Cancer Science
Japanese Cancer Association JCA
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Review Article

Tumor-derived spheroids: Relevance to cancer stem cells and clinical applications

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Key words

Cancer stem cell, chemoresistance, solid tumor, three-dimensional culture, tumor-derived spheroid

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Funding Information

Ministry of Education, Culture, Sports, Science and Technology of Japan; National Cancer Center; Japan Society for the Promotion of Science; Japan Agency for Medical Research and Development.

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Received October 31, 2016; Revised December 13, 2016; Accepted December 28, 2016

Cancer Sci **108** (2017) 283–289

doi: 10.1111/cas.13155

Recently, many types of *in vitro* 3-D culture systems have been developed to recapitulate the *in vivo* growth conditions of cancer. The cancer 3-D culture methods aim to preserve the biological characteristics of original tumors better than conventional 2-D monolayer cultures, and include tumor-derived organoids, tumor-derived spheroids, organotypic multicellular spheroids, and multicellular tumor spheroids. The 3-D culture methods differ in terms of cancer cell sources, protocols for cell handling, and the required time intervals. Tumor-derived spheroids are unique because they are purposed for the enrichment of cancer stem cells (CSCs) or cells with stem cell-related characteristics. These spheroids are grown as floating spheres and have been used as surrogate systems to evaluate the CSC-related characteristics of solid tumors *in vitro*. Because eradication of CSCs is likely to be of clinical importance due to their association with the malignant nature of cancer cells, such as tumorigenicity or chemoresistance, the investigation of tumor-derived spheroids may provide invaluable clues to fight against cancer. Spheroid cultures have been established from cancers including glioma, breast, colon, ovary, and prostate cancers, and their biological and biochemical characteristics have been investigated by many research groups. In addition to the investigation of CSCs, tumor-derived spheroids may prove to be instrumental for a high-throughput screening platform or for the cultivation of CSC-related tumor cells found in the circulation or body fluids.

Solid tumors grow in a 3-D conformation surrounded by a variety of non-tumor cells and ECM that collectively constitute the tumor microenvironment. Under these conditions, tumor cells tend to be exposed to suboptimum growth conditions, such as hypoxia or low nutrient levels, and influenced by cell–cell contacts or a variety of signals from the surrounding tumor and non-tumor cells. In conventional 2-D monolayer cultures, most of these environmental cues are missing. Hence, it is difficult to faithfully reconstitute the tumor microenvironment in conventional cultures, and the biological characteristics of original tumors may be lost because of the cellular adaptation required for survival *in vitro*. With this in mind, many types of *in vitro* 3-D culture systems have been developed to recapitulate *in vivo* growth conditions and study the broader aspects of tumor biology.

One of the representative 3-D culture methods for cancer cells is the tumor-derived spheroid culture. In this culture, primary cancer cells with stem cell-like features are expanded *in vitro* as floating spheres. Here we provide a brief overview of multiple 3-D *in vitro* culture systems of cancer cells and describe the major discoveries from studies using tumor-derived spheroids.

Classification of 3-D Culture Models of Cancer Cells

Three-dimensional culture systems of cancer cells have been developed for distinct and overlapping purposes. These methods differ in terms of cancer cell sources, protocols for cell handling, and the time intervals required for establishing 3-D cultures. Detailed overviews of these 3-D methods were previously published.[1–4] Here we describe four representative methods for the 3-D culture of cancer cells (Table 1).

