3B). Eventually, LT22s were more resistant to Cisplatin than other subpopulation

cells. This study suggests that CSCs marker expression is not perpetual during the culture.

The distinction between GBM-derived CD133⁺ and CD133⁻ cells was investigated in stem cell-permissive medium, grown as neurosphere-like and adherent, respectively.^[151] Magnetic cell separation (Figure 3D) revealed that the CD133⁺ subpopulation was asymmetrically divided, and the yielded cells expressed markers showing characteristics for all three neural lineages (oligodendroglial, astroglial, and β III-tubulin). Moreover, 2–5% of CD133⁺ tumor cells formed spheres from a single cell within 42 days. In contrast, CD133⁻ GBMs displayed a lower proliferation index, and GeneArray analysis revealed that 117 genes were differentially expressed from CD133⁺ tumor cells. These data suggest that CD133⁺ CSCs perpetuate a subset of primary BMs.

According to relevant literature, some transport proteins like MDR1 and ABCG2 have been upregulated in CSCs than in normal stem cells.^[152] Transport proteins can excrete Hoechst33342 dye and cancer drugs from cells, and the resulting side population (SP) cells exhibit a low degree of staining and are identified by flow cytometry. Wang et al. demonstrated that SP cells effluxed Hoechst 33 342 when transplanted into NOD/SCID mice.^[153] Moreover, SP cells were more resistant to chemotherapy and radiotherapy based on the dye efflux properties of ATP-binding cassette (ABC) transporters and smoothed protein expressions. They also showed that SP cells in human nasopharyngeal carcinoma cell line CNE-2 had stem cell characteristics *in vitro* and high tumor-forming ability *in vivo*. Essentially, a high level of cytokine 19 expressions revealed by immunofluorescence and resistance of chemotherapy and radiotherapy found in SP cells were due to ABC half transporter member 2 of G family protein and smoothed protein expression, respectively. The significance of SP cells was investigated in the last two decades, and studies found that many normal tissues contain SP cells, such as skeletal muscle,^[154] kidneys,^[155] and breast mammary epithelia.^[156] These cells share various stem cell properties, including a long lifespan, quiescence, and particularly resistance to drugs via ABC transporter expressions.

Originally, the ALDEFLUOR assay was developed to detect ALDH activity in hematopoietic cells,^[157] but now it is used for the detection of CSCs in various tumors, including Ewing's Sarcoma,^[158] breast cancer,^[159] lung cancer,^[160] colon cancer,^[161] and ovarian cancer.^[162] CSC population in Ewing sarcoma family tumors (ESFT) cell lines and xenografts were identified based on high expressions of ALDH.^[158] Ewing's sarcoma contained an ALDH^{high} population of chemotherapy-resistant cells that retain sensitivity to EWS-FLI1 oncoprotein inhibition. ALDEFLUOR⁺ population, derived from normal and malignant mammary tissue, presented stem cell properties *in vitro* and *in vivo*.^[159] In other words, cancer stem-like cells, isolated from human breast tumors (Figure 3C) based on ALDH activity, propagated as tumoroids. The tumor-initiating capacity and the self-renewal capacity of these cells were maintained even after serial passages. ALDEFLUOR⁻ cells failed to generate tumors, where ALDEFLUOR⁻ tumors ceased growing after three passages. Results showed that ALDEFLUOR⁺ cells contain cells with stem cell properties, while ALDEFLUOR⁻ cells contain progenitor cells that can undergo limited growth but not self-renewal.

Alternative ALDEFLUOR assay was implemented via flow cytometric isolation. CSCs were isolated based on the enzymatic activity of ALDH (Figure 3E), and implantation of these cells in NOD/SCID mouse generated tumors.^[162] Furthermore, ALDH⁻ cells generated tumors with 20% efficiency, while ALDH⁺ cells formed tumors dose dependently with 80% efficiency. Moreover, with other canonical markers, CD133 increased tumor-initiating ability up to 100%. Thus, ALDH shows the potential to be a specific marker for identifying, isolating, and tracking human ovarian CSCs during ovarian cancer development.

Considerable progress has been made in the identification or isolation of CSCs by investigating the radio-resistance of CSCs. CSCs have significant resistance to radiotherapy and can repair the double-stranded DNA that is broken by radiation. Lung cancer A549 cells and breast cancer SK-BR-3 cells repaired their broken double-stranded DNA when given a dose of 3–4 Gy for 12 days.^[163] While SOX2, Oct4, and CD133 expressions were not generally correlated with radio-resistance, the presence of ALDH1, a candidate marker of CSCs, in subpopulations demonstrated increased radio-resistance. Another candidate marker, CD133, was highly expressed in a subpopulation of GBM multiforme, an aggressive brain tumor. Likewise, the population of CD133⁺ cancer-initiating neural stem cells (NSC) showed resistance to gamma radiation via preferential activation of the DNA double-strand break (DSB) response machinery.^[164] Hence, these candidate markers can be used to develop research on screening CSCs with radiation resistance.

Nowadays, scientists carefully analyze 1) sphere-forming ability, 2) surface marker expressions, and 3) tumorigenesis of CSCs following xenotransplantation to identify and confirm CSCs from a heterogeneous tumor cell population. Therefore, a comprehensive phenotypic and genotypic analysis of CSCs *in vitro* after the rapid expansion is essential to confirm the stage of CSCs. Traditional approaches are time consuming and costly, leading to inaccurate patterns of the parental tumor. We believe that advanced materials can capture and/or isolate these elusive cells from peripheral blood and tumor biopsy for large-scale high-throughput applications and drug screening.

3. Expansion of Rare Cancer Cells

Primary cancer cells can be expanded by *in vitro* or *in vivo* approaches. However, rare cancer cells are susceptible to the outside of their microenvironment, and low abundance is the current obstacle. Thus, these hurdles make it difficult to maintain both CTCs and CSCs *in vitro* culture. It is crucial to amplify all CTCs and CSCs from the specimen to view distinct tumor relapses due to tumor heterogeneity. Cell–cell and cell-ECM interactions play a crucial role in cancer progression and metastases within the TME. For example, tumor-associated adipocytes are involved in tumor angiogenesis by secreting adipokines, which include growth factors, hormones, and cytokines.^[165] Engineered adipocytes delivered ruminic acid (RA) and doxorubicin prodrug (pDox) with a reactive oxygen species (ROS)-cleavable linker at the tumor site.^[166] Consequently, antitumor effects mediated by tumor-associated adipocytes succeeded by exploiting fatty acid-binding protein 4-mediated lipid transportation. RA-containing adipocytes downregulate PD-L1 expression of tumor cells, allowing infiltration of effector T cells. PD-L1 and PD-L2 are

expressed in many cell types, including tumor cells and tumor stroma. In the TME, immune cells play a vital role in cancer cell proliferation and invasiveness. Cancer-associated fibroblasts (CAFs) can significantly promote tumorigenesis^[167] via cytokine secretion such as stromal cell-derived factor 1 (SDF1), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and hepatocyte growth factor (HGF).^[168] Cytotoxic T-lymphocyte-associated antigen 4 (CTLA4; also known as CD152) and programmed cell death protein 1 (PD1; also known as CD279) are two immune-checkpoint receptors widely studied for cancer immunotherapy.^[169,170] With the advances in technologies, single-cell isolation and analysis are promising techniques for improved cancer diagnoses and treatments. Particularly, single-cell RNA sequencing (scRNA-seq) enables us to understand intra-tumor heterogeneity and comprehensive gene expression of CTCs^[171] and CSCs.^[172] Single-cell drug screening has shown promise for drug screening and developed for tailored medicine.^[173] However, single-cell drug screening technology is impossible to reflect tumor heterogeneity, cell–cell interactions, and cell–ECM interactions. More recent work in this area extends the methods by using “Organs-on-a-chip,” known as microphysiological systems.^[174] Drug development is slow and costly due to the lack of preclinical models that can be used for drug testing. Dynamic cell culture system changes the physiological maturation and function of organoids during in vitro culture. Furthermore, coupling this technology with gene editing^[175] offers a potential way to establish advanced cancer treatments and other diseases treatment.

