NEOPLASIA
www.neoplasia.com

Volume 17 Number 1 January 2015 pp. 1–15 1

Spherical Cancer Models in Tumor Biology[1]

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Abstract

Three-dimensional (3D) *in vitro* models have been used in cancer research as an intermediate model between *in vitro* cancer cell line cultures and *in vivo* tumor. Spherical cancer models represent major 3D *in vitro* models that have been described over the past 4 decades. These models have gained popularity in cancer stem cell research using tumorospheres. Thus, it is crucial to define and clarify the different spherical cancer models thus far described. Here, we focus on *in vitro* multicellular spheres used in cancer research. All these spherelike structures are characterized by their well-rounded shape, the presence of cancer cells, and their capacity to be maintained as free-floating cultures. We propose a rational classification of the four most commonly used spherical cancer models in cancer research based on culture methods for obtaining them and on subsequent differences in sphere biology: the **multicellular tumor spheroid model**, first described in the early 70s and obtained by culture of cancer cell lines under nonadherent conditions; **tumorospheres**, a model of cancer stem cell expansion established in a serum-free medium supplemented with growth factors; **tissue-derived tumor spheres** and **organotypic multicellular spheroids**, obtained by tumor tissue mechanical dissociation and cutting. In addition, we describe their applications to and interest in cancer research; in particular, we describe their contribution to chemoresistance, radioresistance, tumorigenicity, and invasion and migration studies. Although these models share a common 3D conformation, each displays its own intrinsic properties. Therefore, the most relevant spherical cancer model must be carefully selected, as a function of the study aim and cancer type.

Neoplasia (2015) 17, 1–15

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[1]Work of the laboratory on spheres is supported by a Genevieve and Jean-Paul Driot Transformative Research Grant, a Philippe and Laurent Bloch Cancer Research Grant, a Hassan Hachem Translational Medicine Grant, a Sally Paget-Brown Translational Research Grant, the Institut National du Cancer and Cancéropôle Ile de France (COLOMETASTEM grant), and GEFLUC (Grant 5/188).
Received 2 May 2014; Revised 29 November 2014; Accepted 4 December 2014

1476-5586/15
http://dx.doi.org/10.1016/j.neo.2014.12.004

Introduction

Solid tumors grow in a three-dimensional (3D) spatial conformation, resulting in a heterogeneous exposure to oxygen and nutrients as well as to other physical and chemical stresses. Proliferation and hypoxia are mutually exclusive *in vivo,* except in areas subjected to transient changes in perfusion where nonproliferating but viable hypoxic tumor cells have also been identified [1]. This diffusion-limited distribution of oxygen, nutrients, metabolites, and signalling molecules is not mimicked in two-dimensional (2D) monolayer cultures [2]. In addition to possible induction of chemical gradients in 3D structures, it is now well admitted that the 3D cell–cell interaction *per se* influences cell structure, adhesion, mechanotransduction, and signaling in response to soluble factors which in turn regulate overall cell function in ways that differ dramatically from traditional 2D culture formats [3]. Thus, the study of cells in a 3D context can provide insights not observed in traditional 2D monolayers. To successfully investigate the pathobiology of human cancer, it is necessary to maintain or recreate in culture the typical 3D architecture of the tissue. To date, numerous 3D models have been specifically developed in cancer research to take into account these tumor architectural features in biological processes to as great an extent possible. These models are based on different approaches as illustrated by the multicellular tumor spheroid model (MCTS) [4], organotypic slices of cancer tissue [5], multilayered cell cultures [6], and scaffolds [7]. Continuous progress in tissue engineering, including development of various 3D scaffolds and bioreactor systems, has improved the diversity, fidelity, and capacity of culture models for use in cancer research [8].

The 3D microenvironment enables mimicking the different types of cell heterogeneity observed *in vivo* in different contexts. Thus, 3D systems formed only by cancer cells and homotypic cell–cell adhesion may display different phenotypes like those of quiescent *versus* proliferating cells depending upon the chemically induced gradients [2]. More sophisticated 3D systems combining cancer and stromal cells could emphasize the importance of heterotypic cross talk [9,10].

Among the numerous 3D models, we focus here only on spherical cancer models. All these spherelike structures are characterized by their well-rounded morphology, the presence of cancer cells, and the capacity to be maintained as free-floating cultures. Consequently, multilayered tumor cell cultures, tumor slices, organoids, or 3D cultures within reconstituted basement membrane do not fit in with these features and will not be described here (for a review on 3D models, [2,9]).

Spherical cancer models other than the MCTS model have been described and used in cancer research. Initially, development of the MCTS model was largely due to the work of Sutherland's group in the early 70s [11,12]. A decade later, the group of Rolf Bjerkvig introduced a new model of sphere referred to as the organotypic multicellular spheroid (OMS), easily achieved by the simple cutting of cancer tissues [13]. Histologically, the OMSs closely resemble the tumor *in vivo,* with the presence of capillaries maintained for several weeks in culture [14]. The 2000s witnessed the emergence of a new 3D sphere model, the tumorospheres, for studying and expanding the cancer stem cell (CSC) population. More recently, tissue-derived tumor spheres (TDTSs) were obtained by partial dissociation of tumor tissue, enabling maintaining cell–cell contact of cancer cells [15,16]. Originally, such structures had been observed in a limited number of studies performed for *in vitro* human colon cancer cell lines establishing [17–19]. Thus, TDTSs have been largely characterized for colorectal cancer, as demonstrated by the work of Kondo's group on cancer tissue–originated spheroids (CTOSs) [16] and that of our group on colospheres [15,20]. However, TDTSs could also be obtained from dissociation of various types of cancer tissues including lung, bladder, prostate, or breast cancer tissue and uveal melanoma (personal observation and [16,21]).

At present, given the rapid development of CSC as spheres and the absence of a well-defined terminology for spherical cancer models, clarification of the different spherical cancer models already used and of their application to cancer research is warranted. Thus, we present here the different terms used to designate these 3D systems, their culture techniques, and their major characteristics. We will then review their major fields of application to cancer research: chemotherapy and radiotherapy cell responses, tumorigenicity, migration, and invasion processes.

It is now possible to classify all spherical cancer models into four groups: 1) **multicellular tumor spheroids**, generated in nonadherent conditions from single-cell suspension; 2) **tumorospheres**, models of CSC culture and expansion; 3) **tissue-derived tumor spheres**, formed only by cancer cells after partial dissociation of cancer tissues; and 4) **organotypic multicellular spheroids,** generated by cutting cancer tissue under nonadherent conditions.

Terminology

Historically, the multicellular tumor spheroid model was introduced by radiobiologists in the early 70s and was mainly developed via a wide diversity of cancer cell lines [22]. However, other types of emerging "cancer spheres" have been recently reported and, apart from their 3D morphology, share little with the first MCTS established by Sutherland. Unfortunately, the absence of strict nomenclature by the authors does not enable clear identification of their spheres and leads to some confusions and misunderstandings. The terms "spheroid" and "sphere," respectively, are not consistent throughout the literature, and this is critical to the rational use of spherical cancer models. In general, several terms are used to refer to the culture of well-rounded 3D cancer structures: spheroids, organoids, and spheres (Table 1). For example, "organoid" (meaning miniorganlike) should be dedicated to 3D culture of normal cells and tissue [23–25], but this term has also been used for the 3D structure spontaneously formed by colon carcinoma cell line LIM1863 [26], for 3D cultures of tumor cells embedded in basement membrane–like gel [27], and even for the classical model of MCTS [28]. Likewise, the term "aggregate" was primarily used to describe loose packages of cells and to distinguish them from compact spherical cultures [29]. Unfortunately, some so-called "spheres" and "spheroids" in the literature are no more than loose aggregates that easily detach, cannot be manipulated or transferred, and lack not only true spherical geometry but possibly also cell–cell and cell–matrix interactions, impacting biological properties.

