# Three-Dimensional Spheroids in Cancer Research

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Three-dimensional (3D) cultures of cancer cells that better recapitulate in vivo cell environments emerged as scientifically accurate and low cost cancer models for preclinical screening and testing of new drug candidates before moving to expensive and time-consuming animal models.

Keywords: 3D cultures ; tumor microenvironment ; tumor spheroids ; efficacy analysis ; drug resistance ; cancer therapy

## 1. Introduction

Significant investments are made in cancer research for drug discovery and development. Yet, the approval rate (≤5%) of drugs that reach the clinic remains very low[1][2]. Typically, anticancer compounds are tested in two dimensional (2D) cell culture models, that involve a panel of cancer cell lines, such as those used by the US National Cancer Institute[3]. Drugs that show promising cytotoxicity in 2D in vitro system progress to animal models of human cancers (mainly mice) for anti-tumor efficacy testing[4]. Unfortunately, most of the promising preclinical drugs have no or weak efficacy in real patients with tumors, resulting in a significant delay of anticancer drug development[5]. One of the main factors underlying this poor success is the inadequacy of the preclinical 2D cultures and animal models to recapitulate the human tumor microenvironment (TME). TME is a complex and heterogeneous structure made of cellular (e.g., transformed epithelial cells, fibroblasts, infiltrating lymphocytes, mesenchymal stem cells, endothelial cells) and non-cellular (e.g., extracellular matrix—ECM, growth factors, cytokines and chemokines) components, with a critical role in cancer development and progression[6][7]. The 2D culture systems lack the structural architecture and the microenvironment of the tumor, and display altered gene expression and activation of cell signaling pathways, compared to the in vivo tumor tissues (Table 1)[8][9][10]. Besides the associated higher cost and ethical issues, animal models also display significant limitations and poorly reflect the proprieties of human tumors. For instance, the stromal component of the xenograft is not of human origin, the rate of growth is higher in xenografts (doubling time of a few days) than in primary human tumors (doubling time of a few months), and, thus, they often tend to respond better to anticancer drugs[11].

**Table 1.** Differences between conventional 2D monolayer and 3D spheroid cultures

| Cell Culture System | Advantages | Disadvantages |
|---|---|---|
| 2D cultures | • Fast replication;<br>• Low cost;<br>• Easy to manipulate;<br>• Establish long-term cultures. | • Homogeneity in oxygen and nutrients perfusion;<br>• Decreased cell–cell and cell–ECM interactions;<br>• More susceptible to pharmacological action;<br>• Poor cell differentiation;<br>• Faster proliferation than in vivo tumors.<br>• Modified genetic profile when compared to in vivo tissue. |

| Cell Culture System | Advantages | Disadvantages |
| --- | --- | --- |
| 3D cultures | • Heterogeneity in oxygen and nutrients perfusion;<br>• 3 different layers (proliferation, quiescence and necrosis zones) resembling the in vivo tumors;<br>• Increased cell–cell and cell–ECM interactions;<br>• Mimic drug penetration in the tumor.<br>• Recapitulate the genetic in vivo profile. | • High cost;<br>• Greater difficulty in carrying out methodological techniques. |

Therefore, the development of preclinical models that better recapitulate patient tumor and microenvironment represents a promising challenge to improve the success rates in anticancer drug development. Since the discovery of the importance of the extracellular matrix (ECM) in cell behavior, it became clear that three-dimensional (3D) cell culture systems offer an excellent opportunity to recapitulate the real avascular tumor, by allowing cancer cells to be cultured, either alone or in co-culture with other cell types, in a spatial manner reminiscent of the structural architecture of the tumor that provides cell–cell and cell–ECM interactions, thereby mimicking the native tumor microenvironment (Table 1) [12][13][14][15]. Hopefully, besides circumventing the barriers and limitations imposed by 2D monolayer cultures, 3D cell culture models could reduce or, ideally, replace the use of animal models, thereby resolving the associated ethical and cost issues[16][17]. Here, common 3D cell culture methods are highlighted, the characterization tools for the evaluation of the targeted effect are reviewed, with focus on multicellular tumor spheroids (MCTS) and their applicability in cancer research.

## 2. Application of 3D Cultures in Anti-Cancer Drug Discovery and Delivery

The capacity to reproduce the in vivo 3D tumor environment such as cellular heterogeneity, gene expression patterns, cell differentiation, generation of hypoxia, activation of cell signaling pathways, and cell–cell and cell–ECM adhesions, are amongst the many advantages that prompted the use of spheroids for in vitro evaluation of chemoresistance, migration and invasion, and other aspects of tumor biology (e.g., cancer stem cells/tumorigenicity, hypoxia and tumor metabolism). We will focus on chemoresistance and migration/invasion, and provide a brief overview on the use of spheroids to study drug delivery. Details of the other aspects were reviewed elsewhere[18][19][20][21].