More recently, organoid technologies gained much attention for both tissue engineering and cancer treatments. In December 2020, bladder assembloids have been developed in vitro that show high similarity to normal bladder and bladder cancer.^[176] The results demonstrate the competence of the 3D bioprinting method for establishing multilayer bladder term as “assembloids” by reconstructing tissue stem cells with stromal components to mimic an organized architecture leveraged by epithelium surrounding stroma and an outer muscle layer. Both normal and cancer bladder assembloids showed characteristics of mature adult bladders and pathophysiological features of urothelial carcinoma, respectively. Assembloids represent mature organ architecture rather than typical organoids, which are often comprised of a single type of cell. This study also shows a great opportunity to replace the dysfunctional bladders and test the anticancer drug for the patients. Moreover, many bioengineering approaches are explored for tumor organoid cultures, including ECM scaffolds (i.e., Matrigels, hydrogels, and collagens),^[176–178] spinning bioreactor,^[176,179] low-adherent culture plate,^[180] 3D bioprinting,^[176,181,182] hanging-drop,^[183] micropatterning^[128,184] and microfluidic.^[185,186] Drug screening and tumor organoid banking have recently been developed for personalized anticancer drugs and new drug testing. Stable and long-term tumoroid cultures from rare cancer cells provide an opportunity for high-throughput drug screening of therapeutic regimens but had limited success until recently. Thus, we propose materials that assist in building an artificial niche to support tumoroid formation (**Figure 4**). Tumoroids can mimic the TME consisting of complicated factors like ECM, secreted factors from neighboring cells, physical factors, and hypoxia.

3.1. CTC Expansion

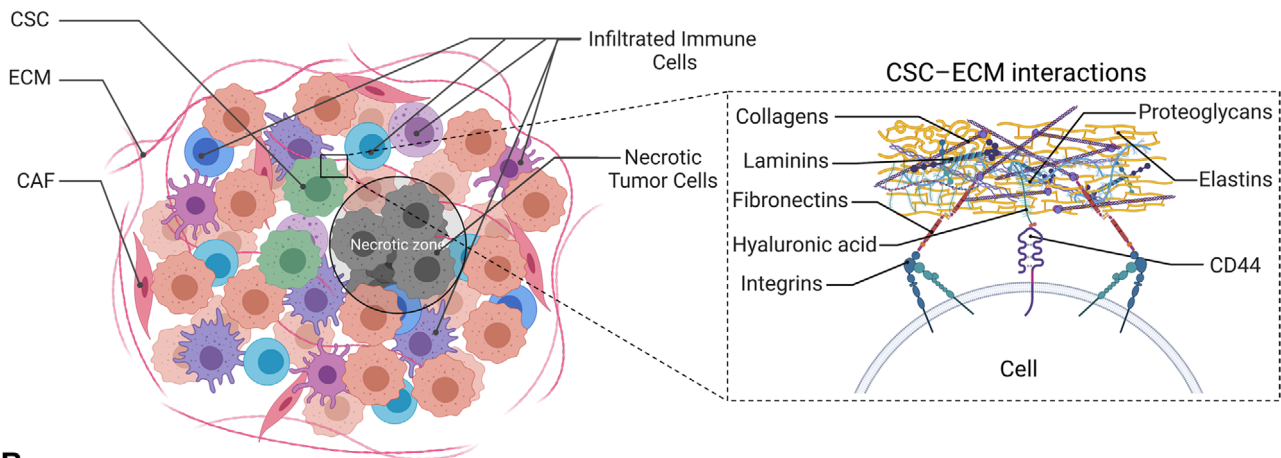
Cancer-related mortality rates remain relatively high because of a high failure rate in anticancer drug development due to insufficient preclinical models. Many CTCs shed from the primary tumor, but only a few survive and colonize successfully in the secondary organ. Human primary cells often require conditioned media with growth supplements^[187] and a defined system, such as a feeder layer,^[188] biomaterial (i.e., scaffold),^[189] and artificial matrixes^[190] to proliferate in vitro (**Table 3**). Optimizing culture conditions may require a substantial amount of time and costs. However, non-primate animal models are the golden standard for many studies, such as drug development and drug delivery studies. These animal models have limited capability to mimic the process of human carcinogenesis, physiology, and progression. Moreover, a substantial number of CTCs, at least >400 CTCs per 7.5 mL blood, is required to give rise to xenotransplants. There are currently some key approaches that play crucial roles in the in vitro CTC expansion into spheres, including serum-free media, hydrogels or Matrigel, feeder layers or co-culture, and ultra-low attached plate (**Figure 5A**). Therefore, establishing a novel approach or platform for expanding rare cells holds the promise for advancing high-throughput screening, tailored therapies, and precise cancer management. The goal here is to summarize previous studies and develop a cost-effective and reproducible method for in vitro culture of CTCs. The number of CTCs isolated from a patient’s blood sample is low, and we need an innovative approach to obtain a large population for molecular characterization.

One of the earliest ex vivo CTC expansions was demonstrated by Zhang et al. in 2013.^[18] Three out of 38 patients with invasive breast cancers were reported. EpCAM[−] CTCs were cultured in stem cell culture medium supplemented with EGF and FGF-2 for the first 7 days, then in EpiCult-C medium from day 8 until day 21 (**Figure 5B**, left panel). Surface marker expressions were assessed, and then ALDH1 and CK5/6/18 possible CTCs markers were expressed on cultured cells (**Figure 5B**, right panel). Additionally, selected markers (HER2⁺/EGFR⁺/HPSE⁺/Notch1⁺), known to cause brain metastasis, have been found in EpCAM[−] CTCs. Xenograft studies showed that these CTCs are highly capable of generating brain and lung metastases, and the presence of HPSE, Notch1, EGFR, and HER2 proteins were also detected in the metastatic lesions in animals.

Furthermore, CTC-derived organoids developed from advanced prostate cancer patients with high CTC numbers were counted in peripheral blood (>100 cells per 8 mL of blood) (**Figure 5C**).^[25] However, CTC-derived organoids were small compared to biopsy-derived prostate cancer organoids. Organoids were maintained in advanced DMEM/F12 medium with growth factor reduced Matrigel. Notably, CTC-derived organoids mimicked primary cancer (prostatectomy specimen) from patients.

In 2014, long-term CTC cultures were established from six patients with estrogen receptor-positive breast cancer.^[17] CTCs were maintained in vitro using a serum-free medium supplemented with FGF, EGF, and B27 under hypoxic conditions (4% O₂) on ultra-low attachment plates for >6 months. One or more CTC cell lines were successfully generated from 6 out of 36 patients, either off therapy or progressing on treatment (**Figure 5D**, upper panel). Cultured CTCs were consistent with standard CTC

A Tumor microenvironment (TME)



B Biomaterials-assistant tumoroids

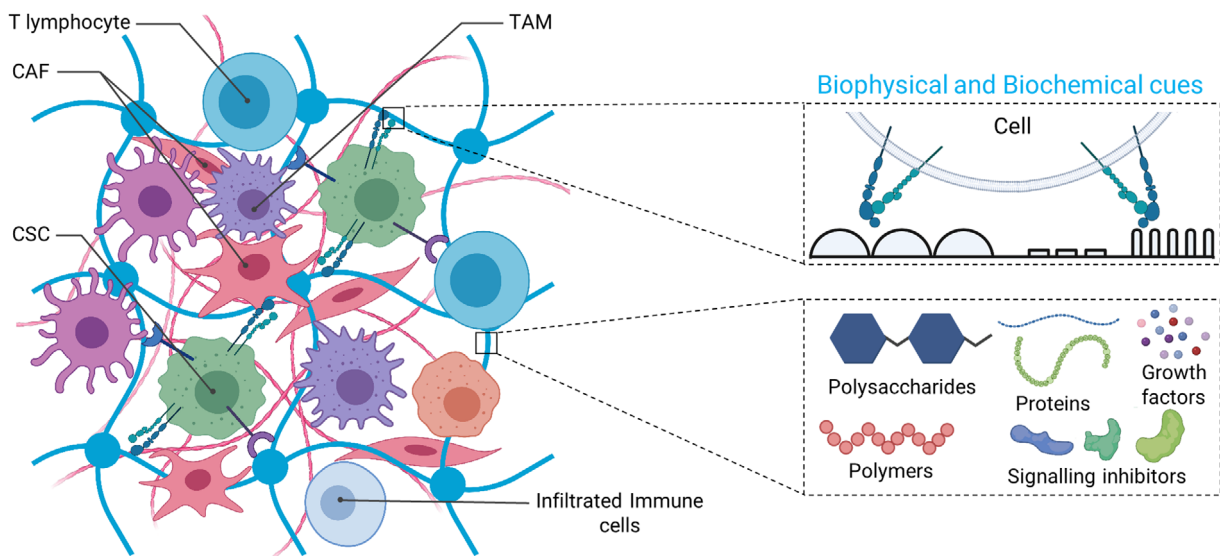


Figure 4. A) Schematic illustration of the tumor microenvironment (TME) and CSC niche. B) Biomaterial-assistant tumoroids composed of various biophysical and biochemical cues and cancer-associated rare cell subtypes could rebuild the primary or secondary tumors in vitro. CSC, cancer stem cell; ECM, extracellular matrix; CAF, cancer-associated fibroblasts; TAM, tumor associated macrophage.

definitions, staining positive for epithelial cytokeratin (>95% of cells) and negative for leukocyte marker CD45 (Figure 5D, lower panel). Three of the five CTC lines were tumorigenic in mice and shared cytological and genomic features with their parental CTCs captured by iChip from patients' blood. In other words, ESR1, TP53, and KRAS mutations were universally present in all CTC cell lines and independently isolated CTCs. The proliferative index of CTC cultures was $\approx 30\%$ by Ki67 staining, and the CTC cultures doubled in 3 days to 3 weeks.