The term "sphere" has been recently applied to normal and CSC culture and expansion. Indeed, the terms "tumorspheres" [30,31], "tumorospheres" [32,33], and "oncospheres" [34] were used to describe CSC spheres issued from different types of cancer having a large panel of derived names. Spheres from brain and breast (normal or cancer) stem cell culture were termed "neurospheres" [35,36] and "mammospheres" [37,38], respectively, related to their tissue of origin. Thus, CSC spheres from colon cancer were referred to as "colon cancer spheres" by the authors who first described them [39].

Table 1. Confusing Terminology to Depict the Different Models of Cancer Spheres.

Cancer Sphere Models	Alternative Names
Multicellular tumor spheroids	Spheroids [171]
	Tumoroids [172]
	Mixed spheroids [173]
	Nodules [174]
	Heterospheroids [175]
	Organoids [28]
Tumorospheres	Spheroids [40]
	Colospheres [43,44]
	Spheres [30,34,36,38,39]
	Tumorspheres [30]
	Oncospheres [34]
	Xenospheres (from patient tumor-derived xenografts) [176]
	Neurospheres (normal and malignant brain) [35,36]
	Mammospheres (normal and malignant breast) [37,38]
	Colon cancer spheres (colon cancer) [39]
Tissue-derived tumor spheres	Colospheres [15,20]
	Cancer tissue–originated spheroids [16]
	Spheroids [21]
Organotypic multicellular spheroids	Biopsy spheroids [85]
	Organotypic spheroids [177]
	Organotypic tumor spheroids [84]
	Fragment spheroids [50]
	Primary spheroids [178]
	Ovarian carcinoma ascites spheroids [48]
	Spherule [47]

However, the latter spheres have also been termed "spheroids" [40], "colonospheres" [41,42], and more recently "colospheres" [43,44].

OMSs are clearly distinct from the classical model of MCTS. OMSs, also designated as "biopsy spheroids" [45], "organotypic spheroids" [13], and "tumor fragment spheroids" [46], are generated directly from cut tissues, whereas MCTSs are established from single-cell suspensions of cancer cells. Ovarian carcinoma ascites fluids can lead to "spherules" [47] or "ovarian carcinoma ascites spheroids" [48], a specific term that prevents confusion with MCTSs from ovarian carcinoma cell lines.

Preparation of Spherical Cancer Models

The four spherical cancer models are derived from various cancer cell sources with different preparation protocols. Medium composition, culture surface, cell density, time required for formation, origin and handling of tumor material are the major parameters (Table 2, Figure 1).

Multicellular Tumor Spheroids

MCTS are generated from single-cell suspension culture in conventional fetal bovine serum (FBS)–supplemented medium without a supply of an exogenous extracellular matrix (ECM). Such cell cultures often originate from cancer cell lines but rarely from tumor cell suspensions derived from tumor tissue. Nevertheless, not all cell lines were able to generate compact MCTSs [49].

Various methods can be used for MCTS cultures (reviewed in [50]), but the core principle remains the same based on anchorage-independent methodology. By providing conditions in which adhesive forces between cells are greater than for the substrate they are plated on, tumor cells were prevented from adhering to underlying tissue culture plastic, with the aim of promoting cell–cell adhesion, leading to well-rounded spherical structure (Figures 1*A* and 2, *A-H*). In this case, traditional culture medium supplemented with FBS is used. Depending on the cell line and system used, MCTSs can be obtained after 1 to 7 days of culture.

Nonadherent conditions could be induced in rotating systems, including gyratory shakers and spinner flasks. These protocols enabled production of large pools of MCTSs of various sizes but necessitated determination of optimal cell concentration, and the cells required vast quantity of media for growth.

MCTS are easily generated by the liquid overlay technique that prevents matrix deposition. Tumor cells are placed on tissue culture plastic (well plates, flasks, or dishes) covered with a thin layer of inert substrate, agar [51], agarose [52], or polyHEMA [53]. This thin film is allowed to dry before addition of medium, in which cancer cells grow without adhering, thus promoting cell aggregation and compaction (Figure 2, *D–F*). Spheroid formation can also be achieved by seeding cells in ultra–low attachment plates without any coating, as the polystyrene surface offers low-adhesion properties [54]. Use of the liquid overlay technique in 96-well plates leads to reproducible formation of one single MCTS per well, homogeneous in size. A small working volume and possibly automation make these cells ideal for high-throughput screening [49].

Another method, the "hanging drop method," has been described for generating MCTSs (Figure 2, *A–C*) [55]. Drops of cell suspension up to 30 μl in size are deposited on a dish lid. Upon inversion of the tray, cells accumulate at the free liquid–air interface to form a single MCTS. This method avoids coating of plates and effects on cells due to substratum contact but requires MCTS transfer for further investigation. The InSphero AG (Schlieren, Switzerland) plate system permits simple transfer of the MCTS from the drop in the well for drug incubation [9,55]. A large amount of regular spheres can be obtained and manipulated in this way.

Table 2. Methods Used to Generate the Different Cancer Sphere Models.

Tumor Sphere Model	Tumor Material	Culture Conditions	Time Required for Tightly Packed Sphere Formation	References
Multicellular tumor spheroids	Single-cell suspension from permanent cancer cell lines (rarely from dissociated cancer tissue)	▪ Medium with FBS w.o. any additional growth factor ▪ Nonadherent conditions (inert matrix agarose like polyH, agarose; hanging drop; spinner)	1-7 d	[49,50,55]
Tumorospheres	Single-cell suspension from permanent cell lines, tumor tissue, or blood	▪ Serum-free medium with EGF and FGF-2 ▪ Low-attachment plastic ▪ Clonal density ▪ Potential preliminary cell sorting	5-7 d until 1-2 mo	[38,40,67,71,112]
Organotypic multicellular spheroids	Cut and minced tumor tissue	▪ Medium with FCS and nonessential amino acids w.o. any additional growth factor ▪ Nonadherent conditions	2-5 d until 12-18 d	[83,84]
Tissue-derived tumor spheres	Partially mechanically or enzymatically dissociated tumor tissue	▪ Medium with FCS w.o. any additional growth factor (colospheres) or serum-free medium with EGF and FGF-2 (CTOSs) ▪ Culture-treated plastic then non- adherent conditions	1-3 d	[15,16,20,21]

FGF-2, fibroblast growth factor 2; *w.o.*, without.

