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ABOUT COVER

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Central nervous system tumors and three-dimensional cell biology: Current and future perspectives in modeling

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Abstract

Central nervous system (CNS) tumors are a variety of distinct neoplasms that present multiple challenges in terms of treatment and prognosis. Glioblastoma, the most common primary tumor in adults, is associated with poor survival and remains one of the least treatable neoplasms. These tumors are highly heterogenous and complex in their nature. Due to this complexity, traditional cell culturing techniques and methods do not provide an ideal recapitulating model for the study of these tumors' behavior *in vivo*. Two-dimensional models lack the spatial arrangement, the heterogeneity in cell types, and the microenvironment that play a large role in tumor cell behavior and response to treatment. Recently, scientists have turned towards three-dimensional culturing methods, namely spheroids and organoids, as they have been shown to recapitulate tumors in a more faithful manner to their *in vivo* counterparts. Moreover, tumor-on-a-chip systems have lately been employed in CNS tumor modeling and have shown great potential in both studying the pathophysiology and therapeutic testing. In this review, we will discuss the current available literature on *in vitro* three-dimensional culturing models in CNS tumors, in addition to presenting their advantages and current limitations. We will also elaborate on the future implications of these models and their benefit in the clinical setting.

Key Words: Central nervous system tumors; Glioblastoma; Three-dimensional modelling; Spheroids; Organoids

Core Tip: Central nervous system tumors present multiple challenges in treatment and patient prognosis. Glioblastoma, the most common adult brain tumor, remains one of the most lethal malignant brain tumors even at the current standard of care. Traditional *in vitro* culturing is limited in recapitulating the features of the *in vivo* tumors. We herein discuss recent advancements in three-dimensional culturing for tumor modeling, their advantages, and limitations, in addition to future perspectives.

INTRODUCTION

Central nervous system (CNS) tumors are distinct neoplastic entities that can either arise from different cells within the CNS (primary neoplasms) or metastasize to the brain from other body organs (secondary neoplasms). In the young population, primary brain tumors are more common than metastatic tumors, whereas metastasis accounts for more than half of those above 40 years of age[1]. The incidence rate of primary CNS tumors in adults in the United States is approximately 30 per 100000 persons[1]. Brain tumors are the most common and the most lethal tumor type among pediatric solid tumors[2]. Of all primary brain tumors, meningiomas and glial tumors (glioblastoma, astrocytoma, and oligodendroglioma among others) account for nearly two-thirds of adult intracranial tumors[3]. Glioblastoma is the most common malignant primary brain tumor in adults with a median age of 64 years[4,5]. Anaplastic astrocytoma and glioblastoma specifically have a 5-year survival rates of 30% and 5.6%, respectively[1].

Despite the advancement in surgical treatment, radiotherapy, and chemotherapy, many high-grade tumors recur and exhibit treatment failure causing high mortality among patients[6,7]. Recent development in cancer research has been revolving around the problem of therapy resistance and the emergence of recurrence in certain tumor types. Accordingly, the idea of cancer stem cells (CSCs) comes into play. Those cells are believed to arise from progenitors that reside within the tumor bulk and are responsible for tumor resistance and recurrence, adding to the complexity of cancer management[8,9]. Those subpopulations of cells are the main reason behind resistance to chemotherapy and radiotherapy. They have the ability to replenish the tumor after tumor resection and standard subsequent therapies[10]. Besides, the presence of CSCs per se might be a main reason behind tumor heterogeneity in different cancers, particularly CNS tumors[11]. Tumor heterogeneity refers to the presence of multiple subpopulations of cells with different genotypic and phenotypic profiles within the same tumor[12]. For example, in glioblastoma, CSCs have the capacity to differentiate into any of the cells that reconstitute the entire cell population that is found within the tumor, such as neurons, oligodendrocytes, and astrocytes[13].

Due to the complexity of CNS tumors, it is crucial to develop models that can recapitulate cancer development and progression and emulate better this disease process *in vitro*. While two-dimensional (2D) cell cultures have been the mainstay in cell cultures techniques for the past couple of decades, it remains challenging to use those models to decipher the *in vivo* behavior of CNS tumors[14]. This is particularly true since 2D cultures hinder the ability to study cell-cell interactions between different subpopulations of cells, as well as cell-extracellular matrix (ECM) interactions and other spatial relationships (Table 1). Moreover, testing therapeutics on models that do not recapitulate *in vivo* tumor behavior may not present the ideal reliable solution that can be applied to clinical settings. Implementing three-dimensional (3D) cell culturing techniques provides compelling evidence to perform more advanced experiments that can yield valuable insights to study human diseases[19,20]. In this review, we aim at recounting the current available literature on 3D culturing models in CNS tumors that allows us to surpass limitations of disease modeling. We also discuss