### 2.1. Chemoresistance

Drug resistance is a major concern responsible for the failure of the current chemotherapeutics and their ability to fight cancer, especially in aggressive and highly metastatic tumors. It is now well established that cancer cells, grown in vitro as 3D spheroids, more accurately mimic the drug behavior in terms of sensibility and resistance than cells grown as 2D monolayers[21]. This difference is probably due to the TME and the spatial organization of the spheroids[22]. Increased cell–cell and cell–matrix adhesions may lead to changes in gene expression. Upregulation of cell–adhesion molecules, such as lumican, SNED1, DARP32, and miR-146a, was reported to increase chemotherapeutic resistance in pancreatic tumor spheroids as compared to 2D monolayers[23]. Fibronectin protected DU145 prostate cancer cell spheroids against ceramide and docetaxel-induced apoptosis through interaction with Insulin like growth factor-1 receptor[24]. A variety of apoptotic stimuli, including combinations of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), ribotoxic stressors, histone deacetylase, and proteasome inhibitors, were reported to be highly effective against mesothelioma cells when grown as monolayers than when grown as multicellular spheroids[21].

Increased resistance to chemotherapeutic drugs in spheroids is attributed to many factors associated with their constitution and organization, such as hypoxia, altered cellular energy metabolism, the acidic microenvironment, the cellular heterogeneity including the presence of cancer stem cells, and cell–cell and cell–ECM interactions [22][24][25][26][27][28][29][30]. The mechanisms by which these factors confer chemoresistance to spheroids were nicely reviewed in[31]. While most studies showed that cells in spheroids are more chemoresistant than cells in 2D monolayers, some studies reported that cells in MCTS are equally or even more sensitive to anticancer agents than their 2D monolayer counterparts. For example, the proteasome inhibitor PS-341 was shown to be equally effective in killing ovarian and prostate tumor cells grown in the form of multicellular spheroids, and tumor cells grown in monolayer cell culture[32].

A number of studies reported that spheroids are also more radioresistant than 2D monolayers. For instance, increased cell compaction increased the resistance of human colon adenocarcinoma spheroids to ionizing radiation[33]. Besides the aforementioned factors, radioresistance may be due to decreased radiation-induced DNA damage as a consequence of lack of oxygen in the spheroids, given that oxygen seems to be required to stabilize DNA damage upon radiation [34][35][36].

### 2.2. Migration and Invasion

The acquisition of motility and migratory ability is an important hallmark of malignant tumors. Common characteristics of solid tumors, such as hypoxia and soluble mediators-mediated interactions with stromal cells, drive tumor cell migration and invasion, through essential steps that involve, amongst others, actin cytoskeleton remodeling, changes in cell–cell and cell–ECM adhesion, and protein degradation of the surrounding ECM[37][38]. Therefore, the success of studying the multistep process of metastasis relies on a 3D microenvironment through which tumor cells can move and disseminate. In this sense, tumor spheroids are viewed as relevant in vitro models for studying invasion and migration processes [19][39][40][41]. For instance, 3D spheroids display adhesion and ECM molecule expression pattern similar to that of the tumor in vivo, and can also induce expression of proteins associated with metastasis[19][40][42]. Importantly, non-tumor cells are also present in the TME and continuously interact, through paracrine signaling, with cancer cells. For instance, fibroblasts were shown to promote contact-dependent cancer cell motility and invasion of 3D spheroids in co-culture with colorectal cancer cells, a finding validated in vivo[43]. Therefore, ideal migration/invasion assays should be performed in 3D co-cultures that also include non-tumor cells, such as macrophages, dendritic cells, endothelial cells, CAFs and immune cells, in order to better simulate the migration and invasion process found in tumor tissues. CAFs, through the release of cytokines and growth factors, together with the other stromal cells, promote the epithelial-mesenchymal transition in heterotypic 3D cell cultures, resulting in tumor development and metastasis [44][43][45][46]. At the same time, endothelial cells in 3D co-cultures tend to accumulate in the peripheral layer, facilitating the adhesion and infiltration of immune cells[47]. In fact, immune cells can secrete interleukin 6 and MMP-9, which cause inflammation, angiogenesis and ECM degradation, thereby promoting tumor invasion and metastasis[48].

Several assays are available to determine the invasion and migration potential of cells in spheroids[19][41]. In the transwell-based or Boyden chamber assays, the spheroids are seeded on the top of a filter coated with a thick layer of ECM-derived components, usually collagen I, and invasion, in response to a chemo-attractant such as growth factors, can be measured by determining the number of cells that move from the top chamber to the lower chamber [19][41][49]. Additionally, the ability of the cells to invade cellular barriers can be determined by adding a layer of fibroblasts or endothelial cells on top of the matrix [19]. This latter is particularly relevant to mimic the ability of cancer cells to cross the blood vessel barrier and to invade deeply the tissues. Alternatively, spheroids can be completely embedded into different matrices, usually between two layers of ECM gel, where cells leave the spheroids and invade the surrounding matrix [50][51]. Sophisticated techniques combined with computerized quantification are now available to reproducibly perform optimized experimental conditions and to calculate the invasive index of cells [19][51][52][53]. For instance, the extent and rate of tumor spheroid invasion, using the 3D spheroid invasion assay, was rapidly and reproducibly measured using imaging cytometer[49]. Spheroid invasion assays can also be used as a metric to measure drug efficacy [50]. For example, lower concentrations of the adjuvant gamma-linolenic acid caused an increase in glioma spheroid invasion, but increased the apoptotic index at higher concentrations[54]. In sum, spheroids have been widely utilized to study the role of mechanisms involved in cellular invasion, and represent a valuable tool for preclinical evaluation of therapeutic agents targeting invasion[50][39][41].