Organotypic multicellular spheroids and organotypic explant cultures are intended to faithfully reproduce the tumor microenvironment. In many cases, these cultures are established after gentle mechanical dissociation of cancer tissues. Cancer cells cultivated by this method are surrounded by non-tumor cells and stromal components that normally exist in the tumor microenvironment. As a result, cancer cells cultivated with these methods generally retain many histological features and the cellular heterogeneity of the primary cancer. Several variations of these methods, such as cancer tissue-originated spheroids, involve the mild dissociation of cancer tissues with mild enzymatic treatments to isolate heterologous cancer spheroids.[5] Notably, cancer tissue-originated spheroids largely maintain the histological features of original cancers in the

Table 1. Representative methods for 3-D cultures of cancer cells

Types of 3-D culture models	Organotypic multicellular spheroids	Multicellular tumor spheroids	Tumor-derived organoids	Tumor-derived spheroids
Source of tumors	Tumor tissues	Cancer cell lines	Dissociated tumor tissues	Dissociated tumor tissues
Clonality of cancer cells	Polyclonal	Monoclonal	Polyclonal/ monoclonal	Polyclonal/ monoclonal
Presence of non-tumor niche cells	+	–	–	–
Presence of serum in culture media	+	+	–	–
Histological preservation of original tumors	++	–	+	–
Enrichment of CSC-like cells	–	±	–	+
Genetic manipulation	±	++	+	+
Application for high-throughput drug screening	–	++	±	±

CSC, cancer stem cell.

absence of non-tumor cells and are capable of propagation after mechanical dissociation.

Multicellular tumor spheroids are typically established from cancer cell lines in conventional media supplemented with FBS, similar to conventional 2-D cultures. Hence, MCTS may be methodologically regarded as an extension of the standard 2-D culture of cancer cell lines. A major difference from 2-D cultures is that cells for MCTS are grown as spheres in a suspension culture or other conditions that promote cell–cell adhesion. In contrast to OMS or related methods intended to preserve the *in vivo* biological features of cancers, MCTS show little histological resemblance to the primary cancer. Despite limited histological resemblance to the primary cancer, cells in MCTS mimic the metabolic and proliferative gradients of *in vivo* tumors and show clinically relevant multicellular chemoresistance.[6] The advantages of MCTS over other 3-D systems, that is, clonality of cells, ease of maintenance, and simplicity of genetic manipulation (Table 1), make this method an appropriate tool for high-throughput drug testing.[7]

Tumor-derived organoids were recently developed by Sato *et al.*[8] and are rapidly gaining popularity as a powerful *ex vivo* model of organogenesis.[9,10] Under specific growth conditions, including basement membrane matrix (Matrigel), Wnt agonists, tyrosine kinase receptor agonists, and bone morphogenetic protein/transforming growth factor-β inhibitors, a variety of tissues were reconstituted *in vitro* in the absence of non-tumor cells.[9] In addition, a multistep carcinogenesis model was developed by introducing sequential oncogene/tumor suppressor gene mutations in non-tumor organoids as an alternative approach to investigate cancer development.[11]

Tumor-derived spheroids, also known as tumorospheres,[2] are the main focus of this review. Tumor-derived spheroids are floating spheres that serve as surrogate systems to evaluate CSC-related characteristics *in vitro*. Hence, the main feature of tumor-derived spheroids is the enrichment of CSCs or cells with stem cell–related characteristics. This feature is distinct from other 3-D methods, that is, OMS and patient-derived organoid cultures, which attempt to faithfully reproduce cancer tissues, including stem cells and their differentiating progenies. Methodologically, tumor-derived spheroids are similar to MCTS in terms of the formation of free-floating spheres. In some cases, the serum-free culture conditions of tumor-derived spheroids were used for MCTS cultures to faithfully reproduce stem cell-like states.

Cancer Stem Cells and Spheroid Cultures

Prevailing CSC models posit that cancer cells are hierarchically organized and that CSCs, which comprise a fraction of cancer cells, are capable of generating entire cancer structures due to their potential for self-renewal and differentiation. However, the identification of CSCs from solid tumors remains evasive, mainly because of the lack of cell-surface markers and plasticity of CSC-related phenotypes. As a result, the identification of CSCs relies on functional assays that determine the capability of CSCs to generate tumors through self-renewal and differentiation, especially *in vivo* tumor-formation assays after transplantation into immunocompromised mice and lineage-tracing assays of *in vivo* tumors generated in genetically engineered mice (Table 2).