Cayrefourcq et al. proved that CTCs isolated from colon cancer patients were able to generate cell lines.^[19] However, the number of CTCs was lower in the peripheral blood of colon cancer patients compared to breast and prostate cancer patients. CTCs were cultured in a 2% serum medium with EGF and FGF2 under hypoxic conditions (2% O₂) on non-adherent plates on the first

day. After a few weeks, the CTCs culture was switched to another growth culture medium to improve cell growth under normoxic conditions (5% CO₂). The established cell line had a tumorigenic potential in immunodeficient mice and resembled the characteristics of original tumor cells from patients with colon cancer. Single-cell transcriptome analyses revealed cells with intermediate phenotype between epithelial and mesenchymal by EpCAM⁺, CK19⁺, E-cadherin⁺, and Vimentin⁻. Most interestingly, CSCs markers, ALDH1⁺, CD133⁺, and Snail⁺, have been observed in CTCs. These results highly indicate that CTCs and CSCs' origin might be the same, even suggesting a positive feedback mechanism between CSCs and CTCs.

A novel in situ approach called 3D co-culture was investigated for expansion and subsequent CTCs capturing by the CTC-chip platform.^[16] CTCs were isolated from stage I lung cancer

Table 3. Expansion medium complement for rare cancer cells.

Cell type	Medium	Supplements	G.F.	Substrate	Incubation	Ref	
Circulating Tumor Cells (blod) (CTCs)							
Breast	DMEM/F12	Day 1-7	<ul style="list-style-type: none"> • Insulin • Hydrocortisone • B27 	<ul style="list-style-type: none"> • EGF • FGF2 	Tissue culture flasks	5% CO ₂	[18]
	EpiCult-C	Day 8-21	<ul style="list-style-type: none"> • FBS • P/S 	-			
	DMEM/F12	Day 22+	<ul style="list-style-type: none"> • FBS • P/S 	-			
Breast	RPMI-1640	<ul style="list-style-type: none"> • B27 • A/A 	<ul style="list-style-type: none"> • EGF • FGF 	Ultra-low attachment plates	5% CO ₂ 4% O ₂	[17]	
Lung	RPMI-1640	<ul style="list-style-type: none"> • FBS • P/S 	-	PDMS Microposts Collagen, Matrigel, CAF	7.5% CO ₂	[16]	
Cancer Stem Cells (CSCs)							
Glioblastoma	Neurobasal media	<ul style="list-style-type: none"> • N2 x 0.5 • B27 x 0.5 	<ul style="list-style-type: none"> • EGF • bFGF 	Tissue culture dishes	-	[205]	
Colon	DMEM/F12	<ul style="list-style-type: none"> • Glucose • NaHCO₃ • HEPES • L-Glutamine • Heparin • BSA • Apotrasferrin • Insulin • Putrescin • Sodium selenite • Progesterone 	<ul style="list-style-type: none"> • EGF • bFGF 	Collagen or Matrigel coated flasks	5% CO ₂	[206]	
Colon	NS-A Basal Medium	<ul style="list-style-type: none"> • L-glutamine • Glucose • Putrescine • Progesterone • Sodium selenite • Insulin • Transferrin sodium salt 	<ul style="list-style-type: none"> • EGF • FGF 	Collagen coated dishes	-	[38]	
Ovarian	DMEM/F12	<ul style="list-style-type: none"> • Insulin • BSA 	<ul style="list-style-type: none"> • EGF • FGF 	Ultra-low attachment plates	-	[208]	

G.F.: growth factors; A/A: antibiotic/antimycotic; FBS: fetal bovine serum; P/S: Penicillin/Streptomycin; BSA: bovine serum albumin; EGF: epidermal growth factor; FGF: fibroblast growth factor; PDMS: Polydimethylsiloxane; CAF: cancer-associated fibroblasts.

patients. Ex vivo expansion succeeded from 14 out of 19 patients when CTCs were co-cultured with CAFs. They were co-cultured on micron posts with 100 µm height consisting of a combination of collagen and Matrigel (Figure 5E). TP53 (tumor protein p53) mutations were characterized in cultured CTCs, and they were identical to those from the patient's tumors. The next-generation sequence (NGS) was investigated to detect matching mutations between cultured CTCs and primary tumors. As a result, CASP8, APC, TP53, and ERBB4 gene mutations matched in three out of the eight paired CTC-tumor samples. Taken together, cancer metastasis may be related to some key genes manifested in CTCs that are involved in cancer progression.

According to our previous studies, a new type of cell culture substrate named binary colloidal crystals (BCCs) or colloidal self-assembled patterns (cSAPs) has shown the potential in rare cell expansion in vitro.^[191,192] In our recent research, we established ex vivo CTC tumoroids using BCCs.^[128] After CTCs

enrichment from peripheral blood from 22 patients with SCLC, CTCs were cultured on BCCs, and spheroids were observed in 14 days and continued to widen. Useful CTC spheroid proliferation was found after 4 weeks, with 82% of patients. Expanded CTCs were characterized as EpCAM⁺, TTF-1⁺, synaptophysin⁺, and CD45⁻. The expressions of N-cadherin and E-cadherin indicated heterogeneity of CTCs. Importantly, in vitro drug sensitivity results recapitulated the clinical response of cisplatin and etoposide. We used this system and generated CTC organoids from 190 patients with 25 different types of primary malignancy at a yield rate of 89%. These CTC organoids were found to retain immunophenotypic and genetic features of the primary tumors (unpublished data).

Almost all studies mentioned above used a defined medium with many growth factors, including EGF, FGF, and B27, and ultra-low attached plates. The sophisticated in vitro model that can recapitulate the biology of CTCs is an urgent demand for