### 2.3. Spheroids and Nanomedicines

Systemic drug toxicity and poor efficacy remain a major concern in cancer therapy due to the lack of selective drug delivery to tumor tissues, stressing the need to improve tumor targeting[55]. Nanomedicines have thus emerged as promising approach to (actively) target tumor and improve drug delivery. These nanostructures are biocompatible, biodegradable, non-toxic, can be prepared on a large scale, can provide controlled drug release, and enhance tissue/cell-specific targeting, in addition to reducing side effects[56][57][58][59][60]. However, despite the promising preclinical outcome that was reported for a significant number of nanotherapeutics, only few nanodrugs reached the clinic and achieved the expected results in patients [55]. Many barriers influence the efficiency of nanomedicine delivery to the target tumor, that are not recapitulated by the 2D monolayer cultures.

Tissue penetration of nanoparticles (NPs) relies on their diffusion capacity through the ECM, which varies in density and size, and is also influenced by cell–cell interactions, necrotic core, hypoxia, and by the intravascular pressure irregularities due to vessel compressions applied by growing tumors[61][62][63]. In this sense, as outlined above, spheroids have gained in popularity over traditional 2D culture systems because their pathophysiological features are close to those of the native tumors, being an excellent model to evaluate nanodrugs and to better predict their clinical outcomes [64][65][66][67].

Consequently, spheroids have been used as valuable tool to study different physico-chemical proprieties of nanocarriers such as chemical composition, size, shape and surface properties, which are crucial for their penetration and antitumor efficacy [65][68][69].

A general observation from studies that used spheroids is that nanoparticles (NPs) penetration is inversely correlated to the particle size[70][69][71][72]. NPs with small size (<100 nm) penetrate deeply and faster in the spheroids and distribute homogeneously, as compared to larger NPs (>100 nm) which remain confined to the superficial layers[70][73][74][75]. However, NPs <50 nm were reported to interact with liver cells, and to be poorly retained in the tumor[76].

The surface charge of NPs also influences their penetration in the spheroids: negatively charged NPs penetrate deeply while their positive counterparts remain at the outer layers[77][78]. Yet, more effective drug delivery is warranted by NPs with positive surface charge due to electrostatic interactions with negatively charged cell membranes. To overcome this issue, it has been proposed the use of pH-responsive negatively charged NPs that can turn to positively charged ones once in contact with acidic conditions (e.g., tumor microenvironment), so that negative surface charge ensures deep penetration in the spheroids, while positive surface charge enables more effective drug delivery[78][79].

Although little information exists on the influence of NP shape on penetration and accumulation in the spheroid, the existing literature indicates that nanorods seem to diffuse more rapidly in spheroids compared to nanospheres, and that short nanorods (400 nm in length) accumulate more rapidly and are better internalized than long nanorods (<2000 nm in length) [80][81][82].

Interestingly, NP penetration into spheroids has been enhanced by modification of the surface coating. For instance, ECM-degrading enzymes such as collagenases have been used to coat NPs of up to 100 nm in size, which demonstrated superior (4-fold increase) penetration over control NPs[74]. Drug efficacy is the most important endpoint of any formulation, and it depends greatly on the penetration and accumulation into the spheroids[69]. In general, nanocarrier formulations with high penetration and accumulation in the spheroids exhibited better antitumor activity[70].

Comparison between NP delivery and efficacy between 3D tumor spheroids and animal models revealed key similarities between the two systems. For instance, the photosensitizer verteporfin encapsulated into lipid nanocarriers strongly reduced tumor cell viability of ovarian spheroid cancer cells, and also inhibited tumor growth in an orthotopic murine ovarian cancer model, when compared to free drug[83]. Similar to in vivo tissues, HepG2 cells in 3D hydrogels were more resistant to biotin-conjugated pullulan acetate nanoparticles (Bio-PA NPs) treatments compared to the 2D system[84]. Moreover, Bio-PA NPs exhibited similar anti-tumor activity in 3D culture cells and in in vivo xenografted hepatic tumor model[84]. Studies also observed that iRGD-conjugated nanoparticles with doxorubicin were able to accumulate with more efficacy and penetrate deeply into tumor in both SH-SY5Y spheroids and H22 tumor-bearing mice, restraining tumor growth in both systems. Overall, this highlights the predictive power of spheroids for in vivo therapeutic efficacy, and their potential as promising alternative to animal models for cancer study, hopefully resolving high cost and ethical issues associated with animal use.

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