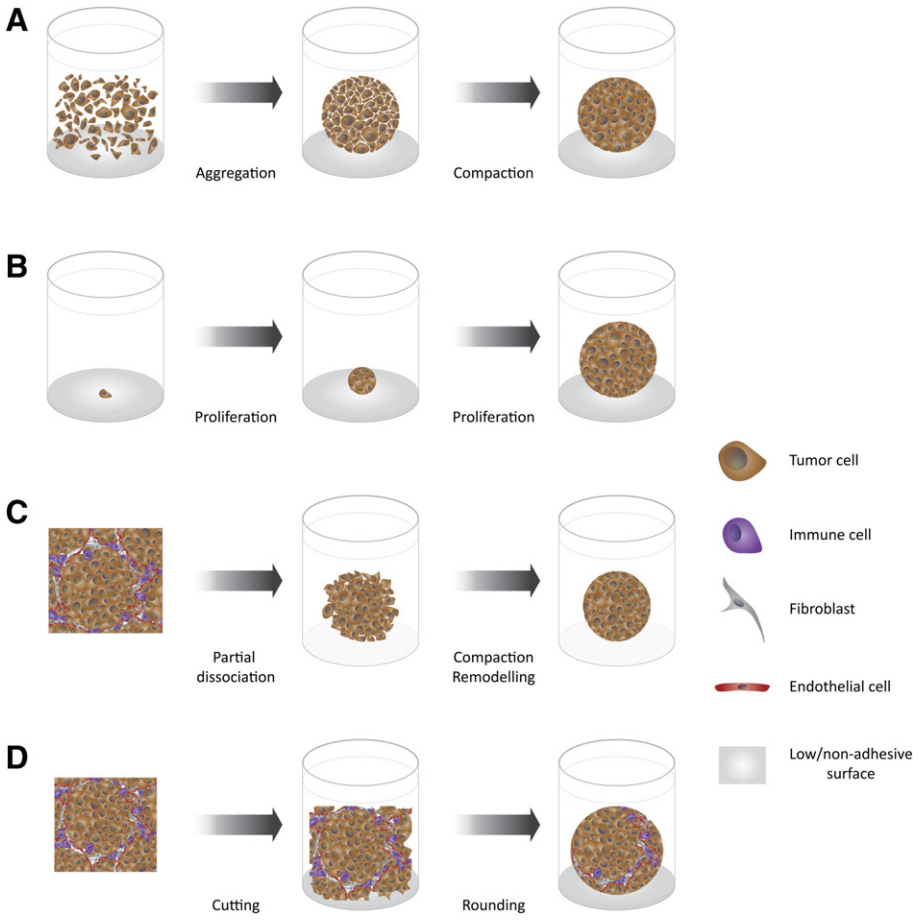

Figure 1. Steps for formation of spherical cancer models. (A) Multicellular tumor spheroids are obtained after aggregation and compaction of cell suspension cultured in nonadherent conditions. (B) Tumorospheres are formed by clonal proliferation in low-adherent conditions and with stem cell medium. (C) Tissue-derived tumor spheres are generated through partial dissociation of tumor tissue and compaction/remodeling. (D) Organotypic multicellular spheroids are formed from cutting tumor tissue in nonadherent conditions that rounded up during the culture.

More recently, several studies reported production of MCTSs using microcapsules with alginate-based membranes (Figure 2, *G–H*) [56,57]. Tumor cells are encapsulated in cellulose-based microparticles, with a narrow size distribution, via a peroxidase-catalyzed reaction in a water-immiscible fluid under laminar flow. Next, the microparticles are coated with an alginate-based gel several dozen micrometers thick via the same enzymatic reaction in water-immiscible fluid. Finally, the cellulose-based microparticles are degraded using cellulase to prepare MCTS formation [58]. The alginate encapsulation approach enables preparation of MCTSs in large quantities of a well-defined size, compatible with high-throughput screening [59]. This method also enables studying cancer cell lines unable to form MCTS by the techniques described above. However, the alginate membrane would reduce oxygen, nutrient supply, and contact between cells, and this membrane might introduce a bias. To address the latter issues, Alessandri et al. developed an aqueous core enclosed by a hydrogel shell in gentle, oil-free conditions. The permeability of the gel allowed free flow of nutrients into the capsule and cell proliferation in a scaffold free-environment [59]. This promising approach, inspired by flavor pearls from molecular gastronomy, must nevertheless prove its biological relevance.

Likewise, some artificial MCTSs have been obtained using methylcellulose, a temperature-sensitive polymer used in several neural tissue engineering applications [60] that gathers the tumor cells.

Whereas the first MCTSs formed by monoculture of cancer cells mimicked micrometastasis or tumor emboli, heterotypic MCTS rapidly appeared to take into account the presence of noncancerous cells in tumor tissue. Diverse spheroid coculturing strategies of tumor and stromal cell types lead to the study of heterologous interactions in tumor tissue. The most frequent cell types used for tumor cell coculturing are immune cells, fibroblasts, and endothelial cells. Stromal cell suspensions can be cocultured with compact MCTS, leading to invasion of MCTS (immune cells [61–63], endothelial cells [64]), or they can be seeded together in starting cell suspensions with tumor cells [9]. Mixed MCTS testing with tumor and endothelial cells for the study of tumor angiogenesis had had little success [65] until Timmins et al. obtained *in vitro* microvascularized tumors [64]. They introduced endothelial cells into preformed HCT116 MCTS in hanging drops; the endothelial cells formed secondary aggregates and then migrated into the MCTS, establishing tubular networks and luminal structures.

The MCTS model has now been well characterized, and new protocols for obtaining them have been reported [55,59]. The latter are obtained from a wide range of cancer cell lines and are currently used in numerous cell biology studies. Micropatterning and

