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Review

Organotypic Models to Study Human Glioblastoma: Studying the Beast in Its Ecosystem

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SUMMARY

Glioblastoma is a very aggressive primary brain tumor in adults, with very low survival rates and no curative treatments. The high failure rate of drug development for this cancer is linked to the high-cost, time-consuming, and inefficient models used to study the disease. Advances in stem cell and *in vitro* cultures technologies are promising, however, and here we present the advantages and limitations of available organotypic culture models and discuss their possible applications for studying glioblastoma.

INTRODUCTION

Glioblastoma (GB) is the most aggressive primary brain tumor in adults (47.7% of cases) (Ostrom et al., 2019; Adamson et al., 2009). Unfortunately, there are no curative treatments for this disease and, despite intensive research, patient survival is normally less than 2 years (Stupp et al., 2017). Cancer drug development has an extremely high failure rate, and less than 10% of the drugs tested in patients reach the market (Waring et al., 2015; Lenz and Stintzing, 2014; Wen et al., 2014; Maeda and Khatami, 2018). This problem is particularly evident in GB, given that only moderate therapy improvements have emerged in the last 20 years (Stupp et al., 2017). One hallmark of GB is its ability to shape the cellular and extracellular environment in a dynamic process over the course of tumor development. Studies have shown that tumors closely interact with their surrounding microenvironment (neighboring cells) (Quail and Joyce, 2017; Jung et al., 2019; Wei et al., 2019). These interactions and modifications of the tumor microenvironment are considered potentially druggable by targeted means and/or chemotherapies. Testing new candidates is very costly and time-consuming (Moffat et al., 2014, 2017; Dimasi et al., 2016; Kondo and Inoue, 2019). The cost per drug is estimated at $1.2–1.3 billion (Dimasi et al., 2016), and, for biologicals (i.e., human antibodies and proteins), it takes approximately 8 years to complete the overall process (Dimasi et al., 2010). For small molecules, the development time is around 12 years, and more recent cost estimates are $2.4 billion and above (Meigs et al., 2018). About 95% of potential anticancer drugs entering clinical development fail, compared with an average of 90% of compounds in all therapeutic areas (Kola and Landis, 2004). Recent assessments found only a 3.4% overall success rate in clinical trials (Wong et al., 2019). The lack of correlation between animal models and classical *in vitro* models of human diseases indicates that suboptimal models are being used to study human pathophysiology and contribute to the high failure rate in drug development (Akhtar, 2015; van der Worp et al., 2010; Hartung, 2013). A strong selection bias in the choice of therapeutic targets, often driven by presence in rodent genomes and the resultant widespread annotation in databases, contributes to the lack of translation (Maertens et al., 2020). Undeniably, animal studies, reviewed elsewhere (Kijima and Kanemura, 2017; Miyai et al., 2017; Patrizii et al., 2018; Hackam and Redelmeier, 2006; Shanks et al., 2009), have brought valuable results and advances in the field (e.g., the shift from mouse genetic models to patient-derived xenograft [PDX]). Species differences between rodents and humans, however, limit the predictive value of mechanistic studies for drug screening.

New approaches, such as *in vitro* models relying on molecular pathways of human toxicity, have been proposed to overcome drug development inefficiencies (Pamies and Hartung, 2017; Hartung, 2013; Hartung et al., 2017). Due to the limited availability of human tissue, especially from the brain, reliable human *in vitro* models are of utmost importance. The simplicity of *in vitro* models makes them cheap and fast and rarely raises ethical concerns. They allow the study of specific molecular mechanisms that would be difficult to examine in a full organism. Scientists have employed clonally derived 2D monolayer cultures from patients' tumors, such A-172, U-87, U-373, LN-229, and U-251, for more than 50 years (Westphal and Meissner, 1998; Mark et al., 1977; Westermark et al., 1981; Bigner et al., 1981). However, it is difficult

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to determine the clinical relevance of these studies, as GB lines tend to accumulate mutations over time and lose the similarity to actual GB tumor cells (Patrizii et al., 2018). GB shapes the cellular and extracellular microenvironment by close interaction with neighboring cells (Diao et al., 2019; Xiao et al., 2017), creating a particular niche that regulates metabolic needs, immune surveillance, survival, invasion, as well as cancer stem cell (CSC) maintenance (Hambardzumyan and Bergers, 2015). Despite the interactions between microglia and GB being the most studied (Roesch et al., 2018; Matias et al., 2018; Gutmann and Kettenmann, 2019), other cells in the microenvironment have shown to play an important role in tumor progression (Neftel et al., 2019). For example, GB cells engage in synaptic communication with the surrounding neurons, increasing neuronal excitability and favoring tumor progression (Venkatesh et al., 2019; Neftel et al., 2019). 2D GB cultures and traditional tumor organoids are not optimal models for discovering drugs that target these interactions between GB and brain cells, as they do not mimic the tumor microenvironment. Bioengineering has advanced modeling of the tumor microenvironment, but most models do not reflect healthy tissue responses (Wolf et al., 2019).