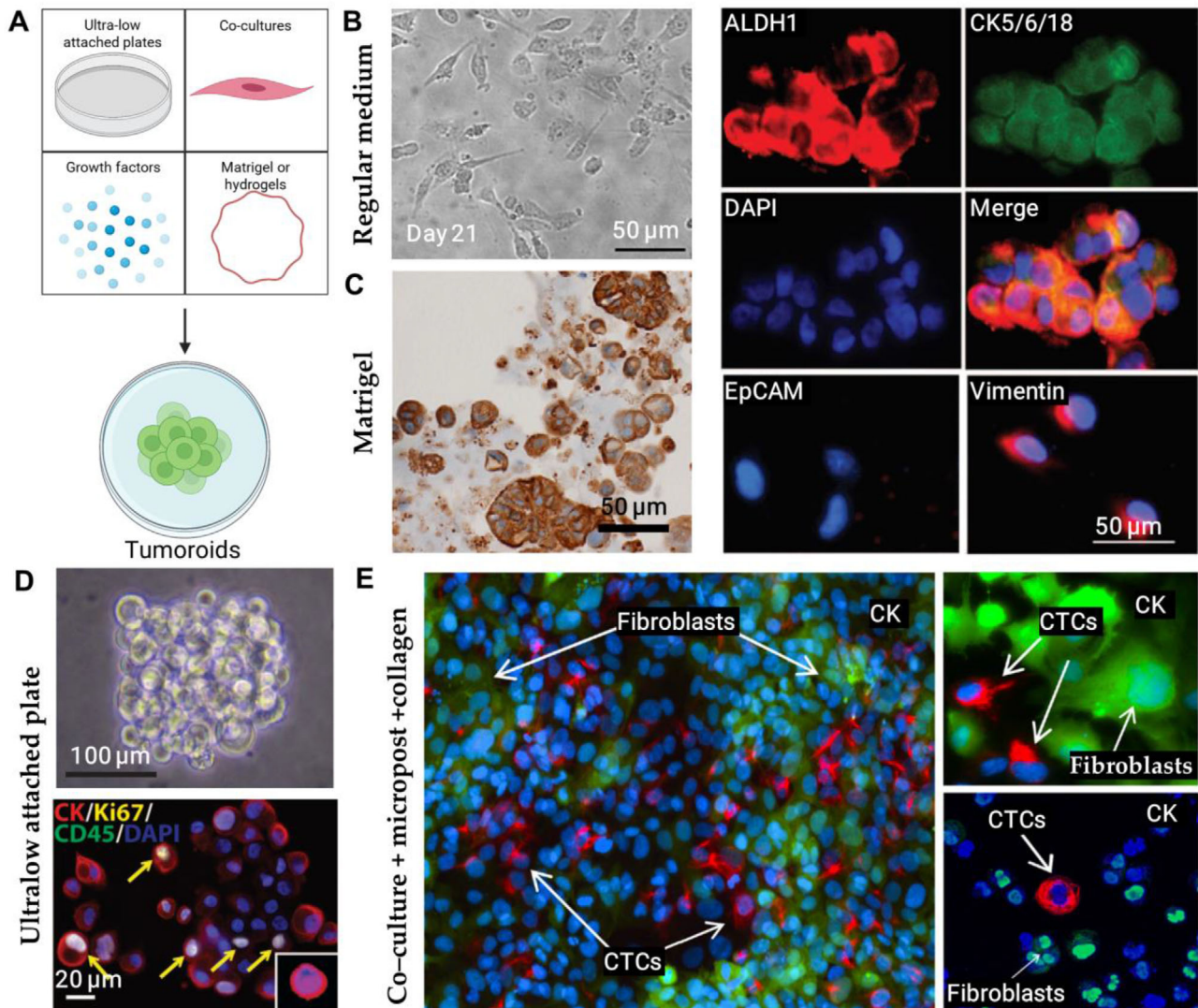


Figure 5. In vitro expansion of CTCs and marker expressions of CTCs from various cancer patients. A) Schematic illustration of material-assisted in vitro CTCs expansion. B) Bright-field microscopy images of CTCs isolated from breast cancer patient grown for three weeks. EpCAM⁻ CTC characterization and culture from three patients. Immunofluorescence staining of potential CTCs (EpCAM⁻/ALDH1⁺/CD45⁻) for ALDH1, CK5/6/18, and vimentin expressions. The merged panel consists of ALDH1/CK/DAPI-positive IF staining. Adapted with permission.^[18] Copyright 2013, American Association for the Advancement of Science. C) Immunohistochemistry staining against pan-cytokeratin of circulating tumor cell-derived organoids from patients with advanced prostate cancer. Following CTCs isolation cells were washed and seeded in growth factor-reduced Matrigel. Reproduced with permission.^[25] Copyright 2014, Elsevier. D) Phase-contrast images of non-adherent breast cancer CTC cultures were grown in ultra-low attachment plates under hypoxic conditions (4% O₂). Non-adherent CTC cultures were grown in ultra-low attachment plates. Surface markers CK, Ki67, and CD45 expressions and nuclei staining DAPI were present under hypoxic conditions (4% O₂). Reproduced with permission.^[17] Copyright 2014, American Association for the Advancement of Science. E) CTCs were captured and expanded from patient samples. CTCs were expanded by co-culturing with fibroblasts on microposts and characterized with CK7/8 (red) surrounded by GFP-fibroblasts. Adapted with permission.^[16] Copyright 2014, Impact Journals.

insight and characterization of CTCs. Among various biological tissues to generate organoid cultures, CTC-derived organoid culture with the assistance of engineered materials is a promising approach for preclinical applications. Hence, we propose that signaling can cause reciprocal interactions between the ECM molecules and the cells from structured materials and support in vitro expansion by emulating CTCs niche in the culture system.

Another approach to expand CTCs is the patient-derived xenotransplantation (PDX) into immunodeficient mice. Baccelli et al.

reported that metastasis-initiator cells might have EpCAM^{low}, C-met^{high}, and CD47^{high}, CD44^{high} phenotypes and metastatic potential in bone, lung, and liver of NSG mice after xenotransplantation of breast cancer patient CTCs.^[193] A subset of CTCs was identified as EpCAM⁺, CD44⁺, CD47⁺, and C-met⁺. Some patients, who were correlated with many metastatic sites and lower overall survival rates, had EpCAM⁺ CTCs, but not all. However, xenografting was successful for only advanced-stage patients with high CTCs numbers. Hodgkinson et al. presented that morphologically and genetically identical tumors (with SCLC patients'

CTCs) were formed in immunocompromised mice.^[194] CTC-derived explants (CDXs) were reflected in the donor patient's response to chemical therapy using platinum and etoposide. Also, the similarity between isolated CTCs and corresponding CDX was revealed by copy number aberration (CNA) analysis. Biopsy collection from SCLC patients is challenging, and the highest CTC counts from SCLC are from all solid tumor patients.^[195] However, SCLCs still provide the best functional model for developing PDX. Therefore, CTC-derived tumoroids pave the way for developing a sustainable patient-derived model for personalized medicine.

The significance of EpCAM⁺ CTCs in cancer metastasis has been proven experimentally.^[196] Rossi et al. showed a greater success rate of CTCs survival and growth in xenotransplants than those previously presented in breast cancer and hepatocellular carcinoma.^[197] The CTCs isolated from metastatic prostate cancer patients by EpCAM enrichment through the CellSearch system survived and grew in a xenograft assay. Interestingly, 100% (8 out of 8) of human CTCs were found in murine peripheral blood, 75% (6 out of 8) in murine bone marrow, and 6 out of 8 murine spleens were positive human cytokeratin. Altogether, this study emphasized that as few as 50 EpCAM⁺ CTCs could initiate tumors in mice; therefore, phenotypic characterization of CTCs has taken attention to further xenograft studies.

Currently, no study directly compares xenografts and cultured CTCs freshly isolated. Recent progress in the fabrication of defined hydrogel and structured substrate systems opens up the avenue for maintaining inherently vulnerable cell types, high-throughput screening, and downstream molecular analysis with tunable biochemical and biophysical properties. A new family of nanotextured surfaces called monolayer BCCs have been recently developed for stem cell differentiation and cell reprogramming.^[191,198] The advantages of these new substrates are 1) tunable structural symmetry (i.e., ordered or random) and 2) facile surface modification. We believe that this new type of surface can facilitate CTC expansion and cancer research soon.

3.2. CSC Expansion

Cells communicate through cell junctions and dynamically interact with ECM, soluble factors, and hormones using their receptors.^[199] ECM plays a pivotal role in normal stem cell^[200] growth and differentiation and cancer progression^[201] via biochemical and mechanical cues. Interestingly, a growing body of evidence suggests that the CSC niche is similar to the stem cell niche, regulating self-renewal and differentiation.^[54,202] The surrounding microenvironment or niche regulates CSCs' stemness and proliferation via multiple sources of cytokine production. For example, the ECM plays an essential role in anchoring CSCs to their niches.^[203] Hyaluronan (HA) plays a crucial role in CSC niches, and the HA-mediated CD44 interaction supports tumor progression by revealing the similarity between CSCs and normal stem cells. Thus, the CSC niche mimics HA-based multilayer films, creating different surface properties.^[204]

Presently, successful *in vitro* CSC expansion is composed of three main components, including serum-free media with growth factors, hydrogel-like materials, or ultra-low attached plates (Figure 6A). Recently defined mediums for the expansion

of CSCs are summarized in Table 3, which maintain a sphere-like phenotype when used with ultra-attachment plates or polymer-coated plates. Ricci-Vitiani et al. expanded CD133⁺ colon cancer cells for long-term (more than 1 year) *in vitro* as undifferentiated tumoroids in a serum-free medium, and cells showed the same morphological and antigenic pattern of the parental tumor.^[38] CD133⁺ colon cancer cells were organized into spheres on ultra-low attachment plates using a defined medium. Once the culture medium was replaced by the standard medium (absence of growth factors and addition of 5% FBS), the CD133⁺ expression was significantly downregulated in colon cancer cells, acquiring an adherent morphology. Moreover, CD133⁺ cells lost their ability to initiate a tumor in immunocompromised mice under standard cell culture conditions. This result suggests that colon cancer-initiating cells need to remain undifferentiated (spheres) to maintain tumorigenic potential.