Table 1 Comparison between two-dimensional and three-dimensional tumor cell culture systems[14-18]

	2D tumor cell cultures	3D tumor cell cultures
Time required	Few days	Few weeks
Physiological relevance	Does not simulate *in vivo* tumor	Simulates *in vivo* tumor
Cell-cell and cell-ECM interactions	Low to no interactions	High level of interactions
Cell morphology	Flat cells expanding on 2D surface	Preserved *in vivo* cell shapes and growth patterns; multilayer growth
Oxygen and nutrients perfusion	Homogeneous	Heterogeneous due to the three-dimensional geometry
Response to drugs	More susceptible to drug actions	More resistant to drugs with a similar drug penetration profile to *in vivo* tumor counterparts
Gene expression	Many differences compared to *in vivo* tumor counterparts	Similar to *in vivo* tumor counterparts
Differentiation	Poor	Well differentiated cells
Cost	Cheap	Expensive
Technique difficulty	Low	High

2D: Two-dimensional; 3D: Three-dimensional; ECM: Extracellular matrix.

the advantages and limitations of different 3D modeling techniques, particularly spheroid and organoid culturing, and elaborate on the future implication of those models in the clinical setting.

3D SPHEROID MODELS

Despite the advancement in medicine and development of new therapies for brain tumors, resistance to therapy and recurrence still represent main challenges. Heterogeneity and tumor initiating cells, known as CSCs, are considered to be the main reason behind cancer initiation, progression, metastasis, resistance, and recurrence [21]. Molecular interactions within the CSC niche are highly correlated with the treatment escape strategy. Immune reactions between CSCs and cytokines, such as interleukin-6, interleukin-4, and transforming growth factor-β along with epithelial-to-mesenchymal transition[22] and other processes implicated in cancer progression represent key players in tumor resistance to conventional treatments[23,24]. Similarly, CSCs rely on several dysregulated pathways to maintain stemness and differentiation into diverse cancerous cells. Such path ways include Wnt, Notch, Hedgehog, phosphatidylinositol 3 kinase/Akt, nuclear factor kappa B, and janus kinase/signal transducer and activator of transcription pathways among others, and are believed to instigate a major effect on tumor initiation and resistance[25]. Therefore, targeting this subpopulation of cells may reduce its stemness and hence the growth, migration, and resistance of the tumor, which will enable a better control over the outcome post-treatment[21]. In gliomas, the interaction of glioma stem cells with their surrounding environment, including the ECM, the disorganization of the brain vasculature, and the hypoxic and acidic milieu in necrotic areas, all combine to maintain the growth and self-renewal of glioma stem cells[26]. Interestingly, a subpopulation of stem/progenitor-like cells in human gliomas was identified as tumor-initiating cells expressing *A2B5*, a glial progenitor marker, and *CD133*, a stem cell marker[27].

To explore further the role of CSCs and eventually develop novel therapies targeting this subpopulation of cells in brain tumors, several methods of isolating them using specific biomarkers (Table 2) have been designed. Also, the need to mimic the ECM along with other factors found within the tumor niche became a necessity. More importantly, 2D assays failed to serve as screening tools for studying brain tumor growth and progression[34], particularly with the lack in the ability to assess cellular interactions using those models[35]. Therefore, 3D culture systems, including hanging drops, spheroids, and organoids among others (Figure 1), were developed to suit better research purposes in studying patient-derived samples[36] (Table 3).

Table 2 Cancer stem cells biomarkers in brain tumors

Markers	Ref.
CD133	Gonçalves *et al*[12], Singh *et al*[13], Ogden *et al*[27], and Li *et al*[28]
A2B5	Ogden *et al*[27],
CD24	Gonçalves *et al*[12]
Aldehyde dehydrogenase (ALDH)	Gonçalves *et al*[12]
CD15	Li *et al*[28], and Son *et al*[29]
ABCG2	Li *et al*[28], Bleau *et al*[29], and Kondo *et al*[30]
Nestin	Rahman *et al*[32], and Pollard *et al*[33]
SOX2	Rahman *et al*[32], and Pollard *et al*[33]
CD44	Rahman *et al*[32], and Pollard *et al*[33]
OLIG2	Rahman *et al*[32], and Pollard *et al*[33]