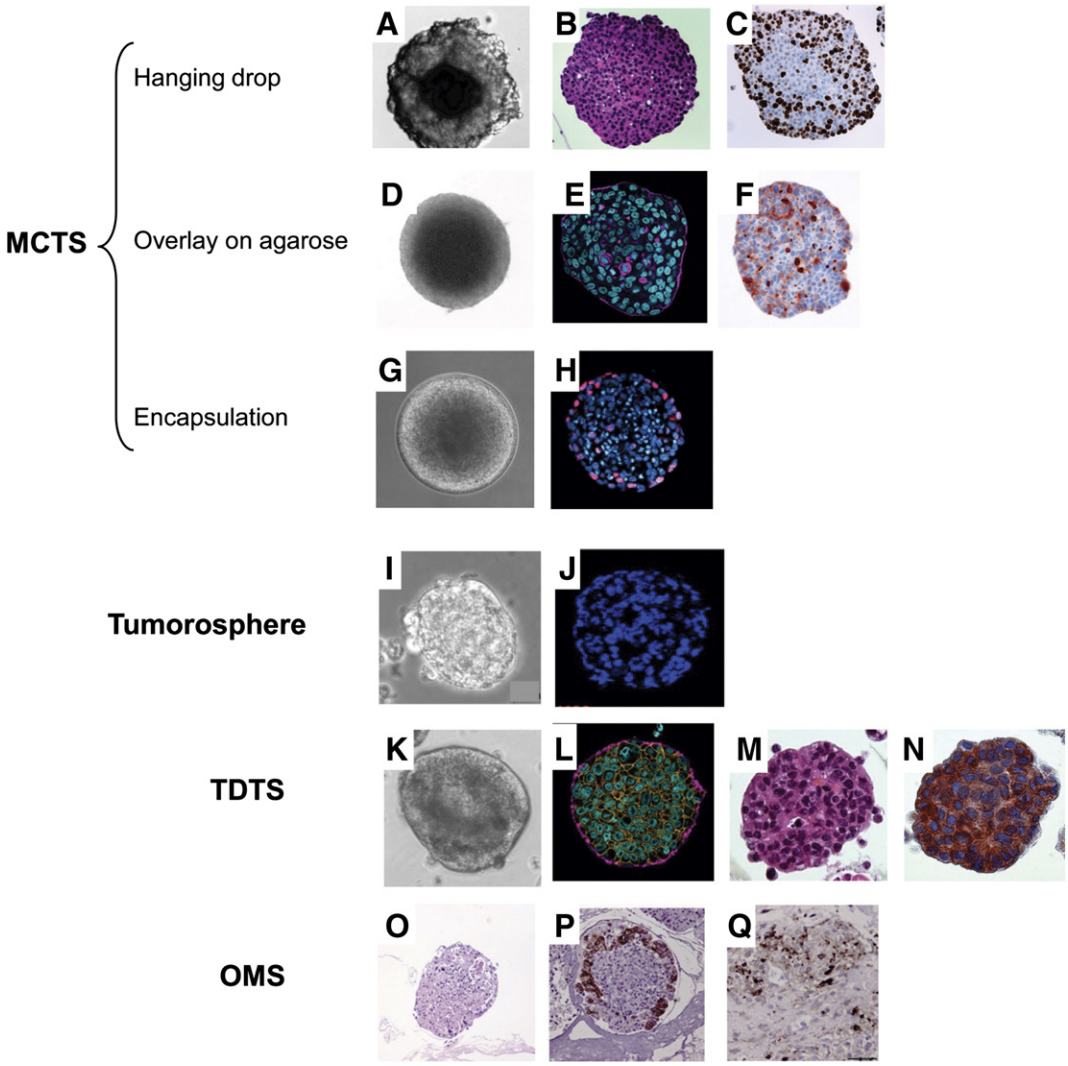

Figure 2. MCTSs, tumorospheres, TDTSs, and OMSs form very tightly packed spherical cancer structures. MCTSs could be obtained by different techniques. (A) Phase-contrast micrograph of MCTS formed by the hanging drop method with human breast cancer MCF7 cells cocultured with normal human dermal fibroblasts; (B) Hematoxylin staining and (C) anti-Ki67 immunostaining of MCTS formed by human colorectal cancer HCT116 cells.(D, F) MCTS formed by human colon cancer HT29 cells on agarose: phase-contrast micrograph (D) and immunostaining against carcinoembryonic antigen on paraffin-section (F). Confocal picture (E) of human colorectal MCTS stained with DAPI (blue) and phalloidin (magenta) according to confocal staining protocol described in [171]. (G) Phase-contrast micrograph of encapsulated MCTS obtained with mouse colorectal cancer CT26 cells. (H) Confocal images of CT26 encapsulated MCTS after cryosection and immunolabeling for DAPI (blue), KI67 (magenta), and fibronectin (red).Phase contrast microscopy (I) and anti-CK20–stained section (J) of tumorosphere from patient colorectal tumors. Nuclei in blue (DAPI), no CK20 staining.TDTS derived from colorectal cancer tissue (K–N): phase-contrast micrograph (K), confocal (L) DAPI (blue), phalloidin (magenta), anti–E-cadherin (yellow). Hematoxylin–eosin staining (M) and anti-CK20 immunostaining (N).Hematoxylin–eosin staining (O), anti-CK20 and anti-CD68 immunostaining (P) in OMSs derived from patient colorectal tumors.CK20 is an intermediate filament protein whose presence is essentially restricted to differentiated cells from gastric and intestinal epithelium and urothelium.Source of pictures:(A-C) Courtesy of Jens M. Kelm, InSphero AG, Schlieren, Switzerland; (G–H) Alessandri K, Sarangi BR, Gurchenkov VV, Sinha B, Kießling TR, Fetler L, Rico F, Scheuring S, Lamaze C, Simon A, Geraldo S, Vignjevic D, Doméjean H, Rolland L, Funfak A, Bibette J, Bremond N, and Nassoy P (2013). Cellular capsules as a tool for multicellular spheroid production and for investigating the mechanics of tumor progression in vitro. *Proc Natl Acad Sci U S A* 110, 14843–14848 [59]; (I) [40]; (J) Vermeulen L, Todaro M, de Sousa Mello F, Sprick MR, Kemper K, Perez Alea M, Richel DJ, Stassi G, and Medema JP (2008). Single-cell cloning of colon cancer stem cells reveals a multi-lineage differentiation capacity. *Proc Natl Acad Sci U S A* 105, 13427–13432. Copyright (2008) National Academy of Sciences, U.S.A. [110]; (O–Q) [84].

microfluidics technologies offer the exciting prospect of standardized MCTS mass production to tackle high-throughput screening applications using more sophisticated MCTS coculture models, more closely reflecting tumor tissues composed of tumor and various stromal cell types.

Tumorospheres

CSC culture as a free-floating sphere (tumorosphere) was first described in brain tumors by Singh et al. [36]. Initially, the quantification and characterization of such floating spherical aggregates had been developed for normal neural stem cells grown

as neurospheres, in which a single cell is able to give rise to a sphere by clonal expansion [35,66]. In the ensuing years, tumorospheres were developed from a wide range of solid tumors, including breast [38], lung [67], colon [39], prostate [68], pancreas [69], and ovarian [70] cancers, under that same assumption that "sphere assays" enable measuring self-renewal capacity.

Methods for CSC isolation and expansion as spheres do not greatly differ from one cancer tissue origin to another. The first step requires mechanical and enzymatic dissociation of the tumor sample in single-cell suspensions. CSC culture can also be performed on cancer cell lines, facilitating the dissociation step. Recently, circulating breast tumor cells from patients have been reported to proliferate as tumorospheres. Consequently, the first step aims here to depleting other blood cells using a microfluidic technology [71]. Next, the cell suspension is cultivated at low density in specific medium called "stem cell medium" in low-adherent conditions to promote extensive proliferation as clonal nonadherent spherical clusters (Figure 1*B*). Stem cell medium is devoid of FBS and supplemented with several factors that favor stem cell growth, including basic fibroblast growth factor and epidermal growth factor (EGF). Hydrocortisone, insulin, and progesterone, known to induce stem cell proliferation, can be added to the medium, as is with the case for heparin, which stabilizes the association between basic fibroblast growth factor and its receptor [72]. Likewise, optimal growth as spheroids has been obtained with leukemia inhibitory factor [73]. Depending on the cancer type, other growth factors can be preferentially added, like Wnt3A for colorectal cancer via the inhibitory effect of Wnt on enterocyte differentiation [74]. Moreover, inhibition of adhesion induces death through anoikis in nonmalignant and differentiated cells. Under these culture conditions, undifferentiated tumor cells proliferate and grow as floating clusters termed tumorospheres (Figure 2, *I–J*).

Intermediate sorting steps on the basis of putative CSC markers by fluorescent-activated cell sorting or magnetic-activated cell sorting can be performed after dissociation so as to enrich the CSC population and eliminate stromal cells. To detect these CSCs, several markers have been proposed depending on the tumor type, including CD44, CD133, aldehyde dehydrogenase, CD90, ABCG2, and ABCB5 (ABC for ATP-binding cassette), with some of them being more specific than others, but no single marker yet exists that has established itself for identifying CSCs with adequate sensitivity and specificity [75].

As pertinently discussed by Pastrana et al. [76], the tumorosphere forming assay must be carefully interpreted. It is widely admitted that each tumorosphere is derived from a single cell and is therefore clonal. Nevertheless, various cell densities are used if cells need to be seeded at extremely low densities to avoid cell fusion and aggregation [77]. Indeed, in-depth analyses in normal neurospheres clearly demonstrated that neurospheres were not clonal aggregates because of fusion-induced "growth" [77]. Semisolid methylcellulose used to grow putative clonal tumorospheres [78] also failed to prevent cell aggregation [77]. In addition to cell density, critical parameters such as medium composition, volume, surface area of the culture dish, and duration are also to be taken into account.