More complex models that better mimic human brain physiology have been developed in the last decade (Pamies and Hartung, 2017). GB spheroids, as an alternative to traditional cell culture methods, have been used extensively in recent years (Avci et al., 2015a, 2015b; Fan et al., 2015; Nguyen et al., 2016). These models better reproduce many functional and structural properties of solid tumors *in vivo* (Li and Kumacheva, 2018) due to the cell-cell interactions, facilitated by the 3D structure, between the various cell types composing the GB tumors (Alepee et al., 2014). GB spheroids, which allow the use of high-throughput readouts, have been successfully utilized for drug screening (Ferreira et al., 2018). Nevertheless, patient-derived GB spheroids do not model the tumor microenvironment.

The recent development of patient-derived GB organoid cultures (GBO) now mimics tumor heterogeneity. In this case, GB tumors are grown using brain organoid (cerebral organoids) culture methods (Hubert et al., 2016) instead of regular spheroids. Beginning with tumor-initiating CSCs, this method allows us to imitate the heterogeneity in the state of cellular differentiation and the repartition of oxygen and nutrients found in GB tumors (Hubert et al., 2016). Thus, this model better recapitulates inter- and intra-tumoral heterogeneity and maintains many key features of GBs, and, to some extent, the tumor structure itself (Jacob et al., 2020). GBOs are an excellent tool for drug screening and can be rapidly deployed to investigate patient-specific treatment strategies (Jacob et al., 2020). However, because of the lack of surrounding healthy tissue, interactions between tumor and its healthy tissue environment is not possible. GBO models have been reviewed elsewhere (Jin et al., 2020).

Advances in stem cell technologies —including microfabrication, perfusion, and 3D cultures (among others)— have led to new *in vitro* models in GB research and have been reviewed elsewhere (Caragher et al., 2019; Xiao et al., 2017; Robertson et al., 2019). These models have become novel tools in very different scientific areas (Kaushik et al., 2018; Rossi et al., 2018; Bar-Ephraim et al., 2019). Organotypic models (also called 3D models or organoids), in particular, have shown great promise. In this review, we will summarize the various 3D human organotypical models that have been used to study GB, together with some of their advantages and limitations.

We define GB-brain organotypic cultures (GB-3DBrain) as 3D, human stem cell-derived models that introduce a GB tumor via genetic editing or by co-culture with GB cells. Microfluidic organotypic cultures will not be included in this review. To the best of our knowledge, only six articles have been published on this topic (Nayernia et al., 2013; Ogawa et al., 2018; Bian et al., 2018a, 2018b; Plummer et al., 2019; Linkous et al., 2019; Krieger et al., 2020).

ORGANOTYPIC CULTURE MODELS

The terms *organoid* or *organotypic culture* need to be carefully considered, as both GBOs and new central nervous system (CNS) stem cell-derived 3D models share the same terminology. The first team to generate GB-3DBrain was Preynat-Seauve and collaborators (2013). They introduced GB cells into their human ESC-derived organoid—one of the first human brain organoids to be reported (Preynat-Seauve et al., 2009; Nayernia et al., 2013). The protocol for this GB-3D brain model was also recently published in detail (Cosset et al., 2019). The authors were interested in studying nervous tissue/tumor interaction. By using microarray technologies, they found that type 1 interferon response and extracellular matrix-related genes regulation were significantly correlated with patient survival (Nayernia et al., 2013). However, this article and its novel