Table 3 Characteristics of three-dimensional modeling technologies

Modeling technology	Methods of generation	Applications	Limitations
Spheroids[37-39]	Static suspension; Hanging drops; Spinner and rotational bioreactor; Magnetic levitation; Microfluidic system; Gel embedding (Matrigel, *etc.*)	Radioresistance through hypoxia and cell-cell contacts; Chemosensitivity and drug screening; Migration and invasion; Propagation and analysis of CSCs	Tumor heterogeneity; Immune system response; Interaction with normal non-tumor cells; Lack organ-like histology
Organoids[14, 17,37,40,41]	Culturing done on matrices (Matrigel, collagen I, hyaluronic acid, *etc.*); Addition of culture supplements including FGF, EGF, Noggin, N2, B27, *etc.*	Disease mechanism; Drug discovery and toxicology; Developmental, stem cell biology and regenerative medicine; Infectious disease	Oxygen and nutrient distribution underdeveloped; Cellular microenvironments are challenging to replicate; Imaging difficulties; Expensive and time consuming assay

CSC: Cancer stem cell; EGF: Endothelial growth factor; FGF: Fibroblast growth factor.

The increasing demand for spheroid models may be attributed to the characteristics offered by these models, such as the relatively decreased cost when compared to animal models, in addition to the resolving of ethical issues attributed to animal use [18]. Heterogeneity of spheroid models, oxygen gradient, and cellular interactions are few common characteristics found between spheroid models and normal *in vivo* conditions[18]. For such reasons, culturing cells shifted from the conventional 2D systems to the upgraded 3D models as a promising tool to simulate the growth and interaction of tissues, whether cancerous or not, under natural conditions. As such, spheroid models became widely known for their role in research as tools for anti-cancer drug screening and discovery[18,42,43].

Several cancers are hard to culture using conventional methods due to their infilt-rative and heterogenic characteristics. Therefore, recreating tumors by growing tumor spheroids from either cell lines or primary cells aims to solidify the efforts made in understanding cancer resistance[44], in addition to exploring molecular pathways contributing to tumor invasiveness, recurrence, and metastasis. Interestingly, the response of both cell lines and patient-derived tumor cells differs between 2D and 3D culture systems. Gomez-Roman *et al*[45] showed that three patient-derived glioblastoma cell lines had a similar radiosensitive result in 2D and 3D cultures. However, the effect of several drugs on the same cell lines differ on clonogenic survival in 2D *vs* 3D systems. In another study, when pediatric CNS tumor cells were cultured in neural stem cell media, generated cells expressing stem cell markers were able to undergo differentiation and when injected in immunocompromised mice and zebrafish, were able to initiate a tumor[46]. These findings suggest that 3D models represent an ideal *in vitro* culturing method to understand tumorigenic aspects of brain tumors, in addition to targeting CSCs.

Using 3D cultures has led to the discovery of several molecular pathways as novel therapeutic targets in brain tumors[19,20,47]. As such, Narayan *et al*[48] used 3D spheroid model to show that the inhibition of AKT using MK2206 reduced spheroid
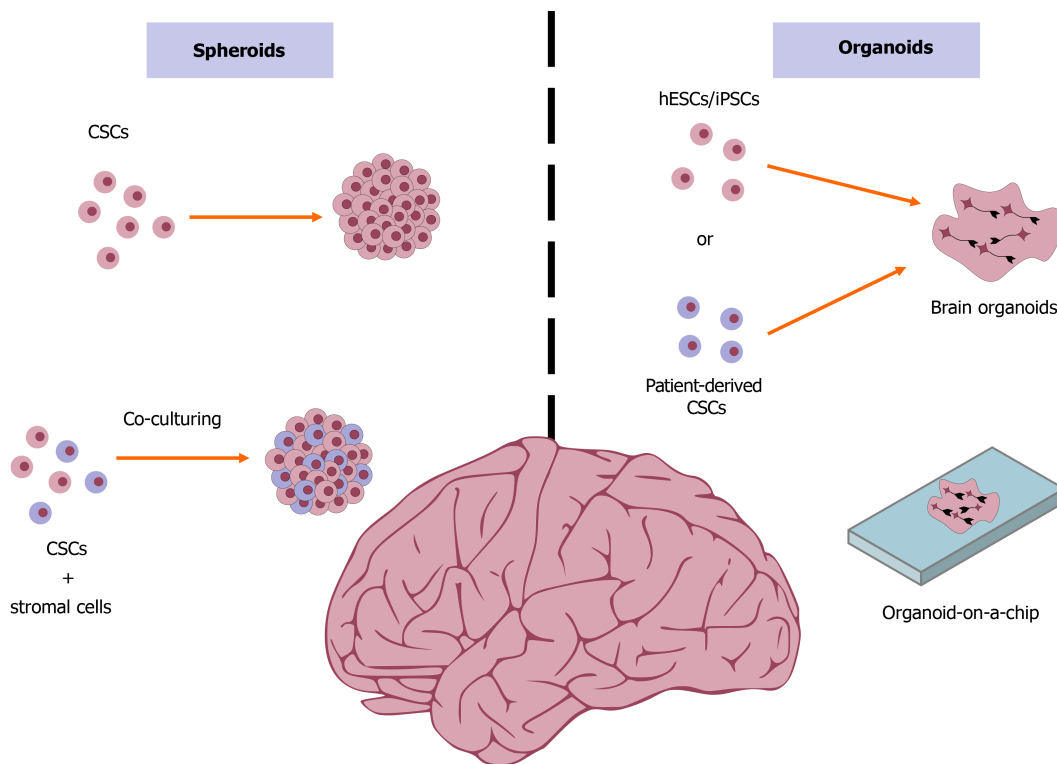
Figure 1 Schematic illustrating the implication of three-dimensional cell culturing to model central nervous system tumors. CSCs: Cancer stem cells; hESCs: Human embryonic stem cells; iPSCs: Induced pluripotent stem cells.