Tissue-Derived Tumor Spheres

In contrast to MCTS obtained from single-cell suspensions, TDTSs are obtained from partially dissociated cancer tissue. This model includes CTOSs [16], MARY-X spheroids [79], and our colospheres [15,20]. Colospheres were generated by finely cutting the tissue sample with a scalpel blade and then crushing it with a striated plunger from a disposable syringe. The resulting pieces are cultivated in a cell culture–treated flask with classical medium supplemented in FBS. Colospheres are formed in 1 day after cell remodeling and compaction (Figures 1*C* and 2, *K–N*) and are isolated by passing the bulk in cell strainers. This TDTS model was obtained in around 50% of colorectal tumor specimens and was related to tumor aggressiveness [15]. Nevertheless, the frequency of colospheres attained 95% (19/20) in colorectal cancer patient–derived xenografts (personal data). Colospheres can be kept for at least 2 weeks in culture in growth medium and nonadherent conditions to prevent plastic attachment [20]; moreover, they can be cryopreserved as whole sphere structures in freezing medium (personal data). Only tumor tissue dissociation produces colospheres; none were obtained from nontumoral tissue counterpart, showing that strong interaction between epithelial cells is not sufficient to give rise to TDTS.

As performed for generation of CTOSs, an intermediate step of incubation with liberase after mechanical dissociation is required. Next, aggregates are filtered by a cell strainer before culture in suspension with stem cell medium (without FBS and supplemented in growth factor) or in Matrigel with a medium supplemented with FBS. CTOSs were observed in 98% of samples, including endoscopic biopsy samples [16]. These structures can be passaged by dividing them mechanically, allowing expansion *in vitro*. Similarly to colosphere protocol, nontumoral colonic mucosa never gave rise to CTOS.

MARY-X spheroids were obtained from a patient inflammatory breast cancer xenograft. Upon mincing, the tumor cells are released into the medium supplemented with FBS as sheets of cells and single cells to form tight, compact clumps or aggregates of cells. These structures can be maintained in culture for periods of up to 3 months [80].

Organotypic Multicellular Spheroids

The OMS model is very similar to the explant model, which consists of culturing *ex vivo* fragments of tumors [81,82] without dissociation, in contrast to TDTSs. The method for establishing OMS in culture from tumor tissue is simple. The tumor fragment is cut with a scalpel into pieces (0.3 to 0.8 mm in diameter) and incubated in tissue culture flasks previously coated with 0.75% agar in medium. Growth medium is supplemented with 10% FBS and with an excess of nonessential amino acids. Tissue fragments are cultured for 12 to 18 days [83] or 2–5 days [84] until they round up to form OMS (Figures 1*D* and 2, *O–Q*). At that time, they can be isolated from tissue debris with a pipette by visual control using a microscope. The OMS can be further cultured in new agar-coated flask with medium. The growth medium of the overlay suspension is usually changed once a week, and the OMSs can be kept in culture for several weeks [13,83]. OMSs can be stored as frozen stocks. As a typical example, cryopreserved glioma OMSs remain viable, retain their histological characteristics, and display only minor phenotypic and genotypic changes after thawing [85].

OMSs have been successfully generated from glioblastoma [86,87], meningioma [88], mesothelioma [46], head and neck squamous tumor [89], lung cancer [90], bladder cancer [91], and colorectal cancer [84]. Spheroids isolated from ovarian carcinoma ascites fluid are a special case in the OMS family. Unlike the other OMSs, they are not generated after tissue processing but are isolated directly from patient effusions [92].

Biology of Spherical Cancer Models

Multicellular Tumor Spheroids

MCTSs can attain 1 to 3 mm in diameter [93,94] and are somewhat compact depending on the cancer cell line [29]. In large MCTSs (>500 μm diameter), a necrotic core is surrounded by a viable rim, with an inner layer of quiescent cells and an outer layer of proliferating cells, as described in microregion of tumor *in vivo*. Such tumor cell heterogeneity is due to growth factor deprivation, nutrients and oxygen gradients, and catabolites accumulation.

Moreover, the dynamic of the MCTS growth rate reproduces that of solid tumor *in vivo* characterized by an early exponential phase followed by a period of delayed growth. Whereas monolayer cultures grow exponentially, MCTS and *in vivo* tumor are characterized by exponential cell proliferation followed by a phase of declining growth rate associated with an increase in nonproliferating and necrotic cells [4]. Ewing tumor MCTSs have been demonstrated to be more closely related to patient tumors in their proliferative index but also to cell morphology, cell–cell junctions, and ERK1/2 MAPK and PI3K ± AKT pathway activation [95].

Spatial organization of MCTSs is based on cell interactions differing from those existing in flat monolayer culture. Indeed, differential expression of proteins implicated in cell–cell and cell–matrix interactions has been revealed through monolayer–MCTS transition. Thus, in several studies, E-cadherin was found to be overexpressed in MCTSs compared to monolayer cells [96–100], in the manner of CD44 and EpCAM in gastric cancer MCTSs [29]. In contrast, integrin β1, β4, and α6 subunits were downregulated in MCTSs from epidermoid carcinoma cell line A541 [101]. Dynamic analysis of hepatoma MCTS formation has shown the fundamental role of E-cadherin and β1-integrin in cell aggregation and MCTS compaction [102].

Synthesis of ECM components and their receptors can be largely influenced by culture conditions [103]. Thus, a thick ECM-like filament network has been reported to be associated with the surface itself. Moreover, the presence of ECM components (fibronectin, laminin, collagen, and glycosaminoglycans) has been demonstrated in MCTSs from human thyroid cancer and glioma cell lines [104].

MCTSs at least partially recapitulate differentiation of the parent tumor (Table 3), in contrast to monolayer cultures. Generally, cells are more strongly differentiated in MCTS cultures than in monolayer cultures. Outer layers of MCTS from hepatoma cell lines are differentiated into smooth tightly packed polarized cells [102], and microvilli were observed on the surface of MCTSs from colon cancer cell lines [15,105]. Furthermore, MCTSs contain pseudoglandular structures with lumen, very similar to the characteristics of the original adenocarcinoma specimens [106]. Similarly, features of histological differentiation characteristic of primary ovarian carcinomas are not present in monolayer cultures but are restored in MCTSs [107].

Table 3. Comparison of Various Tumor-Related Parameters in the Four Cancer Sphere Models.

	Multicellular Tumor Spheroids	Tumorospheres	OMSs	TDTSs
Cancer sphere culture				
Success rate of initiation	+	+	+/–	+
Ease of maintenance	++	+	+/–	+/–
Genetic manipulation	++	+	ND	ND
Sphere composition				
Tumor heterogeneity	+/–	–	++	++
Tumor–stroma interaction	+	–	++	–
Immune system	+/–	–	+	–
Characteristics of original tumor	+	–	++	++
Application fields				
Tumor growth	++	+	+	+/–
Survival	++	–	+/–	+
Hypoxia	++	–	+	+
Cancer stemness	–	++	+/–	–
Migration/invasion	+	–	+	+
In vivo tumorigenicity	+/–	++	+	+
Personalized medicine	+	+	++	++
High-throughput drug screening	++	+	–	+/–
Low-throughput drug screening	++	++	+	++
Radiosensitivity	++	+/–	+	ND

Parameters are appreciated as best (++), suitable (+), possible (+/–), and unsuitable (–). *ND*, Not determined.

Tumorospheres

Little is known about the biology of tumorospheres; indeed, contrary to the use of other sphere models, that of the tumorosphere does not seek to mimic cancer tissues but rather to study CSC properties (Table 3): it is admitted that tumorospheres do not fully replicate the 3D structure and environment of an *in vivo* tumor [108].

The CSC model postulates that tumors are organized hierarchically with a subset of rare tumor cells, which possess self-renewal and multilineage differentiation potential. Because of the general lack of cell-surface markers and the absence of a distinct and discernible morphological phenotype related to the instability of the CSC phenotype, CSCs have typically been defined and studied on the basis of functional assays in relation to these properties. The gold standard for evaluating the presence of CSCs is transplantation of a few cells into an immunocompromised mouse: CSCs have the unique capacity to form tumors in serial xenotransplantation assays after injection of low number of cells and to reestablish, at each *in vivo* passage, the hierarchical cell organization and heterogeneity of the parental tumor [75]. *In vitro* methods have been developed as attractive surrogate assays to measure their ability to form *in vitro* tumorospheres (clonogenic) when plated at low density in nonadherent cultures in "sphere"-forming assays. Histological examination of tumorospheres demonstrated the absence of nonneoplastic cells in patient-derived tumor spheres obtained without sorting step [109].