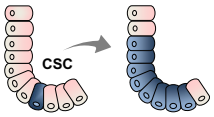
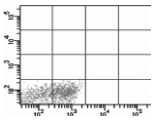
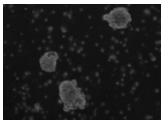
In the 1990s, Dick and his colleagues[12]discovered CSCs in hematopoietic cancers. Subsequent studies reported that cells with CSC-like properties were present in solid tumors, including glioma, colon cancer, and breast cancer.[13] These findings prompted researchers to establish methods to propagate CSCs in solid tumors *in vitro*. Because it had been known that normal neural cells can be propagated in sphere cultures, it is not surprising that spheroid cultures of cancer cells were first achieved with glioma CSCs *in vitro*.[14] Following these studies, spheroid cultures were established from cancers of breast, colon, ovary, and prostate (see below). Although there are some reservations as to the extent of which sphere formation reflects the CSC phenotype,[15] the capability to form spheroids *in vitro* is regarded as a convenient surrogate to evaluate the functionality of CSCs because of the propensity of stem cells to propagate as spheroid bodies.[16] Thus, tumor-derived spheroid cultures have been closely tied to *in vitro* studies of cancer stemness and regarded as one of criteria for CSCs.[17]

Eradication of CSCs is likely to be of clinical importance due to their association with the malignant nature of cancer cells. It was hypothesized that CSCs are related to the chemoresistance and metastasis of cancer, and some reports indicate that CSCs show higher resistance to chemotherapeutic agents than most tumor cells.[18,19] Hence, a great deal of research on tumor-derived spheroids has been directed toward investigating the chemoresistance of CSCs with the hopes of elucidating the refractory nature of solid cancers.

Spheroid Cultures From Primary Cancers

The sources and types of cancer cells for tumor-derived spheroids vary. However, the general procedures for the *in vitro*

Table 2. Common experimental methods used to evaluate the major criteria for cancer stem cells (CSCs)

Criteria for CSCs	Experimental system			
	Tumor formation assays	Lineage tracing experiments	Flow cytometry analyses	Tumor-derived spheroids
				
In vivo tumorigenicity	+	+	–	–
Self-renewal	+	+	–	+
Capable of differentiation	+	+	–	±
Expression of specific CSC markers	+	+	+	+
Capable of spheroid formation	–	–	–	+

expansion of CSCs as spheres are similar and based on the unique property of stem/progenitor cells to survive and grow in the form of spheroid bodies in serum-free media. Tumor tissues are typically subjected to mechanical/enzymatic dissociation, filtration, or flow cytometry to obtain single-cell suspensions. Next, these cells are suspended in serum-free media supplemented with growth factors, such as epidermal growth factor and fibroblast growth factor, in non-attachment plates (Fig. 1).

Using spheroid culture methods, putative CSCs or cancer cells with stem cell-like properties have been isolated and expanded from tumors. However, the parameters used for these experiments did not necessarily follow the rigorous experimental conditions defined for CSC cultivation, and thus, the results should be carefully interpreted. For example, cell density is a critical parameter to evaluate self-renewal because a higher density tends to cause cell aggregation and compromise clonal conditions for cell growth. Passage numbers may also affect the results because initial cell populations capable of spheroid formation may include transient amplifying cells.[16] In many reports, these parameters were not mentioned in spite of their importance to evaluate the CSC-related characteristics. However, sphere size may merely reflect the proliferation rate of other cells rather than CSC traits, although this parameter was often used as one of the criteria for CSCs. Because of these variations in experimental parameters, it is difficult to compare the results from different studies, especially those on different cancer types. Nevertheless, it is likely that the findings described below will serve as a starting point for future *in vitro* studies of CSCs and related cells.