growth and sensitized spheroids as well to both radiation and chemotherapy (Temozolomide; TMZ), while the synergistic effect was not observed in 2D. Our group also evaluated the potential anti-tumor effect of Tideglusib, an irreversible glycogen synthase kinase-3β inhibitor drug, on human neuroblastoma cells showing that this drug significantly hindered the neurospheres formation eradicating the self-renewal ability of highly resistant CSCs[49]. We also demonstrated that Tideglusib alone and in combination with radiation significantly decreased the sphere formation of glioblastoma cell lines by targeting and reducing their CSC population[20]. On another note, since drug repurposing has recently emerged as a promising approach to target CSCs and overcome therapy resistance by identifying novel therapeutic strategies for cancer[47], we demonstrated that Metformin and Ara-a are both effective in the treatment of glioblastomas and neuroblastomas, *in vitro*, by targeting their cancer stem/progenitor cell population[47].

Glioma organotypic multicellular spheroid models were used to study lymphokine-activated killer (LAK) cell infiltration and toxicity using LAK cell therapy. Results showed that those cells caused intense cellular damage to the glioma spheroids, and hence, the model offered a solid base on which LAK cell therapy studies are based [50]. Moreover, Matrigel™, a gel-like substance used as a 3D culture matrix to recapitulate the tumor microenvironment, was used to assess the invasiveness of embedded patient-derived glioblastoma cancer cells. Results conveyed that invasion in Matrigel™ increased in rigidity-independent cell lines[51]. Moreover, the use of spheroid culturing model enabled researchers to assess the effect of protein inhibition on the outcome of sphere formation ability. For instance, lentiviral short hairpin RNA knockdown of *PAR1* in a glioma cell subpopulation expressing *A2B5+* was able to reduce sphere formation, and consequently the growth and self-renewal as well[52]. Also, novel microRNAs were identified as molecular targets for glioblastoma by assessing the migration of patient-derived spheroids in serum-free conditions, where miR-32 and miR-222 were associated with decreased migration[53]. Generally, the spheroid model is widely used in cancer research to enrich the cancer stem/progenitor cell subpopulation and to enhance drug discovery using a realistic *in vitro* model before expanding to the relatively expensive *in vivo* research. Accordingly, a chemosensitivity assay has been developed for patient-derived hepatocellular carcinoma spheroids in which a 3D co-culture system was established to enable drug screening and hence an optimized treatment for every patient[54]. Likewise, patient-derived non-small cell lung cancer cells were expanded in culture for more than 120 d

using the 3D spheroid model, in an attempt to establish a drug screening prototype [55]. Our group recently derived CSCs (as spheres in 3D cultures) from Kras-mutant lung adenocarcinoma cells and used whole-transcriptome sequencing to identify gene features differentially expressed in CSCs showing that those cells are associated with an augmented malignant phenotype including stemness, tumor-promoting inflammation, and anti-oxidant responses[56].

Although the 2D culture system was the predominant model in the previous research era, several attempts are taking place to shift from this conventional method towards a more advanced and realistic 3D model for culturing cell lines or patient-derived cells. In addition to the cell-cell interaction, cell-environment interactions and cellular heterogeneity represent an important aspect of the 3D culturing technique. Therefore, recapitulating the extracellular environment can provide better analysis of tumorigenic behavior to different treatment regimens[17]. However, even though 3D culture systems are more physiologically relevant than 2D and currently represent the transition between conventional culturing methods and *in vivo* experiments, several limitations still accompany those techniques. For instance, in spheroid formation, the distribution of oxygen, nutrients, and waste is still underdeveloped[14]. Also, cellular microenvironments are challenging to replicate in certain 3D methods, in addition to the difficulty in imaging that requires high standard and specialized microscopes, such as laser scanning confocal microscopes. Moreover, the dissociation of spheroids to single cells requires to proceed with several experiments, which represents one of the issues facing 3D cultures[17]. Last but not least, there is no unique and standard protocol for 3D culture formation, and there is a difficulty in obtaining spheres from cells extracted from certain tissues, while the most significant disadvantage remains the time consumption necessary to perform and analyze such assays[40].