The CSC capacity for multilineage differentiation was assessed *in vivo* by testing the ability to reproduce tumor heterogeneity in a xenograft assay and/or *in vitro* by testing the capacity of tumorospheres to differentiate under differentiating conditions. In stem cell medium, patient-derived tumorosphere cells maintain their sphere morphology and remain poorly differentiated, with little or no expression of differentiation markers that include cytokeratin (CK) 20 in colon cancers [110]; GFAP or β-tubulin 3 in brain tumors [36]; CK18, CK14, and α-SMA in breast cancers [38]; and CK7 and CA-125 in ovarian cancers [70]. After withdrawal of growth factors and addition of 10% FBS on tissue-culture–treated plastics or in Matrigel, floating cells were able to adhere and to strongly differentiate with expression of differentiation markers. In contrast, the addition of myofibroblast-derived factors prevented differentiation of colon CSCs [111].

It is noteworthy that single neurospheres from normal brain contain stem cells, progenitors, and differentiated cells, as observed in the tumorospheres. Tumorospheres are not homogeneous structures enriched with undifferentiated cells but rather comprise a range of morphologically distinct entities displaying inter- and intrasphere molecular heterogeneity, including variable expression of markers of differentiation [112]. Several studies have demonstrated clonal

heterogeneity among tumorospheres. Thus, in the case of patients with colorectal cancer, three different types of CSC, all undifferentiated, were resolved on the basis of clonal tumorosphere cultures from individual patient tumors (one cell per well was seeded in a 96-well plate) [109]: 1) a rare subset of CSCs that maintained tumor growth on serial transplantations; 2) a subset of tumor-initiating cells with limited self-renewal capacity; the latter contribute to tumor formation only in primary mice and are therefore not consistently defined as CSCs; and 3) a more latent subset of CSCs apparently activated in second or tertiary transplantation assays. Likewise, in PTEN-deficient glioblastoma, a series of phenotypically distinct self-renewing cells was observed in both CD133+ and CD133− fractions [113]. Clearly, the tumorosphere assay selectively enriches for the growth of CSC, although it is noteworthy that these spheres also contain more differentiated tumor cells [114].

A recent work [115] reports that the tumorosphere assay enriches CSC population in a cell line–dependent manner and that the conventional monolayer culture might maintain a CSC phenotype more effectively than the tumorosphere depending on the cancer cell line.

To date, no study has precisely determined how many cells within tumorospheres are actually CSCs: the gold standard for evaluating the presence of CSC remains a comparison between a putative CSC population and unselected cancer cells in *in vivo* assays. Readout of the tumorosphere assay involves the number and size of the spheres; thus, the evaluation of the CSC presence is subject to discussion. Indeed, quiescent stem cells may not divide to form tumorospheres because the assays used do not provide as-yet-unknown key components of the *in vivo* niche required for activation of dormant stem cells. Likewise, the size may simply reflect growth factor responsiveness.

Tissue-Derived Tumor Spheres

Initially, the observation of such structures was reported in a limited number of studies performed for the *in vitro* human colon cancer cell lines establishing [17–19]. Thus, TDTSs have been largely characterized for colorectal cancer, as demonstrated by the work of Kondo's group on CTOSs [16] and that of our group on colospheres [15,20]. However, TDTSs were also obtained from dissociation of various types of cancer tissues including lung, bladder, prostate, and breast cancer tissue and uveal melanoma (personal observation and [16,21].

TDTSs are formed by tissue remodeling and compaction after partial tissue dissociation and are exclusively composed of tumor cells (Figures 1*C* and 2*L*) without nonneoplastic cells [15,16,21,80]. This might be explained by strong cell–cell interactions between carcinoma cells. However, the interaction between epithelial cells could not *per se* explain the formation of TDTS because none are obtained from nontumoral mucosa. Thus, interactions between tumor cells and nonneoplastic cells might be lost after partial dissociation, resulting in the absence of stromal cells in TDTSs. Indeed, E-cadherin has been shown to be involved in cell–cell interactions within TDTSs [16,20,80,116], whereas E-cadherin/β-catenin complexes were shown to be tethered to the cytoskeleton. Because this organization has been demonstrated to strengthen cell–cell adhesion in other systems [117], it can be postulated that the same is true for TDTSs. Through their strong cell–cell interactions, colospheres, MARY-X spheroids, and CTOSs escape anoikis and may remain viable for several weeks in culture.

A major trait of TDTSs is their capacity to recapitulate avascular tumor microregions. Colospheres and CTOSs have been shown to mimic the parent tumor (Table 2) in terms of histological characteristics, gene expression profiles, mutations in key genes, and tumorigenic and metastatic properties [15,16,20,21,80]. Thus, MARY-X spheroids display polar architecture and internal lumenlike structures (canalis) coated with microvilli as observed in lymphovascular emboli *in vivo*. This type of architecture is not found in MCF-7 MCTSs [80]. In the same manner, colospheres retain the differentiation level of the parent tumor, including glandularlike structures and mucus production, in contrast to MCTSs formed by cancer cell line from the same parent tumor [15]. Moreover, after 1 week of culture, fractions of proliferating cells in colospheres are approximately the same as observed *in vivo*, whereas MCTSs from cancer cell line possess extensive proliferative capacity, probably because of selective pressure induced by culture (personal data). However, colospheres show little volume growth even after 15 days in culture. A potential explanation would be that active cell proliferation is compensated by substantial cell death and/or cell shedding. In most CTOSs, the increase in size slowed down after about 14 days of culture in stem cell medium through AKT pathway activation. As in the *in vivo* situation, proliferating cells predominantly localize to the outer rim of the growing CTOSs [16].

Organotypic Multicellular Spheroids

From the various *in vitro* sphere models described here, OMSs seem to be the 3D model which is closest to *in vivo* tumors, in light of the absence of any dissociation process for obtaining these spheroids (Table 2).

This model is very close to both organ culture of tumors, wherein a small piece of tissue is cultivated on the surface of the medium in a moist gas phase [118], and the explant model, which consists of culturing tumor fragments completely immersed in medium [119]. Precise characteristics of OMS have not yet been reported, and most information on this model was obtained by Rolf Bjerkvig's group [83].

The morphology of OMSs is usually similar to that of the original tumor tissue. OMSs recapitulate the original heterogeneity of the tumor; they maintain the presence of macrophages and preserve vessels with striated fibers of collagen in association with fibroblasts (Figure 1*D*) that surround vascular elements [13]. Maintenance of the stromal component within the OMS clearly makes OMSs distinct from the TDTSs. It is noteworthy that OMSs obtained from glioma biopsies show wide variation in central necrosis formation. In these spheroids, cell cycle analyses reveal that the fractions of proliferating cells (S and G_2M) in the glioma OMSs after 3 weeks of culture are approximately the same as those observed in the tumors *in vivo*. Even after prolonged culture (~70 days), the cell cycle was unchanged. Similarly, OMSs from bladder cancer display cell cycle distribution which is similar to that observed in original tumors [83]. Furthermore, mitotic figures are frequently observed in OMS, demonstrating their growth capacity. Nevertheless, as described for colospheres, no significant volume growth was observed after several weeks in culture, suggesting cell loss and/or cell shedding in these structures. This was demonstrated for one glioblastoma in which cell shedding from each OMS was found to be approximately 31 cells per hour [13].