The phenotypic and genotypic differences of tumor stem cells have been shown in distinct culture conditions. CSCs derived from GBMs closely mimicked the genotype, gene expression, and biology of the parental tumor cultured in the serum-free medium rather than those in the regular medium.^[205] In particular, tumor stem cells cultured in serum-free medium showed consistent telomerase activity indicating cell divisions, whereas telomerase activity was lost in serum-containing medium. Besides, single nucleotide polymorphism (SNP) analysis and spectral karyotyping analysis revealed a homozygous deletion at chromosome 9, loss of chromosome 10q, trisomy of chromosome 7, and local amplification of EGFR locus. Moreover, after 1 year of culture in serum-free culture conditions, both polyclonal and monoclonal populations of glioma cells precisely mirrored their parental tumor genotype. In contrast, glioma cells cultured in serum-containing medium underwent substantial genomic rearrangements after ten passages represented by pseudotetraploidy (chromosomally abnormal) and a loss of heterozygosity deletion of the entire chromosome 4 and chromosome 17.

A subpopulation of human colon carcinoma CD133⁺ cells grew *in vitro* as undifferentiated tumor spheroids (Figure 6B, left) and initiated tumor growth in immunodeficient mice.^[206] Xenografts resembled the original human tumor, maintaining the rare subpopulation of tumorigenic CD133⁺ cells. Colon carcinoma samples were dissociated into single cells and cultured in a serum-free medium containing EGF and FGF-2 to obtain the sphere-like culture. Spheroid cultures were all characterized by the round cell shapes and were negative for surface markers (CDX2, CK20, and CK7) but positive for CD133 (Figure 6B, right).

Eramo et al. expanded a rare population of tumorigenic cells from small cell and non-small cell lung cancer patients.^[207] After 1–2 months, all four subtypes of CD133⁺ cells formed spheres in a serum-free medium containing EGF and bFGF (Figure 6C). *In vivo* studies exhibited that generated tumors in mice were phenotypically identical to the original tumor. Interestingly, after differentiation, lung cancer CD133⁺ cells acquired the specific lineage markers while failing the malignant capability together with CD133 expression level.

The self-renewing stem cell population was isolated from solid ovarian tumors, using a method termed anchorage-independent (i.e., stem cell-selective) culturing breast cancer-initiating cells.^[208] Primary tumor specimens were obtained, dissociated,

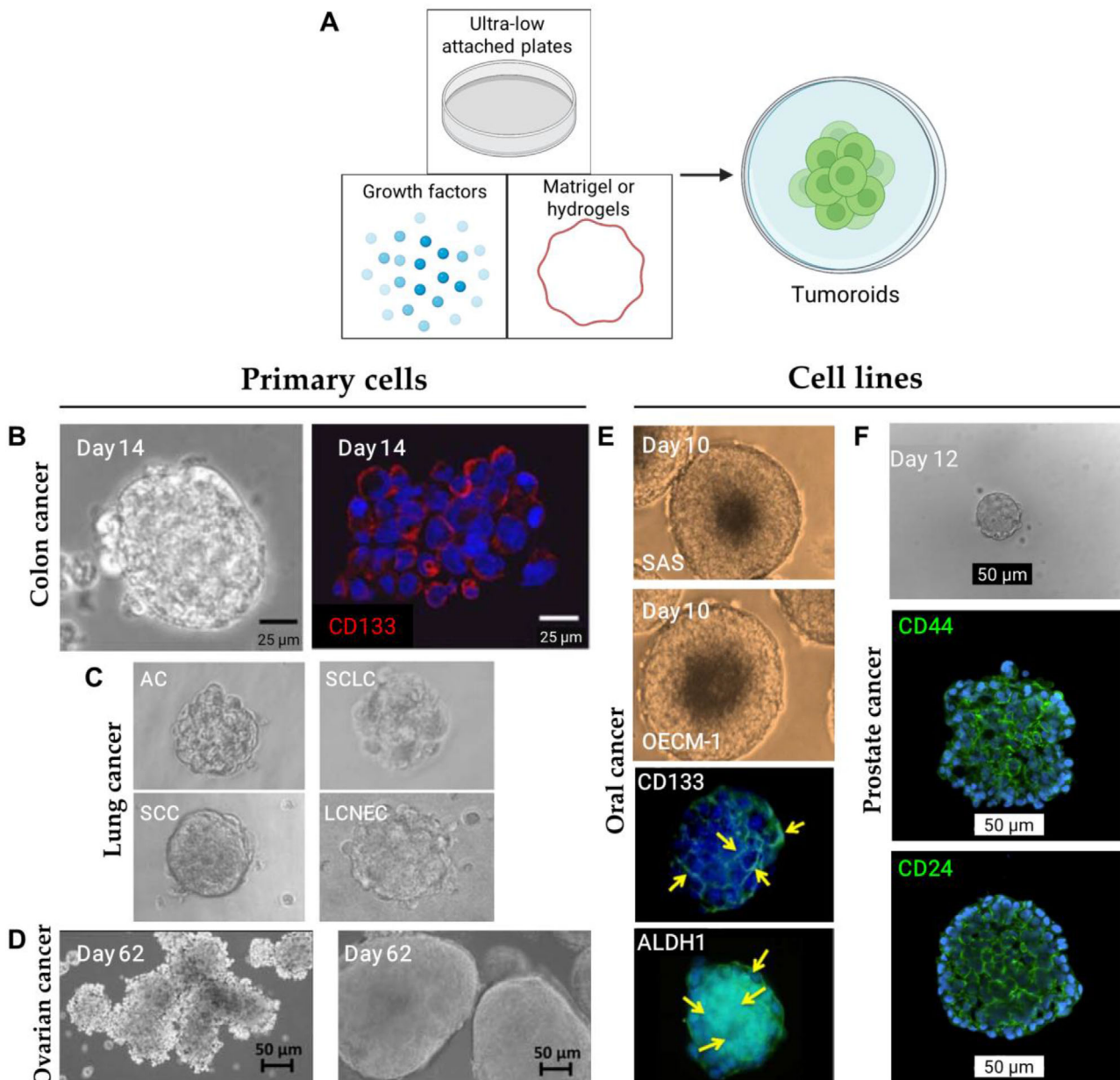


Figure 6. In vitro expansion of CSCs and marker expressions of different types of tumor cells. A) Schematic illustration of material-assisted in vitro CSCs expansion. B) CSCs morphology on Matrigel after 14 days (left), isolated from the colon adenocarcinoma patient. Colon cancer spheroids expressing CD133 (red) and nuclei (blue) following 14 days on Matrigel (right). Reproduced with permission.^[206] Copyright 2007, Elsevier. C) Four different subtypes (adenocarcinoma [AC], small cell lung carcinoma [SCLC], squamous cell carcinoma [SCC], and large cell neuroendocrine carcinoma [LCNEC]) of lung cancer cells were grown on non-treated tissue culture flasks for 1–2 months. Adapted with permission.^[207] Copyright 2008, Springer Nature. D) Distinct morphological spheres expanded from different ovarian cancer patients. The spheres were plated in ultra-low attachment plates and cultured under serum-free conditions. Adapted with permission.^[162] Copyright 2011, John Wiley & Sons. E) Oral squamous cell carcinoma SAS and OECM-1 cell lines were cultured on non-adhesive agarose thin films coated culture plastics (upper panel), expressing CD133 and ALDH1 surface markers (lower panel). Reproduced with permission.^[36] Copyright 2012, PLOS. F) DU145 prostate cancer cells were cultured as suspension spheres in a 24-well tissue culture plate for 12 days, expressing CD44 and CD24 surface markers. Adapted with permission.^[210] Copyright 2011, Elsevier.

and inoculated on regular culture plates in a serum-free medium with EGF, bFGF, and insulin. After 1 week, non-adherent spherical clusters of cells were observed. These floating spheres were enzymatically dissociated weekly, resulting in secondary spheres generated from single cells. After ≈ 10 passages, about 1% of the

spheres remained, appearing as distinct prototypical spheroids. Importantly, spheres were expressing some CSCs markers, such as CD117 and CD44. They obtained reasonable spheroids under stem cell conditions, with a $\approx 10^3$ -fold increase after 6 months.

Several primary cancer cell lines were established from ovarian cancer patients and examined for capacity of self-renewal.^[162] Proper sphere cultures were obtained from the initial bulk cells on ultra-low attached plates after 2 months (Figure 6D), which showed a strong self-renewal capability in vitro. Essentially, in vitro cultured primary cells developed tumors in NSG mice after inoculation. Following stem cell marker analysis revealed that there were appreciable ALDH, CD133, and ABCG2, but slight levels of CD44 and CD117.

Cancer cell lines behave as primary CSCs during in vitro culture; however, they are not ideal cells to study CSC biology. A non-adhesive culture system was used to generate spheres from the oral squamous cell carcinoma SAS and OECM-1 cell lines (Figure 6E, upper panel) within 5 to 7 days.^[36] Spheres expressed putative stem cell markers, CD133 and ALDH1 (Figure 6E, lower panel), and displayed tumor-initiating and self-renewal capabilities. Both SAS and OECM-1 spheres presented non-adhesive properties when DMEM and RPMI1640 mediums were used, respectively.