3D ORGANOID MODELS

Basic science research on brain tumors is challenged mainly by the absence of reliable models that mimic human response. Organoids, which are 3D cell cultures grown *in vitro* to imitate organs, have emerged as promising tools for preclinical tumor modeling and experimentation[57] (Tables 4 and 5). Human neuroepithelial stem cells derived from induced pluripotent stem cells of patients with Gorlin syndrome were used to model medulloblastoma (MB) upon orthotopic transplantation in mice[66]. Genetically engineered cerebral organoids were used to model brain tumor formation and progression[38] and recapitulate the key aspects of malignancy in gliomas[58]. In cancerous organoids, oncogenes could be amplified using transposons, and mutations are induced in tumor suppressor genes using sequence editing techniques (CRISPR-Cas9). In a study by Bian *et al*[38], *MYC* gene amplification yielded CNS primary neuroectodermal tumor. Also, combinations of mutations associated with glioblastoma generated glioblastoma-like neoplastic cerebral organoids (neoCORs). These genetically engineered neoCORs allowed for the study of tumorigenesis and the different genetic aberrations that may be found within tumor cells[38]. In addition, neoCORs were found to emulate the structural organization of *in vivo* tumors while also containing both tumor cells and normal CNS cells.

Medulloblastoma, an aggressive brain tumor of childhood, causes the highest morbidity and mortality rates among cancer patients of the pediatric patient population[67,68]. Being a heterogeneous tumor, MB constitutes several subgroups: Wnt, Shh, Group 3, and Group 4[69]. Among the molecular subgroups, Group 3 MB known as the *MYC* group, is characterized by *c-MYC* amplification and is associated with a poor prognosis wherein approximately 50% of patients have metastatic disease at the time of diagnosis[59,69]. Differential expression of tumor oncogenes such as the overexpression of *c-MYC* with either *GFI1* overexpression or *p53* loss is shown to induce MB tumorigenicity[59]. Yet, neither patient-specific MB models nor specific therapy for Group 3 MB patients exists. Ballabio *et al*[59] showed that *OTX2/c-MYC* together constitute a novel driver gene combination required for MB tumorigenesis. In their study, human induced pluripotent stem cell-derived cancer organoids were established to model Group 3 MB. These organoids mimicked Group 3 genetic alterations as they overexpressed *GFI1/c-MYC* and *OTX2/c-MYC* gene combinations. Besides, DNA methylation signature and Group 3-specific markers analysis proved that the generated organoid-based MB model recapitulates several features of human Group 3 MB. In addition, SMARCA4, a chromatin modifier, reduced Otx2/c-MYC tumorigenesis *in vivo* and in human cerebellar organoids. On the other hand, treatment with Tazemetostat, a *EZH2*-specific inhibitor, reduced *OTX2/c-MYC* tumorigenesis in