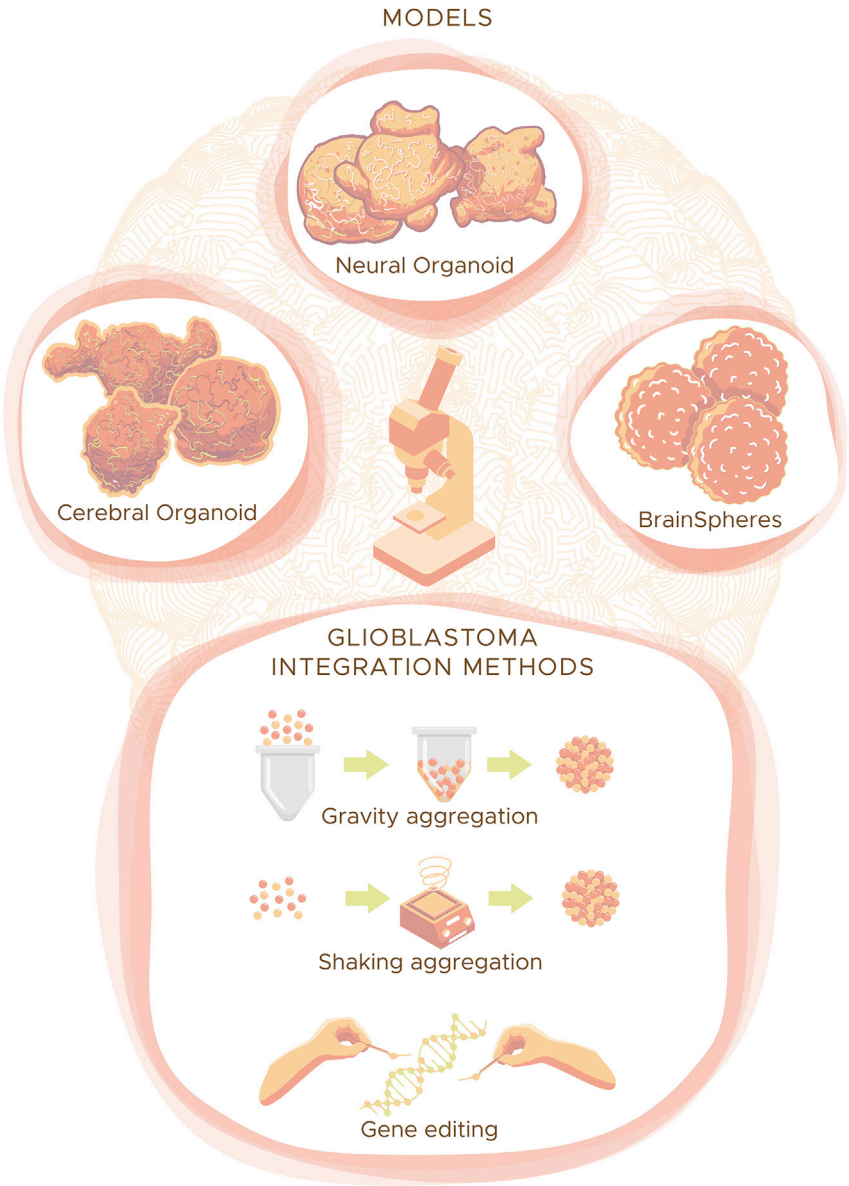

Figure 1. Organotypic Models Used to Generate GB-3DBrain and Methods to Introduce Glioblastoma Cells

cell culture approach went largely unnoticed and unrecognized—until this publication, it received only 15 citations. It was not until 2018 that other research followed the same approach.

The majority of human stem cell organotypic cultures used to generate GB-3DBrain models are the so-called *cerebral organoids* (Lancaster et al., 2013), either by using the exact protocol (Bian et al., 2018a; Ogawa et al., 2018) or by adding minor adaptations (Krieger et al., 2020; Linkous et al., 2019). The exception were Nayemia and collaborators, who used a different type of organotypic culture (the neural organoid) (Nayernia et al., 2013), and Plummer and collaborators, who utilized a model called BrainSpheres (Plummer et al., 2019) (Figure 1).

Lancaster and collaborators' cerebral organoids are 3D models derived from induced pluripotent stem cell (iPSC) by a 21-step protocol, using techniques such as aggregation, Matrigel droplets formation, and spinning technologies (Lancaster and Knoblich, 2014) (Figure 2). These 3D brain tissues mimic endogenous