Liu et al. demonstrated that hydrogel materials like 2.5% hydroxypropyl methylcellulose (HMC)-agarose established CSC cell line by emulating brain tumor niches.^[209] Human GBM cell line, U-87 MG, cultured on a 2.5% HMC-agarose-based culture system, exhibited the highest spheroids number and largest size after 8 days of culture. CD133 expressions of GBM cells after six days of culture in 2.5% HMC-agarose-based culture system was 60%, which was relatively higher than the control group expressing only 15%. Interestingly, spheroids extracted from a 2.5% HMC-agarose-based culture system showed high Oct4 stem cell marker expression compared to no expression on TCPS substrate. Additionally, cells on a 2.5% HMC-agarose-based culture system had the highest chemoresistance. The live cells were greater than 80% even at the high dose of 500 μM temozolomide for 72 h. In terms of gene profiling, the expression of the ABCG2 gene was upregulated after culture in a 2.5% HMC-agarose-based culture system.

Another GBM-derived CSCs study showed that neurosphere-like CSCs were cultured under medium conditions favoring the growth of neural CSCs.^[151] Briefly, CSC cultures were characterized according to their growth pattern; 11 out of 15 primary GBMs contained a significant CD133⁺ subpopulation that displayed neurosphere-like morphology after 3–4 weeks of primary culture. In contrast, adherent spheres (4 out of 15) were not associated with CD133 expressions. So, in primary GBMs, at least two different CSCs behave differently under conditions suitable for NSC culture.

A subpopulation of DU145 prostate cancer cells grew as spheres^[210] (Figure 6F, upper panel) under defined serum-free conditions and displayed CSC markers CD44⁺ and CD24⁺ (Figure 6F, lower panel). DU145 cells were cultured in a serum-free medium containing BSA, B27 and lacking vitamin A, supplemented with recombinant EGF at 10 ng mL⁻¹, in a regular 24-well culture plate. As a result, mature DU145 spherical cells were able to initiate xenograft tumors better than monolayer cells, and spherical cells possessed self-renewal capacity, displaying considerable increases in proliferation.

During embryogenesis, EMT and the reverse process called the mesenchymal–epithelial transition (MET) play a crucial role.^[211–213] Advanced genetic research revealed that embryonic

transcription factors gave malignant features, such as invasiveness, motility, and apoptosis resistance, to neoplastic cells.^[214–222] For example, the expression of WNT2 in pancreatic cancer cells suppressed anoikis by enhancing anchorage-independent sphere formation and metastatic tendency.^[223] EMT induced detachment of tumor cells from a primary site into the blood circulation and enabled CTCs to enter the bloodstream.^[224] Expressions of EMT markers were associated with invasion, migration, and resistance to anoikis and apoptosis, where all may be required for the survival and dissemination of CSCs and CTCs.^[225,226] The distinct roles of CSCs in cancer progression can be studied by a variety of complementary in vitro approaches. Molecular-level study on expanded CSCs offers the great possibility of generating precise targets for cancer that may overcome drug resistance and effectively combat the process of tumor metastasis.

4. Current Status in Cancer Therapy

4.1. Current Cancer Treatments

Due to cancer metastasis and recurrence, cancer is by far the deadliest disease in many countries. Modern medicine is also continuously developing and innovating to overcome cancer. This section summarizes the existing tumor treatment options and the principles of treatment in common tumors.

Cancer treatments can be divided into systemic and local treatments. Systemic therapy involves preoperative adjuvant therapy and chemotherapy or targeted therapy after tumor metastasis or recurrence. On the other hand, local treatment refers to the treatment of detected tumor lesions, including resection, stereotactic ablation radiotherapy (SABR), thermal ablation, radiofrequency ablation, cryotherapy. The local treatment has an excellent effect on tumors with a straight edge and no spreading, especially for cancers insensitive to radiotherapy and chemotherapy, such as liver cancer.

In cancer therapy, preoperative systemic therapy is beneficial to tumor surgery. For example, breast cancer patients can be cured after systematic treatment with endocrine hormones. Excision is the preferred local treatment if the tumor is resectable after preoperative adjuvant therapy. Noisy resectable pancreatic cancer patients are randomly given neoadjuvant treatments before an operation, and then the possibility of surgical resection is evaluated. Only 5% of small-cell lung cancer patients are detected early, and their tumors are surgically removed in the early stages I–II of NSCLC. In contrast, the rest of the patients miss the optimal surgical period. Therefore, early cancer screening is an integral part of cancer prevention and treatment, and the screening process should fully consider the benefits and risks of future treatments. Taking NSCLC as an example, biopsies are not necessarily required for patients at stage I or II for treatment decisions because biopsies delay treatment and increase cost and operation risks. In the case of surgical intolerance, patients are evaluated following preoperative concurrent chemoradiotherapy. If patients have inappropriate medical conditions, adjuvant therapy can be performed by surgery and chemotherapy. SABR or complete radical radiotherapy along with chemotherapy may also be used. If chemoradiotherapy is given as the initial treatment, the amount of radiotherapy needs to be considered.

After stage IV or metastasis of cancer, it is necessary to perform a comprehensive patient analysis. For instance, molecular detection is required to determine the histological subtypes of metastatic NSCLC. Based on the mutated genes, corresponding drug targeting mutations will be selected first, such as erlotinib (inhibitor of the EGFR tyrosine kinase). Depending on the patient's condition after drug administration, other targeted drugs or local treatment (i.e., SABR or surgery) are given. For breast cancer patients after metastasis, chemotherapy can be alternated with targeted therapy in addition to endocrine therapy when specific gene (e.g., BRCA1) mutations are detected. A systematic treatment scheme is selected for metastatic hepatocellular carcinoma patients based on the patients' liver function. For example, sorafenib is used on patients with Child–Pugh grade A liver. Overall, targeted drugs play a dominant role in the treatment of cancer metastasis.

Radiotherapy, chemotherapy, targeted therapy, and local therapy are often combined. For instance, chemoradiotherapy can be classified into alternating therapy, sequential therapy, and synchronous therapy. Similarly, as essential means of cancer treatment, immunotherapy also has a chemotherapy-target-immunotherapy combined regimen. Among them, the degree of adverse reactions of immunotherapy is positively correlated with the curative effect. With the deepening understanding of immunotherapy's mechanism, immunotherapy can even be applied to stage I or II cancer. More cancer treatments are being developed, such as proton knives for cutting double-stranded DNA in cancer cells and in vitro tumor organoids for drug screening. With the continuous innovation of technology, precise and personalized medicine is the future of cancer therapy.

4.2. Patient-Derived Xenografts

Patient-derived xenografts (PDX) is an old approach that researchers developed since the first attempt of human cancer transplantation into an animal in 1775. The early tumor xenografts were established in immune-suppressed hosts in the 1950s by Helene Toolan.^[227] The advantages of using PDX are being able to model using various cancers, consisting of tumor heterogeneity, and cause high tumor–stroma interactions. Although not all patient-derived tumors can successfully be engrafted into mice, the success rate of PDX establishment is increased because of the establishment of immunocompromised mice.^[228] Animal models have provided some valuable insights into cancer biology, but they are time consuming. Moreover, most importantly, these animal models usually cannot reflect accurate pathogenic processes in patients. For instance, human cancers have complicated histological structures due to genetic and phenotypic heterogeneity, and they do not develop well into cancers in a genetically engineered mouse model.^[229]

A number of cancers, such as colorectal,^[230,231] pancreatic,^[232,233] breast,^[234,235] lung,^[236] skin,^[237] head and neck,^[238] prostate,^[239] and ovarian cancer^[240] has been established in PDX models. Complete recapitulation of patient's tumors in the PDX model remains as a tackle. Few limitations of PDX models have been revealed by Morgan et al., who reported that out of the 14 total mutations detected in the primary tumors, only 6 mutations were detected in the corresponding PDXs, and

four additional mutations that arose in early passages of PDXs were not present in the primary NSCLC.^[241] Moreover, NSCLC gene expression studies on PDXs confirmed that downregulation of genes corresponding to cell adhesion and immune response pathways was observed in human-derived tumor-associated cells. These results indicated that the PDX deviates from the original tumor over time.