Table 4 Organoid and organoid-on-a-chip models

Ref.	Model system	System cell origin	Tumor type	Relevant genes	Results summary
Bian *et al* [38], 2018	neoCOR	hESCs	CNS-PNET; GBM	Amplified expression of MYC; Differential expression of GBM associated gene aberrations (GBM-1, GBM-2, and GBM-3)	neoCOR used to test gain- and loss of-function mutations, singly or in combination; generation of CNS-PNET or GBM xeno-transplantable tumors
Ogawa *et al*[58], 2018	Human cerebral organoids	hESC	GBM	RAS activation and TP53 deletion	Generation of tumors in cerebral organoids using CRISPR/Cas9 technology; tumors exhibited invasive phenotype and replicated the hallmarks of tumorigenesis *in vivo*
Ballabio *et al*[59], 2020	Human cerebellar organoids	Human induced pluripotent stem cell (iPSC)	Medulloblastoma (MB) subgroup 3	Overexpression of GFI1/c-MYC (GM) and OTX2/c-MYC (OM) gene combinations	OM as a novel driver gene combination required for Group 3 MB tumorigenesis; GM and OM overexpression induces tumor formation in mouse cerebellum; SMARCA4 and Tazemetostat reduces OM tumorigenesis
Linkous *et al*[60], 2019	GLICO	hESCs; iPSCs	GBM	-	GLICO recapitulate primary human GBM with in a primitive brain microenvironment; GSCs exhibit high resistance to drug and radiation-inducedgenotoxic stress; GSCs form tumor by relocating to the human cerebral organoid, invasion and proliferation within themicroenvironment of the GLICO
Akay *et al*[61], 2018	Microfluidic chip	Patient primary human GBM multiforme specimens	GBM	-	Generation of brain cancer chip that exhibit diffusion prevention mechanism to culture GBM-patient derived 3D spheroids; treatment with TMZ and bevacizumab (Avastin, BEV) in combination enhanced GBM cell death compared to TMZ alone
Ayuso *et al*[62], 2017	Microfluidic chip	U-251 MG human GBM cell line	GBM	-	Generation of microfluidic device to behavior models that simulate blood flow through the tumor; deprivation of nutrients and oxygen induces pseudopalisade formation; pseudopalisading process renders GBM cells to become of more aggressive behavior
Cui *et al* [63], 2018	Microfluidic chip	GL261 and CT-2A mouse glioma cell lines	GBM	-	Generation of microfluidic angiogenesis model that simulate GBM tumor angiogenesis and macrophage-associated immunosuppression within GBM tumor microenvironment; GL261 and CT-2A GBM-like tumors promote angiogenesis through driving M2-like macrophage polarization; TGF-b1, and surface integrin (avb3) endothelial-macrophage interactions regulates inflammation-mediated angiogenesis through Src-PI3K-YAP signaling; inhibition of integrin (avb3) and cytokine receptor (TGFb-R1) repress GBM tumor neovascularization
Lin *et al* [64], 2018	Microfluidic chip	Patient derived GSCs	GBM	-	Generation of glioma perivascular niches on a chip; Perivascular niches maintain the pluripotent state of GSCs; Stronger chemoresistance of GSCs against TMZ associates with endothelial cell co-culturing, GSCs neurosphere formation and the expression of 6-O-methylguanine and Bmi-1 gene
Yi *et al* [65], 2019	Bio-printed chip	Patient primary human GBM specimens	GBM	-	Generation of complex cancerous-tissue constructs constituting brain ECM composition, oxygen gradient-generating system, cancer-stroma structure; exhibited patient-specific response upon the treatment with drug combinations, chemoradiation and TMZ

2-D: Two dimensional; 3-D: Three dimensional; CNS-PNET: Central nervous system primitive neuroectodermal; CSC: Cancer stem cell; GBM: Glioblastoma; GLICO: Cerebral organoid glioma; GSCs: Glioma stem cells; hESC: Human embryonic stem cells; NeoCOR: Neoplastic cerebral organoid; PI3K: Phosphatidylinositol 3 kinase; PNET: Primary neuroectodermal tumor; TGF: Transforming growth factor; TMZ: Temozolomide.

ex vivo cultures and the established human cerebellar organoids[59]. Therefore, human organoids-based models might truly represent useful tools to investigate the molecular mechanisms underlying cancer development, drug screening, and therapy.

Among the cell types essential for tumor pathogenesis are the endothelial cells. In glioblastoma, for instance, the tumor favors tissue invasion along with existing vasculature[70]. However, their absence limits glioma natural history reconstruction in organoids. Co-culturing with endothelial and mesenchymal progenitors would generate more reliable cerebral organoids[71,72]. Similarly, co-culturing with hematopoietic progenitors give rise to microglial cells that mediate brain inflammatory and injury responses[73,74]. In addition to the missing cell types, variability in the organoid generation is another limitation in human cerebral organoids. Brain

Table 5 Features and characteristics comparison between spheroids and organoids

	Spheroids	Organoids
Cells used	Cell lines or CSCs	Embryonic stem cells, induced pluripotent stem cells or CSCs
Physiologic relevance	Lower	Higher
Tumor heterogeneity	Lower	Higher
Technique difficulty	Lower	Higher
Cost	Lower	Higher
Time	Weeks	1-3 mo
Genetic manipulation	Moderately available	Moderately available
Biobanks	Not available (cells are difficult to maintain long-term)	Available
Advantages	Cost effective; Highly accessible; Good for high throughput drug testing	Retains tumor heterogeneity; Better simulation of the physiological environment
Disadvantages	Hard to maintain long-term; Not as representative of the physiologic environment	More complex; Higher failure rate; May give variable results

CSC: Cancer stem cell.