Tumor cells that propagated as OMSs from biopsies show a remarkable stability in ploidy even after long culture periods [83]. Moreover, genomic profiles of OMSs from glioblastomas are genetically stable and more representative of the parent glioblastoma than short-term primary cultures [120]. Nonetheless, this type of spheroid has been reported in only a few types of cancer.

Applications

Radioresistance

Radioresistance was the first application scope to be studied using the MCTS model (Table 3). This powerful model is particularly well adapted to ionizing radiation studies because tumor sensitivity to ionizing radiation is controlled by parameters that include intercellular contact and communication, oxygen, damage repair, and apoptosis induction [121]. The first evidence of radioresistance in MCTSs dates back to the early seventies and has been made on rodent cell lines [122]. In general, survival of MCTS cells is better than that of the same cells cultured in monolayers, and growth of MCTSs and tumors *in vivo* after irradiation is very similar. MCTSs from the WiDr colorectal cancer cell line thus appear to accurately model the radiation sensitivity of WiDr tumors compared to cells in a monolayer [123]. Using the MCTS model, resistance to radiotherapy has been explained by hypoxia [124] and cell–cell contacts [122]. Santini and colleagues thus demonstrated that an increase in compaction of HT29 MCTSs was responsible for enhanced resistance to ionizing radiation [117]. Moreover, proposed mechanisms for the "contact effect" include gap junctional "reciprocity," cell shape–mediated changes in (repair-related) gene expression, and alterations in chromatin packaging that influence DNA repair [125]. To our knowledge, no radiosensitivity studies had previously been performed on whole tumorospheres. CSC spheres have been consistently dissociated before radiosensitivity assays [126]. Few radioresponse studies have been performed on OMSs and then only from human glioblastoma. Radiation induces minor effects on glioblastoma OMSs, as observed in *in vivo* situation. In glioblastoma, radioresistance is related to the presence of blood vessels and hypoxia. In contrast, most glioblastoma cell lines cultured as monolayers are radiosensitive [127].

Chemosensitivity

Decades of research have firmly established that, in a preclinical or clinical setting, cancer cells grown *in vitro* as 3D MCTSs more accurately mimic the drug sensitivity/resistance behavior of cancer cells found in solid tumors *in vivo* than cancer cells cultured under conventional 2D monolayer conditions. Experts in the field recommend their use in major programs for drug screening and development and frequently point out the rationale for using MCTSs in antitumor drug testing [49] (Table 3). MCTSs have been and are still being used for modeling and for studying multicellular resistance (for review, [128]) due to different mechanisms: hypoxia [129], alteration of chromatin structure or chromatin packaging [125], apoptosis inhibition [130], cell cycle [131], and permeability [132]. Numerous anticancer therapies have been evaluated in different cancer cell types in MCTS and directly compared to the same cells grown in a 2D monolayer format. Studies showed that tumor cells were less sensitive to anticancer agents when evaluated in MCTSs compared to 2D culture conditions [133]. However, a number of studies indicated that the observed effects of anticancer agents against tumor cells in MCTS culture were equal to, or more sensitive than, the same tumor cell type cultured in a 2D monolayer format [134]. Because of formation of HER2 homodimers, trastuzumab inhibits cell proliferation in SKBR3 and SKOV-3 cells when they are maintained as MCTSs as compared to conventional 2D culture through formation of HER2 homodimers [135]. Activation of HER2 in MCTS led to higher sensitivity to trastuzumab while maintaining MCTS compaction.

Whether cell sensitivity to drugs/compounds is increased or decreased, information obtained from use of these 3D cell cultures in cancer research can potentially provide a more accurate representation of drug/compound activity *in vivo*. In addition, the regular size and well-rounded shape, as well as easy rapid handling, make MCTSs powerful tools for drug testing, facilitating high-throughput screening [9,49,59].

Although recent evidence indicates that CSCs respond to antitumor agents differently *in vitro* and *in vivo* [108], tumorospheres are being increasingly used for studying the response to chemotherapy because the remaining CSCs presumably trigger relapse after treatment termination. Interestingly, Todaro and colleagues demonstrated that tumorospheres from patient colon tumors are resistant to 5-FU and oxaliplatin through autocrine production of interleukin-4 [40]. An original study reports the proof of concept of culturing circulating tumor cells from patients as tumorospheres to perform personalized anticancer drug testing [71] (Table 3). Many studies use tumorospheres derived from cancer cell lines to highlight the resistance of CSC compared to the bulk tumor. Those studies compared the anticancer drug response of tumorospheres to that of adherent cells, referred to here as bulk tumors. However, as in the case for the radioresponse, it is crucial to take into account the 3D aspect of the tumorospheres because the resistance observed in CSCs could be due to multicellular resistance and not to the intrinsic properties of CSCs. Few studies have analyzed the effect of anticancer drugs on OMSs [46,91,136], which could be explained by heterogeneity between OMSs of the same tumor, thus complicating standardization. Glioblastoma OMSs were used to demonstrate that NG2/MPG-expressing tumors were more resistant to doxorubicin, carboplatin, and etoposide [136], and OMSs from human mesothelioma showed resistance to apoptosis after TRAIL-plus-cycloheximide treatment, partly mediated by Akt/PI3k and mTOR pathways [46]. From a personalized treatment perspective, study of the response of TDTSs could predict a patient's tumor response to chemotherapy, and initial studies are promising (Table 3). Growth of CTOSs from colorectal cancer specimens was dose-dependently inhibited by 5-FU, whereas the response of CTOSs differed in individual cases [16]. Using colospheres from colorectal cancer patient–derived xenografts, our group showed a correlation between the *ex vivo* colosphere response to 5-FU or irinotecan and the *in vivo* xenograft response [20].

Migration and Invasion

The multistep process of metastasis can only be successful if the 3D microenvironment is permissive for tumor cell invasion, metastatic dissemination, and metastatic growth. Noncellular components of the tumor microenvironment, such as ECM and hypoxia, critically drive tumor progression via increased ECM deposition, cross-linking, and remodeling [137]. Moreover, cadherins and integrins have been linked to the metastatic process, and adhesion and the ECM molecule expression pattern in the 3D environment resemble those of the tumor *in vivo*. In addition, 3D conformation can induce expression of proteins associated with metastasis, as suggested by enhancement of carcinoembryonic antigen expression in the MIP-101 colorectal cancer cell line grown as MCTSs [138]. Depending on the cell type and tissue environment, cells can migrate in two major ways: individually, when cell–cell junctions are absent, or collectively as multicellular groups, when cell–cell adhesion is retained. This multicellular migration mode, or "collective migration," is commonly used by carcinomas, which retain high or intermediate levels of