Glioma spheroids (neurospheres). The *in vitro* neurosphere assay was initially developed by Reynolds and Weiss[20] to quantify the activity of non-tumor neural stem cells derived from the adult mouse brain. The established neurospheres maintained the capacity to produce mature classes of neural cell types and showed multilineage differentiation after transplantation *in vivo*.[21,22] Subsequently, long-term neurosphere cultures were established from human specimens and showed the capability for migration and differentiation following transplantation into immunocompromised rats.[23] Human neurosphere formation was facilitated by the selection of neural stem cells with the stem-cell marker of central nervous system lineages, CD133.[24]

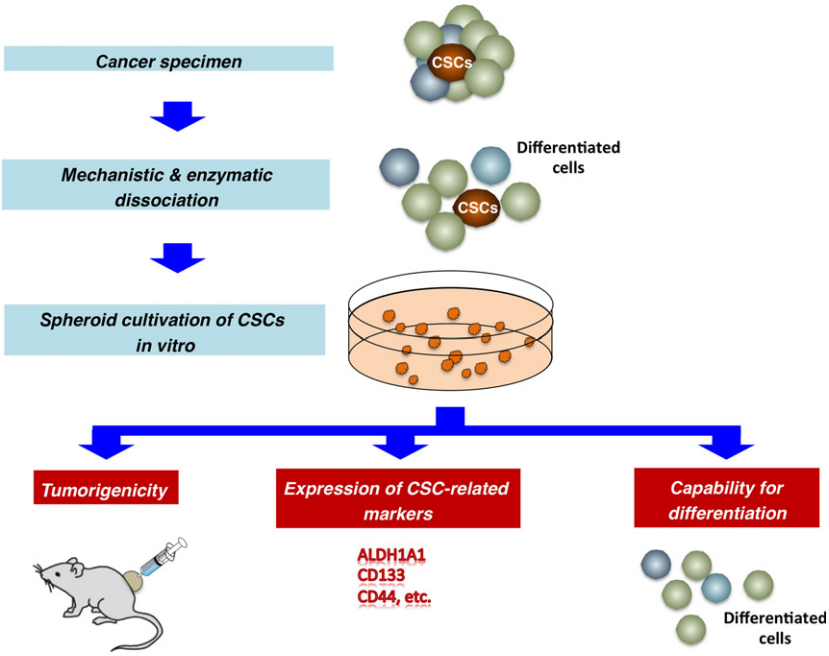


Fig. 1. General protocols for the establishment of tumor-derived spheroids. CSC, cancer stem cell.

Using the techniques used to isolate normal neural stem cells, Dirks and his colleagues[14]established neurospheres from human brain tumors. As is the case for normal neural stem cells, CD133-positive cells from tumors were responsible for neurosphere formation, and the CD133-positive sphere cells showed CSC-like characteristics, that is, self-renewal, proliferation, and differentiation capacities.[14] Interestingly, it was reported that adherently growing CD133-negative cells show stem cell-like properties in a subset of primary glioblastomas, suggesting the existence of at least two different types of CSCs.[25]

Cancer stem cells maintained as neurospheres represent a more reliable model system than cancer cells grown under standard serum-containing culture. Indeed, neurosphere CSCs more closely mimic the genotype, gene-expression profile, and biology of parental tumors.[26,27] In addition to CD133, several other surface markers were used to distinguish neurospheres from glioblastoma CSCs.[28] Neurospheres have been used to investigate stem cell-specific therapeutic targets[29,30] and were adapted to *in vitro* assays to test the efficacy of therapeutic compounds.[31]

Mammalian cancer spheroids (mammospheres). Using the experimental protocol for neurosphere establishment, Wicha and his colleagues[32] established a non-adherent "mammosphere" from human mammary epithelial cells. The established mammospheres were enriched for early progenitor/stem cells and able to differentiate along all three mammary epithelial lineages. After the identification of CSCs from breast cancer,[33] similar protocols for the mammosphere culture were used to establish a long-term mammosphere culture from breast cancer.[34–36] The established sphere cells represent cancer-initiating cells because they expressed stem-cell markers and were capable of forming xenograft tumors in immunocompromised mice.[34] Mammospheres were also established from metastatic cells[37,38] and ductal carcinoma *in situ*.[39]

Mammospheres have been used to examine intertumoral heterogeneity[40,41] and determine the proliferative roles of the interleukin-8/C-X-C motif chemokine receptor 1/2-mediated pathway[38] and insulin-like growth factor 2 pathway.[42] In another study, established mammospheres were used to examine the specific effects chemical compounds have against CSCs.[43] Mammosphere assays were also used for a mouse breast cancer model of Erb-B2 receptor tyrosine kinase 2 expression[44] and p53-deficiency.[45] An investigation of the Wnt/β-catenin signaling pathway[46] and Sox2 expression[47] was carried out using mouse mammospheres.