organoids contain a subpopulation of non-CNS differentiated tissues and do not differentiate completely except for the deepest layers of the human cerebral cortex[58, 75]. However, despite these existing limitations, tumors derived from cerebral organoids are like those generated in mice, transplantable like patient-derived tumors, and support both the proliferation and invasive behaviors upon using patient-derived primary tumor explants as well as established cancer cell lines[58]. Theoretically, to address host-tumor cell interaction in 2D culture, it is possible to co-culture brain cells with patient-derived glioma stem cells. Unfortunately, the resulting disordered mixture of normal cells would lack human ECM and would not represent the carefully organized arrangement of cells in the human brain[60]. However, human glioblastoma can be modeled in a primitive brain microenvironment using human embryonic stem cell-derived cerebral organoids and patient-derived glioblastoma stem cells. By growing patient-derived glioblastomas within the cerebral organoids, Linkous *et al*[60] established a clinically relevant cerebral organoid glioma GLICO model. In addition to being scalable for high-throughput drug screening, their *ex vivo* model closely phenocopies surgical and autopsy specimens. In addition to exhibiting high resistance to drug and radiation-induced genotoxic stress, glioma stem cells were reported to relocate to the human cerebral organoid where they invade and proliferate to form tumors that highly recapitulate patient glioblastomas[60].

On another note, one of the most recent advancements in CNS tumor modeling are the tumor-on-a-chip systems, which have been recently employed in glioblastoma (Table 4). Akay *et al*[61] developed a brain cancer chip able to culture primary tumor-derived human glioblastoma cells as 3D spheroids. In addition, this chip had a multichannel system that allows for targeted drug delivery, which was upgraded from their previous model published back in 2016[61,76]. The authors hope that this new technology would provide clinical benefits to oncologists as a means of studying patient-specific drug responses, in addition to playing a crucial role in improving the current drug testing framework. This system showed a lot of potential; however, it harbors some limitations, as most spheroid systems do, due to the lack of the complete tumor microenvironment, which is known to influence glioblastoma's drug sensitivity and general behavior[26,63,77,78].

In addition, microfluidic chip models were already being investigated as a potential biomimetic model for glioblastoma. Ayuso *et al*[62] developed a microfluidic chip that allowed them to simulate blood flow through the tumor in addition to vessel obstructing events that have been associated with glioblastoma. At around the same time, Cui *et al*[63] published findings on their microfluidic angiogenesis model chip, which allowed them to reconstitute organotypic glioblastoma models in a vascularized microenvironment that simulated immune and vascular conditions. This model

allowed them to study the effects of immune cells and inflammation on the angiogenesis and tumor growth, in addition to possible therapeutic targets. Other researchers were also able to use microfluidic chips to study glioblastoma and the perivascular CSC niches as an important factor in tumor chemoresistance[64]. More recently, Yi *et al*[65] developed a bio-printed glioblastoma-on-a-chip model using patient-derived tumor cells co-cultured with vascular endothelial cells and a decellularized ECM from a porcine brain all compartmentalized in a way to simulate the complex tumor microenvironment. This compartmentalization helps create an oxygen gradient that recreates what is seen *in vivo* in glioblastoma with central hypoxia. Their model was shown to reproduce successfully the structural, biochemical, and biophysical qualities of the original *in vivo* tumor. The model's strength comes with its ability to recreate a tumor with the same chemotherapy and radiotherapy resistance and susceptibility profile as its *in vivo* counterpart. These advancements have made the tumor-on-a-chip model, a fertile field for future studies in personalized medicine and the determination of the optimal treatment regimen for each patient. It may even be possible, soon, to conduct point-of-care testing using this system as it can be set up within 2 wk[65]. While more advanced than other 3D models that do not employ co-culturing, the glioblastoma-on-a-chip still lacks the immune cell component, which is an important aspect of cancer development and progression[79].

Organoids, while still a relatively new field, especially in neuro-oncology, have already proven extremely useful in the understanding of tumor pathophysiology, in addition to their practical clinical uses in testing therapeutics. Having provided a close simulation of the tumor microenvironment, organoids have allowed for the filling of many of the missing knowledge gaps in understanding tumor invasion[58,60,79,80], the role of the immune and vascular systems[10,63,81], the importance of intra-tumor oxygen gradients[77,82], and glioblastoma CSC behavior and role[10,60,83]. In addition, as organoids are becoming better models to recapitulate *in vivo* tumors, therapeutic testing may now provide more reliable and applicable results for clinical settings. They have also taken us a step further towards the use of patient-derived tumors for drug repurposing and drug combination testing to help in the clinical decisions pertaining to drug regimens, saving patients' time and money that could have been wasted on ineffective treatments[38,59,61,80].