4.3. Patient-Derived Tumor Organoids

Patient-derived tumor organoids (PDOs) or tumoroids is a promising approach to expand primary cancer cells and an alternative to replace PDXs due to its display of near-physiologic cellular composition and behaviors. In 2011, for the first time, Sato et al. established human tumor-derived organoids from colon cancer patients using Matrigel as a matrix.^[242] Since then, many attempts have been made to expand patient-derived tumor cells and healthy human tissues as an organoid in vitro culture for high-throughput screening, tissue engineering, and personalized medicine.^[177,243–245] Tumorigenesis is a complicated process driven by specific genetic events at different stages, such as angiogenesis, metastasis, and drug-resistant development. Wetering et al. developed 3D organoid cultures derived from healthy and tumor tissue from CRC patients.^[243] Somatic copy number and mutation spectra of colorectal carcinoma were recapitulated in tumor organoids. Essentially, one of the cultured organoids was sensitively inhibited by a porcupine (Wnt secretion inhibitor) when RNF43 (negative Wnt feedback regulator) mutation was present rather than APC. This observation implied that a small subset of cancer patients with a mutation in RNF43 could potentially use porcupine inhibition as a treatment.

Pancreatic PDO can be generated from freshly resected primary human pancreatic adenocarcinoma (PDAC) and maintained in a defined culture condition.^[244] These PDOs show histoarchitecture, reflect phenotypic heterogeneity of the primary tumor, and exhibit patient-specific physiological changes. These changes include hypoxia, oxygen consumption, and differences in sensitivity to inhibition of EZH2 histone methyltransferase. Particularly, the sensitivity of EZH2 inhibition was different among organoids derived from various patients, associated with H3K27me3 in both tumor organoids and matched patient tumors. These findings suggest that clonally derived organoids can be used to identify the patient-specific sensitivity of novel therapeutic agents.

Moreover, PDO cultures allow us to study the effects of particular oncogenic mutations using genetic engineering. Drost et al. used CRISPR/Cas9 approach to identify key “driver mutations” involved in CRC growth and progression by expanding human primary intestinal organoids.^[246] Their study revealed that accumulated mutations in organoids in the mismatch MLH1 repair gene precisely modeled the mutation profiles observed in mismatch repair-deficient CRC. In a breast cancer cohort, the cancer predisposition gene NTHL1 encoding a base excision repair protein revealed a mutational footprint using PDO culture approaches.

In the native stem cell environment, stem cells receive specific biophysical and biochemical cues from the niche to self-renew, differentiate, and organize into tissues and organs. CSCs are also

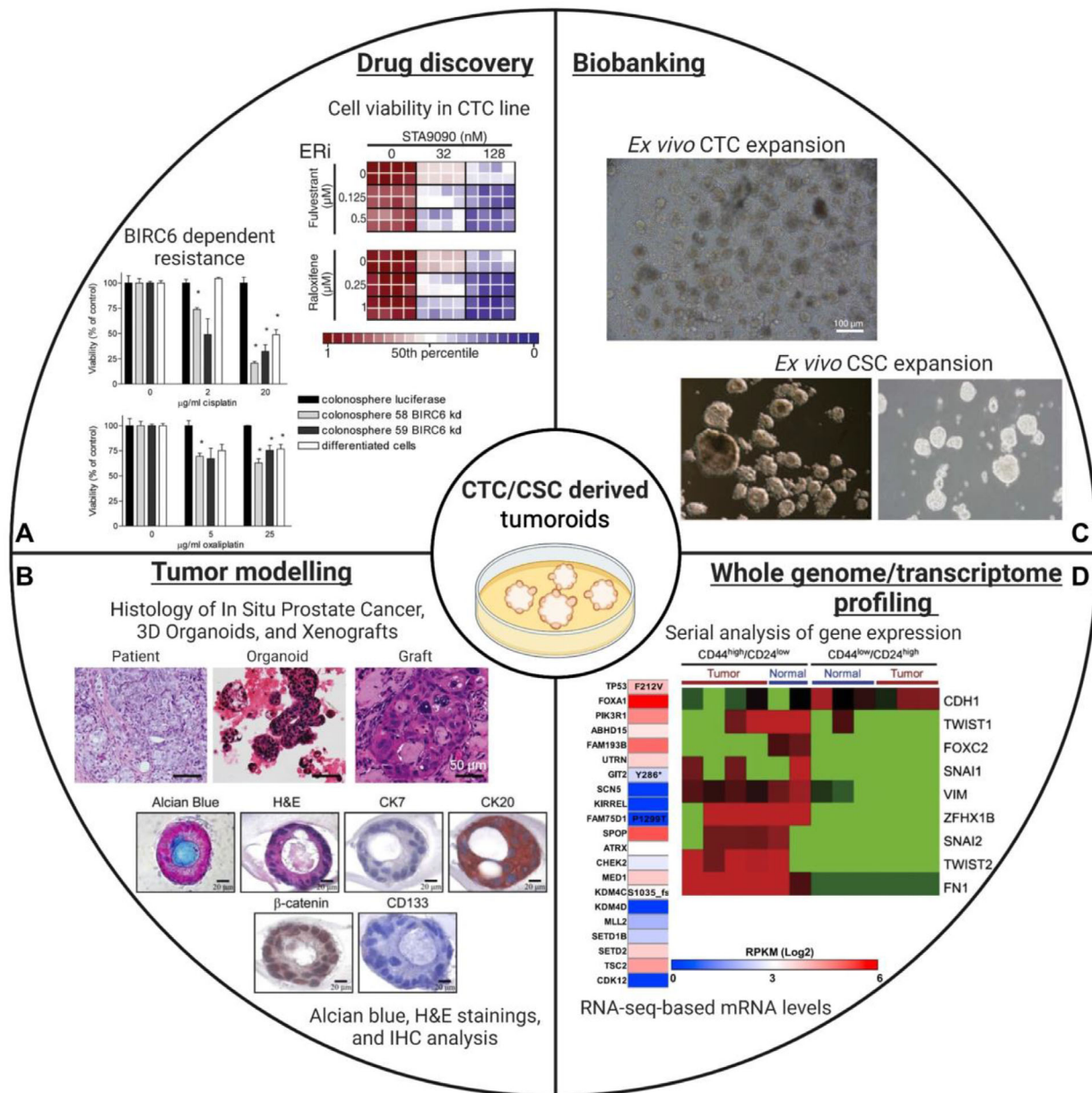


Figure 7. Potential clinical applications of CTC/CSC-derived tumoroids. A) Drug discovery. Reproduced with permission.^[254] Copyright 2011, The American Society for Biochemistry and Molecular Biology. Reproduced with permission.^[17] Copyright 2014, American Association for the Advancement of Science. B) Tumor modeling. Reproduced with permission.^[206] Copyright 2007, Elsevier. Adapted with permission.^[25] Copyright 2014, Elsevier. C) Biobanking. Reproduced with permission.^[128] Copyright 2020, MDPI. Reproduced with permission.^[255] Copyright 2014, American Association for Cancer Research. Reproduced with permission.^[256] Copyright 2009, American Association for Cancer Research. Reproduced with permission.^[225] Copyright 2008, Elsevier. Adapted with permission.^[25] Copyright 2014, Elsevier.

hypothesized as stem cells,^[247] and their microenvironmental niche governs the fate of CSCs through paracrine signals and/or other cellular systems (e.g., immune cells). When CSCs are in their niches, they can proliferate and differentiate into different tumor cells and even self-assemble into structures (organoids), which they are predetermined. Gjorevski et al. mimicked the native intestinal stem cell (ISC) microenvironment using synthetic PEG hydrogels.^[248] Matrix stiffness of 1.3 kPa was required for ISC expansion and proliferation with fibronectin-based adhe-

sion; 190 Pa was recommended for differentiation and organoid formation with laminin-based adhesion. PEG hydrogels contributed to biophysical cues without supplying any biochemical signals. Specifically, on 1.3 kPa substrate, yes-associated protein 1 (YAP) was significantly enhanced, and nuclear translocation was increased, revealing that ISC expansion is a YAP-dependent mechanism. Notably, patient-derived CRC organoids embedded within RGD-functionalized PEG gels mostly survived and continued to expand, indicating that the PEG-based

hydrogels can be adapted to culture human organoids. This result depicts the potential of synthetic materials to mimic native microenvironments of stem cells and CSCs.