Colorectal cancer spheroids (colonospheres). Cancer spheroids from primary colorectal cancer were first established from CD133-positive colon cancer cells cultivated in serum-free medium. The cultivated sphere cells maintained the ability to faithfully reproduce the same histopathological features of the original tumor in immunocompromised mice.[48] These "colonospheres" were used to investigate CSC-related characteristics, such as chemoresistance,[49–53] metastatic capacity,[54] and tumorigenicity at single-cell levels.[55] In addition, the colonospheres were used to evaluate the chemosensitivity of novel compounds targeting the Wnt pathway.[56]

Through the systematic search for compounds that regulate the proliferation of colon cancer spheroids, we showed that the inhibition of ROCK markedly improved the efficiency of sphere formation.[57] The improved efficiency was at least in part attributed to the induced expression of CSC marker CD44v.[57] The established sphere cells were capable of faithfully reproducing the original tumors (Fig. 2).[56,57]

Ovarian cancer spheroids. Ovarian spheroids initially attracted attention due to their morphological resemblance to multicellular aggregates in cancerous ascites[58] rather than their relevance to CSCs. Spheroid growth in ascites can be attributed to a specific environment of malignant ascites that encourages spheroid formation.[59] Dissemination to the surfaces of the peritoneum and other organs within the peritoneal cavity is the most common metastatic pattern of ovarian cancers, and aggregated spheroids may play an essential role through their attachment to the surface of other organs and subsequent formation of multiple disseminated tumors.[60,61]

Bapat *et al.*[62] first reported a spheroid culture from cancer cells in ascites derived from ovarian cancer patients. These spheroids were maintained *in vitro* using 5% FBS and showed that they were capable of forming tumors in mouse xenograft models. Subsequently, spheroid cells were established from

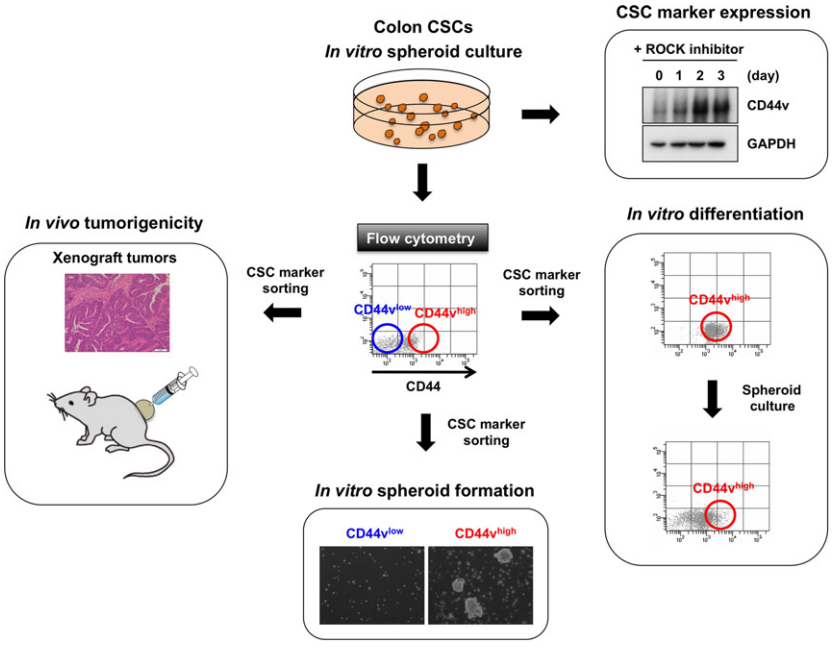


Fig. 2. Evaluation of cancer stem cell (CSC)-associated phenotypes of colon spheroids derived from colorectal cancers. The CSC-associated phenotypes were validated based on CSC marker expression, flow cytometry, *in vivo* tumorigenicity, *in vitro* spheroid formation, and *in vitro* differentiation.