CLINICAL IMPLICATIONS OF 3D SPHEROID AND ORGANOID MODELS

Going from 2D to 3D culture allowed for better understanding of the pathology and the physiology of brain tumors[47]. Tiburcio *et al*[84] proved that it is better to study the effect of hemizygous/heterozygous *IDH1R132H* in 3D cultures, where they proved that in 3D culture, heterozygous cells for the *IDH1R132H* mutation had low malignancy whereas the hemizygous cells fell into a higher malignancy cluster. In contrast, among 2D cultures, both types were presented to have the same malignancy. In addition, the mesenchymal gene set in glioblastoma cells appears to be enhanced only in the 3D models, which is consistent with the fact that 3D cultures are more fit models to study glioma biology.

Moreover, brain tumors like glioblastoma can remodel their microenvironment and their ECM through interactions with basement membrane proteins in order to gain drug resistance against TMZ and increase their survival in its presence. Musah-Eroje and Watson[77] demonstrated that 3D models and assays could be used to characterize brain tumor cells and identify the pathways through which such cells gain their drug chemo-protection and metastasis abilities. However, it is not only the interaction with the microenvironment that makes tumor cells resistant to TMZ but also their interaction with nearby endothelial cells. Towards proving such correlation, a 3D model was used by Lin *et al*[64], where neurospheres formed from glioblastoma CSCs co-cultured with endothelial cells appeared to be more resistant than those not cultured with endothelial cells. A similar study was done by Civita *et al*[85] emphasized the role of astrocyte interaction with glioblastoma cells through the formation of nano tunnels that allows the exchange of undamaged mitochondria and other cellular contents with tumor cells under stress, which in turn promotes chemoresistance against anticancer drugs, including TMZ, vincristine, and clomipramine.

When it comes to establishing treatment protocols from a 2D model, heterogeneity and diversity between patients are some of the biggest challenges. Trying to solve such problem, Skaga *et al*[10] proved that patient-derived glioblastoma tumor spheres maintain the traits and characteristics of the parental tumor, which makes it a good

model to test for patient-specific responses to drugs or to test the sensitivity of patient to a certain drug. However, such models do not account for the interaction between tumor cells and normal cells in the same culture. On the other hand, neoCORs created from induced pluripotent stem cells, have a more organ-like histology since they take into consideration such interactions between transformed and non-transformed cells [38]. In these models, it is possible to test for antitumor drug effect while running a safety test in the same system. In addition, neoCORs could be used to test the vulnerability of subjects to various combinations of driver mutations and improve the process of screening for efficient targeted therapy like immuno- and gene-therapy[38, 86]. Furthermore, Malatesta *et al*[87] used organoids, composed of tumor cells cultured with astrocytes and normal neurons, as a model to prove that the knockdown of the primate-specific long noncoding RNA, *GTT1*, inhibits the growth of tumor cells without impacting normal cells and thus not affecting the organoids' viability, making it a possible glioma-specific therapeutic target. However, most organoid models are limited by their lack of vasculature, and thus the study conducted may not account for glomeruloid microvascular proliferations and perivascular palisading necrosis[38]. Cui *et al*[63] avoided such limitations by producing a more complex 3D angiogenesis model that included immuno-vascular, cell-cell, and cell-matrix interactions with controllable immunosuppressive conditions. In their study, the authors emphasized the roles of macrophages in immunosuppression, in addition to the role of endothelial-macrophage and cell-ECM interactions in controlling malignant angiogenesis in glioblastoma tumors. Furthermore, a new 3D model was established mimicking the *in vivo* microenvironment of glioblastoma tumors, focusing on their potential in screening for novel therapeutics[63].

Brain cancer chips are another form of 3D modeling that have proven to be very effective when it comes to administrating multiple drugs simultaneously and testing their responses on patient-derived glioblastoma cells. In addition, they could be used to do high-throughput drug screening without any technician input, which renders it an efficient method to produce and generate personalized treatment plans in the near future[61,76].

CONCLUSION

In conclusion, recent advancements in cell culturing techniques have led to a trending gravitation away from 2D culturing models in favor of 3D models such as spheroids and organoids. While these new models have historically been more expensive, harder to maintain, and require more specialized skills and equipment[88], such limitations are becoming less relevant as these techniques are becoming more popular and mainstream. Tumor modeling is now a highly evolving field, and the development of 3D culturing techniques has opened the doors to fast-paced developments making this field one of the most dynamic currently in cellular biology. From the first reports of spheroid cell cultures in the 1980s to the recent breakthroughs in genetically-engineered brain organoids and patient-derived models, current tumor modeling techniques have brought us closer than ever to the era of personalized medicine and point-of-care tumor models for therapeutics testing. The benefit of 3D modeling and cell culturing does not stop only at oncological studies but has also been already used and paved the way for breakthroughs in other fields such as regenerative medicine[89, 90]. It remains to be seen whether the benefits of 3D models could be harnessed in clinical scenarios soon; however, with the current pace of breakthroughs and new developments, this future may not be as far off as one might believe.

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