Gao et al. used 3D organoid systems to successfully culture both prostate cancer biopsy specimens and CTCs for a long period.^[25] CTC-derived organoids and grafts of these organoids into mice resembled primary cancer. mRNA and protein expression analysis revealed that CTCs-derived organoids were positive for SPINK1. Androgen receptor (AR) expression level was relatively lower, but the expressions of selected AR targeted genes, including STEAP1 and TMPRSS2, were the same as those from other organoids (biopsy derived). Complete RB1 deletion was found in CTC-derived organoids by RNA-seq analysis. Copy-neutral genomic rearrangement of the remaining allele was a possible interpretation. All these findings summarize that the characterization of metastatic lesions is increasingly integrated into clinical trials of targeted agents in prostate cancer. The opportunity to generate tumoroids increased this clinical practice and opened the door to investigate tumor genomes from liquid biopsy with rare tumor cells for direct analysis.

Previous studies demonstrated the clinical benefits of 3D tumor models. Two anticancer drugs, 5-fluorouracil (5-FU), a cell growth inhibitor, and tirapazamine, an anticancer drug that functions as a hypoxia-selective cytotoxin, used different modes of action to produce distinct responses in 3D spheroids compared to conventional 2D cell cultures of human epithelial carcinoma cells.^[249,250] The 3D spheroids remained viable after 5-FU treatment, whereas cells grown as 2D monolayers did not survive in this treatment because spheroids had a lower proliferation rate leading to a reduced sensitivity to 5-FU.^[251] 3D cell cultures possess several in vivo features of the original tumor, including cell–cell interaction, hypoxia, drug penetration, response and resistance, and ECM production/deposition.^[252] The potential of spheroid models for the development of new anticancer strategies has been demonstrated over time. Chemo- and radiocytotoxicity are the crucial areas of use for large spheroids since the clinical response to chemical or physical treatments depends on parameters such as oxygen tension, compactness, apoptosis inhibition, damage repair, and permeability.^[253]

Therefore, well-defined and tunable biomaterials that mimic ECM and accurately resemble native TME can generate tumoroids. These tumoroids can recapitulate human tumor biology and can be used for versatile applications, including drug discovery, tumor modeling, biobanking, and whole-genome/transcriptome profiling (Figure 7).

5. Conclusions and Future Perspectives

Humans try to fight or live with cancer cells. We have achieved a certain degree of success, but far from victory. Patient-derived tumor organoids (PDOs) or tumoroids have shown enormous potential in unraveling therapeutic strategies in cancers. Nevertheless, building a tumoroid using CTCs or CSCs is the next challenge due to limited initial cell numbers. Hence, capturing CTCs and CSCs in high efficiency is vital for tumoroids. Expansion of these cells is another challenge. We propose that the improper in vitro microenvironment can rapidly change the phenotypes and slow down the cell cycle of these rare cancer cells. Biophysical and biochemical cues are both critical in the TME; therefore, the

capturing protocol needs to be improved, and optimized physical support is needed for in vitro cancer cell expansion and tumoroid formation.

In vitro expansion of tumoroids has other limitations such as lack of tumor–stroma interaction, tumor heterogeneity, and/or vascularization. Combinational approaches will further facilitate tumoroid formation, such as co-culturing tumor cells with immune cells, endothelial cells, or CAFs or using blood vessel mimetic microfluidic devices. A defined system orchestrated by proper biophysical and biochemical cues can better develop these malignant cells into 3D tumor spheroids, which can be used to pre-screen toxic drugs before chemotherapy and new drug discovery. This technology could reduce financial expense, shorten the therapeutic time, and prolong the lifespan of cancer patients.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

assembloid, cancer stem cell, circulating tumor cell, drug screening, microenvironment

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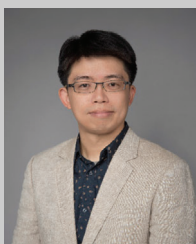
Jua Kim received her B.S. degree in bioengineering from Syracuse University, Syracuse, NY, USA, in 2009. She continued her academic research at Syracuse University and received her M.S. and Ph.D. degrees in 2012 and 2017, respectively. After her short career in industries, she decided to pursue her research as a postdoc scholar under Prof. Peng-Yuan Wang at Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences. Her current research interests are biomaterials and their interactions with the biological system. One of her research areas is manipulating the electrochemical behavior of metallic alloys to reduce biofilms on the metallic surface.



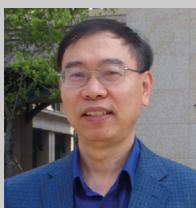
Yanshan Xu received her bachelor's degree in polymer materials and engineering from Jinan University in 2020 and is now studying for her master's degree in materials and chemical engineering from South University of Science and Technology of China. She is a joint student of South University of Science and Technology of China and Shenzhen Institute of Advanced Technology, Chinese Academy of Science, under the supervision of Prof. Peng-Yuan Wang. Her research interest is lung cancer organoid technology and drug screening application in vitro.



Zongyi Yin obtained a master's degree from China Medical University in 2017 and also obtained a surgeon qualification certificate. From 2017 to 2019, he completed the standardized training of residents in the Third Affiliated Hospital of Sun Yat-sen University. From 2019 to the present, he has worked in the General Hospital of Shenzhen University and is currently the attending doctor. His research areas of interest include regulation of the immune microenvironment of liver cancer and the application of Chinese medicine in liver cancer.



Tzu-Ming Liu received his B.S. degree in electrical engineering from National Taiwan University in 1999 and Ph.D. degrees in Photonics & Optoelectronics from National Taiwan University in 2004. Dr. Liu was an assistant professor since 2009 and became an associate professor in the Institute of Biomedical Engineering, National Taiwan University. In 2012, he visited Wellman Center for Photomedicine, Massachusetts General Hospital, USA, and built multicolor infrared femtosecond laser sources for multi-label multi-photon microscopy. In 2016, he moved to the University of Macau as associate professor.



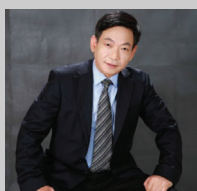
Chu-Xia Deng obtained his M.S. degree from the Institute of Hydrobiology, Chinese Academia of Science in 1984 and Ph.D. degree from the University of Utah in 1992, respectively. After finishing his postdoctoral training in the Harvard Medical School in 1995, he became a tenure-track investigator, and then the senior investigator at the United States National Institutes of Health. Prof. Deng has been serving as the founding dean of the Faculty of Health Sciences, University of Macau, since 2014. His research involves in some chronic diseases, development, ageing, stem cells, and cancers, particularly in the underlying mechanisms for BRCA1-associated breast cancer.



Chang Zou is the deputy director and principal investigator of the Clinical Medical Research Center in Shenzhen People's Hospital. His research focuses on applying new techniques such as single-cell sequencing, spatial transcriptomics, liquid biopsy, CTCs, 3D printing, PDX models in the tumor precision treatment. His work has been published in high-impact journals such as *Molecular Cancer*, *Signal Transduction and Targeted Therapy*, *Pharmacology & Therapeutics*, *Journal of Thoracic Oncology*, *Journal of Experimental Medicine*, *Autophagy*, and so on. Dr. Zou has been funded in more than 20 projects, including the National Science Foundation of China, Key Project of Guangdong-Shenzhen Joint Fund (1M).



Xi Xie received his Ph.D. degree from the Department of Materials Science and Engineering at Stanford University. Then he joined Prof. Langer and Prof. Anderson's lab at the Massachusetts Institute of Technology for postdoc research. Now he is a full professor in the School of Electronics and Information Technology & State Key Lab of Optoelectronic Materials and Technologies at Sun Yat-Sen University. At the same time, he was appointed as adjunct professor in the First Affiliated Hospital of Sun Yat-Sen University. His research is focusing on micro/nano-devices for biomedical applications, including drug delivery systems and biosensors.



Xiaowu Li obtained his medical doctor degree from West China University of Medical Sciences in 1994. He worked as a postdoctoral fellow at Beijing Medical University, and then the Oral Tumor Research Center of the University of California, USA. From 2004 to 2017, he worked in the First Affiliated Hospital of the Third Military Medical University. From 2017 to present, he has been the director and professor of the Department of Hepatobiliary Surgery in Shenzhen University General Hospital. His research field focuses on the mechanism of circulating tumor cells mediated tumor thrombus formation in liver cancer/pancreatic cancer metastasis.



Peng-Yuan (George) Wang is a professor at Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences. He received his M.S. (2006) and Ph.D. (2011) in chemical/biomedical engineering from National Taiwan University. He worked as a postdoctoral fellow and lecturer in Melbourne, Australia. His research focuses on developing biointerfaces for cell manipulation such as stem/cancer cell expansion, stem cell differentiation, and cell reprogramming in precision and regenerative medicine. Dr. Wang has published more than 60 papers and applied/issued over 10 patents. Dr. Wang has received many prestigious awards including the Discovery Early Career Researcher Award (DECRA) from Australian Research Council.