primary ovarian cancer tissues using serum-free media.[63] In addition to spheroid cultures, several groups reported ovarian CSC-like cells using attached culture methods.[64,65] Although these studies were carried out under different culture conditions, the tumorigenicity of the established cells was validated in all cases.

CD44 was initially identified as a CSC marker for a subset of ovarian cancers[63,64] and preferentially expressed in spheres derived from ovarian tumors.[63,65] Subsequently, ALDH and CD133 were found to serve as major biomarkers that can enrich tumorigenic cells from ovarian cancer.[66–69] In fact, the ovarian cancer spheroid cells preferentially expressed these markers[67] and required ALDH activity for their proliferation. The differences among the observed markers may be attributed to the heterogeneity of ovarian cancer CSCs.

We reported that ROCK inhibition in ovarian cancer spheroids, similar to that in colon cancer spheroids, promoted cell survival and propagation.[70] The spheroids showed characteristics as CSCs, including expression of CSC markers, capability for differentiation, and tumorigenicity.[70] Unlike the cases in colorectal cancer spheroids, the dependence on ROCK inhibition was not mediated by CD44v, but rather by expression of other CSC markers, such as ALDH1A1 and Sox2.[70]

Prostate cancer spheroids (prostaspheres). Investigation of the culture conditions for normal prostate stem cells revealed that they could be expanded as spheres (prostaspheres) in the presence of Matrigel.[71] The sphere cells from prostate cancer showed an extensive self-renewal capacity and capability of multilineage differentiation.[71] Prostate sphere-forming cells were associated with basal cell types (Sca-1$^+$) that express Trop2[72,73] and p63-expressing basal cells,[74] or with increased nuclear factor-κB signalling.[75] Using a similar protocol, cancer spheroids were expanded from tumors derived from mouse prostate cancer models and used to establish coculture models with cancer-associated cells.[76,77]

Future Application of Tumor-Derived Spheroid Culture

Our knowledge of the biological nature of solid-tumor CSCs has greatly expanded over the last decade, at least in part through research on tumor-derived spheroids *in vitro*. By exploiting the knowledge gained from emerging *in vitro* cultivation methods, such as tumor-derived organoids, it may be possible to establish better methods for the *in vitro* cultivation of CSCs. Furthermore, the refinement of culture protocols may allow for the use of tumor-derived cultures as a high-throughput screening tool to identify molecules that inhibit CSC proliferation.

Another potential use for tumor-derived spheroid cultures is to expand this method to cultivate CSCs from other types of clinical specimens, such as metastatic foci or body fluids (e.g., ascites, pleural fluid, and circulating blood). Notably, the spheroid cultivation of CSCs from the circulating blood of breast and lung cancer patients has attracted attention as a novel technology to isolate and expand circulating tumor cells *in vitro*.[78–80] In conjunction with emerging liquid biology research, the isolation and expansion of CSC-related cells through sphere formation may be a powerful technology to investigate the original tumors without highly invasive clinical procedures.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research in Innovate Areas from MEXT (16703010), a National Cancer Center Research and Development Fund (28-A-3), Grants-in-Aid for Challenging Exploratory Research and Scientific Research (B) from JSPS (16K15436, 14506268), and Grants-in-Aid from AMED (16cm0106120h0001, 16ck010619 4h0001).

Disclosure Statement

The authors have no conflict of interest.

Abbreviations

ALDH	aldehyde dehydrogenase
CSC	cancer stem cell
MCTS	multicellular tumor spheroid
OMS	organotypic multicellular spheroid
ROCK	Rho-associated protein kinase
Sox	SRY-related HMG-box

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