differentiation [139]. Collectively, these data present spherical cancer models as relevant *in vitro* models for studying invasion and migration processes (Table 3). Numerous studies used MCTS for invasion and migration assays, and recent publications have demonstrated their great advantage in the evaluation of therapeutic agents/drugs with antimigratory properties using rapid highly reproducible 96-well plate–based technique [54,140]. MCTSs can thus be embedded in different matrices [141,142] or seeded on top of ECM [143]. Invasion has also been studied using MCTSs in different coculture systems. MCTSs obtained from the MCF-7 cell line have been confronted *in vitro* with chick heart fragments to study the effect of retinoic acid on the invasion process [144]. Expression of antisense uPAR and antisense uPA from a bicistronic adenoviral construct inhibited invasion of fetal rat brain aggregates by cells from SNB19 MCTSs [145]. Invasion was also assessed using cultures of MCTSs with mouse embryoid bodies [54], precultured reepithelialized endometrial fragments [146], and organotypic brain slice cultures [147]. Interestingly, two different sphere models obtained from the same type of cancer displayed differing invasion and migration properties. In a recent study, MCTSs obtained from the glioblastoma U87MG cells were unable to invade the corticostriatal slice, in contrast to glioblastoma OMSs. The U87MG cell line may have lost the invasive features characteristic of glioblastomas as a result of selective pressures and genetic drift [147]. Likewise, a given 3D model of different tumor grade shows different properties, illustrating a poor artifact effect due to the model used. Thus, OMSs have been shown to mimic properties of their origin tumor. Using coculture of OMSs from brain tumors with fetal rat brain aggregates, low-grade glioma OMSs were less invasive than those obtained from the highly malignant glioblastomas. Indeed, the invasiveness of the glioblastoma OMSs was characterized by a gradual destruction of normal brain tissue by tumor cells, followed by replacement of normal tissue by these cells [148]. OMSs can also be embedded in matrix to study migration and invasion of cells from the OMS. Not all the OMSs from a given glioblastoma sample displayed the same capacity to migrate in or invade ECM [86], reflecting inherent intratumoral heterogeneity. This was confirmed by two studies in which the metastatic potential of ovarian carcinoma ascites OMSs was assessed using adhesion [48] and migration [149] assays in different matrices and in normal human mesothelial cell monolayers. Taken together, these observations strongly suggest that the metastatic potential of OMSs is driven by both their microenvironment and their intrinsic invasive properties.

Studies on the metastatic properties of TDTS have shown that colospheres retain aggressiveness of the parent xenograft tumor in contrast to MCTSs [15,20]. Furthermore, MARY-X spheroids have been shown to mimic lymphovascular emboli *in vivo*, a structure efficient at metastatic dissemination. Similarly to the tumor emboli, MARY-X spheroids display a high level of E-cadherin [150] because of altered trafficking [151].

Despite the emergence of the concept of "migrating cancer stem cells" [152] and the association between EMT and stemness properties [153], tumorospheres are not used in Matrigel assays for dissemination monitoring but rather for differentiation induction, so as to assess their capacity of multilineage differentiation [110].

CSCs/Tumorigenicity

Tumorosphere cultures have gained popularity as *in vitro* assays for propagating and analyzing CSCs (Table 3). As already discussed above, the "CSC sphere assay," derived from the normal neural stem cell assay, should be more carefully interpreted before conclusions can be drawn concerning their stemlike properties. Moreover, the concept of CSC itself remains subject to debate [154], and the difference between the CSC, cancer-initiating cells, and highly proliferating cells is ambiguous.

In breast cancer, use of serum media was demonstrated to promote cell differentiation rather than an undifferentiated state. Clonal dilutions used in tumorosphere-forming assays revealed the intrinsic property of cancer stem/progenitor cells at surviving and growing in serum-free suspension, whereas more differentiated cells undergo anoikis and die under these conditions [32].

Recent data suggest that CSCs depend on a similar, permissive environment, the CSC niche, to retain their exclusive ability to self-renew and to give rise to more differentiated progenitor cells, while themselves remaining in an undifferentiated state [155]. This CSC niche, by analogy with the normal stem cell niche, is defined as a particular location or microenvironment that maintains the properties of stem cell self-renewal and multipotency. The CSC niche is composed of blood vessels, stromal cells, and ECM components but also defined by its 3D organization. Although tumorospheres are characterized by the absence of stromal cells and the lack of exogenous ECM, it has been reported that cancer cells themselves within tumorospheres are able to produce ECM components like tenascin C to partially create their CSC niche [34]. Likewise, culture medium can be supplemented with cytokines like SCF and G-CSF, present in the bone marrow microenvironment, forming a metastatic niche [156].

In general, the 3D local microenvironment in all spherical cancer models could enhance cell survival through strong cell–cell contacts and might, at least in the biggest ones, offer a hypoxic microenvironment favorable to cancer stemness [157]. Nevertheless, we demonstrated using several human carcinoma cell lines that MCTSs are not more tumorigenic than cell suspension when injected into mice ([15] and personal data). However, the size of the injected MCTSs (~150 μm) did not lead to a hypoxia microenvironment, known to be associated with tumor development [158].

TDTSs have been reported to initiate tumors and to reproduce characteristics of the original tumor [16,20,21,159]. Study of the expression of potential CSC markers by colospheres and CTOSs has shown that expression of CSC markers is maintained at a low level as observed in the parent tumors. The CSC character is not lost during culture, although colospheres remain largely differentiated. The presence of several differentiated cell states is important because cooperation between CSC and differentiated cells in drug resistance mechanisms has been recently reported [160]. In contrast, MARY-X spheroids contain a high percentage of cells expressing putative cancer or normal stem cell markers related to the aggressive nature of inflammatory breast cancer [159]. Similarly, OMSs can be cultured without loss of tumorigenicity, even after cryopreservation [85].

Other Applications

MCTS have been used in many other applications, including studies on hypoxia [161], tumor metabolism [162], penetration of adenoviruses into tumors [163], and the effects of mechanical stress upon tumor cells [164]. More complex sphere models have been developed involving coculture with other cell types to study interactions between the tumor and stroma *in vitro*. MCTSs can be cultured with endothelial cells to study angiogenesis (for review, [65]) or with immune system cells (for review, [165]), such as dendritic

cells [166], macrophages [167], monocytes [63], and T lymphocytes [61,62], for studying interactions between tumor cells and immune cells. Moreover, MCTSs containing tumor cells and fibroblasts have been generated to study influence of fibroblasts on the tumor [168,169]. The "minitumor spheroid model," which is more complex, includes endothelial cells, fibroblast and tumor cells [170].

Conclusion

We provide here a rational classification of the four most commonly used spherical cancer models in cancer research: 1) the MCTS model, described since the early 70s and obtained by culture of cancer cell lines in nonadherent conditions; 2) tumorospheres, a model of CSC expansion established in serum-free medium supplemented with growth factors; and 3) TDTSs and 4) OMSs, obtained by tumor tissue mechanical dissociation and cutting, respectively. These models appear to closely resemble each other. Nevertheless, although they share a common 3D conformation, these four models display their own intrinsic properties. MCTSs arise from cell lines from all cancer types and offer a very high level of reproducibility, but considerable debate still continues to surround the usefulness and pertinence as culture-adapted cell line models. Tumorospheres have been proven to be an excellent model for enriching the CSC fraction but not for studying intrinsic properties of CSCs related to their 3D architecture. TDTSs and OMSs are relevant models for mimicking tumors, but they are not susceptible to transfection and standardization. Finally, depending on the study aim and the cancer type, the most relevant sphere models must be carefully selected.

Acknowledgements

Work of the laboratory on spheres is supported by a Genevieve and Jean-Paul Driot transformative research grant, a Philippe, Stéphanie and Laurent Bloch cancer research grant, a Hassan Hachem translational medicine grant, a Sally Paget-Brown translational research grant, the Institut National du Cancer and Cancéropôle Ile de France (COLOMETASTEM grant), and Groupement des Entreprises Françaises dans la Lutte contre le Cancer (grant 5/188). L.B.W. was supported by postdoctoral fellowships from the Association pour la Recherche en cancérologie de Saint-Cloud (ARCS) and the Canadian Institutes of Health Research (CIHR). We thank Jerri Bram for her valuable help. We thank Jens M. Kelm, Pierre Nassoy, Danijela Vignjevic, Connie R. Jiménez, and Uros Rajcevic for sharing pictures of spherical cancer spheres.

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