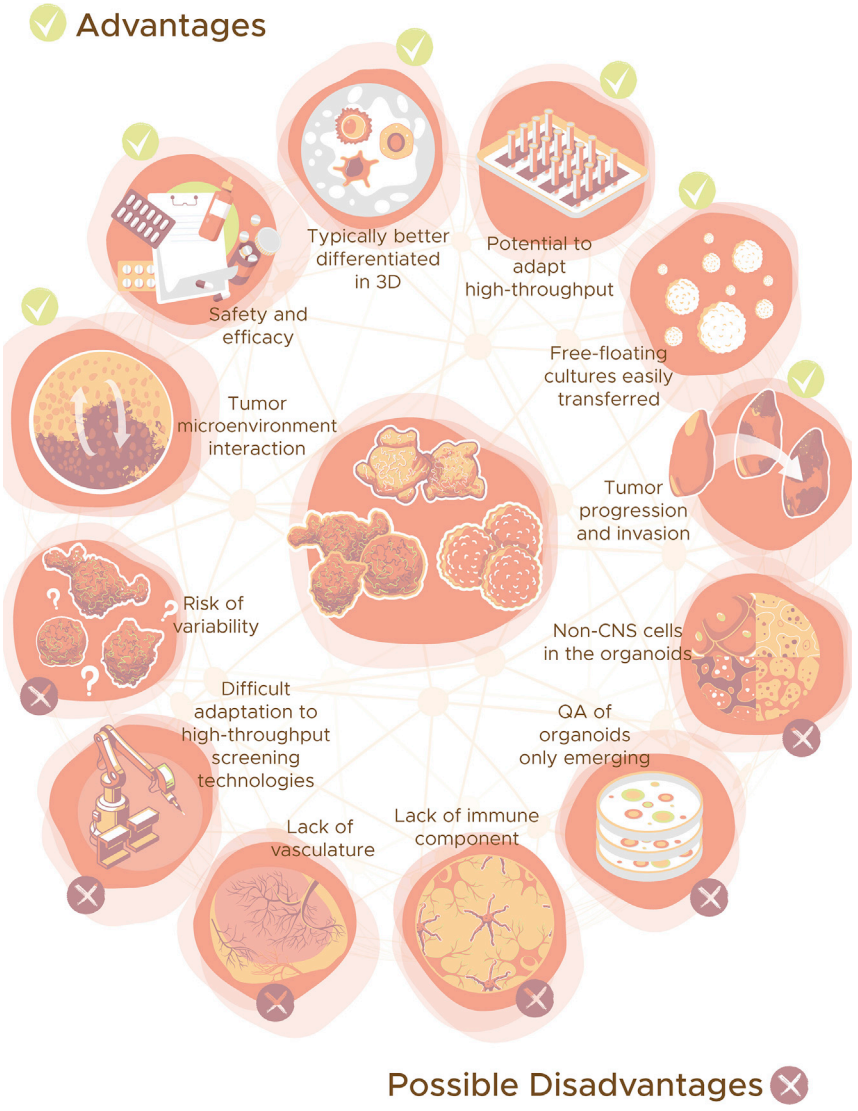

Figure 2. Summary of Advantages and Possible Disadvantages of GB-3DBrain Models

brain development, with the ability to differentiate into parts resembling cerebral cortex, ventral telencephalon, choroid plexus, and retinal identities, among others, within 1–2 months (Lancaster and Knoblich, 2014). Some authors made slight changes to adapt the model to the incorporation of the tumor cells; for instance, the developmental stages when GB are introduced vary from 11 days to 4 months (Bian et al., 2018a; Ogawa et al., 2018).

In the model presented by Nayemia (human embryonic stem cell-derived neural organoids) (Lancaster and Knoblich, 2014; Cosset et al., 2019) (Figure 2), similar approaches added the GB cells at 3 weeks of differentiation. In this type of organoid, structural organization and distribution of neural cell markers in germinal layers/mature tissue (similar to the human fetal brain) (Preynat-Seauve et al., 2009) are also observed.

Plummer and collaborators used a BrainSphere model (Pamies et al., 2017c) (Figure 1). Instead of large organoids with complex structural organization, the authors used a smaller 3D culture (300–350 μm) (Table 1). The rationale for developing this model was to obtain a reliably reproducible *in vitro* model (Pamies et al., 2017c), without necrotic centers that could generate artifacts during drug screening and testing, as well as easily adaptable to high-throughput technologies. The model was shown to produce reasonable

Author	Model name	Type of 3D culture	Days in culture	Size	Cells	Method used to incorporate GB	N* of PSC lines tested	N* of gliobastoma lines	Compounds tested	Endpoint type	High-throughput
Nayernia et al., 2013	NA	Human neural organoid	39	6 mm	hESCs (H1)	Gravity	1	?	None	Immunofluorescence Genomics	NA
Ogawa et al., 2018	NA	Cerebral organoid	Up to 91	> 3 mm	hESCs (H9)	Gene- editing (CRISPR/ Cas9) and Gravity	1	NA	None	Immunofluorescence Genomics	NA
Bian et al., 2018b	Neoplastic cerebral organoid (neoCORs)	Cerebral organoid	> 40	1 - 4 mm	hESCs (H9)	Gene-editing (Sleeping Beauty transposase)	1	NA	5 (afatinib, erlotinib, gefitinib, canertibib and pelitinib)	Images and FACS analysis	NA
Plummer et al., 2019	Glioblastoma BrainSphere (gBS)	BrainSpheres	28 – 56	350 µm	hiPSC	Aggregation	1	1	2 (temazolamide and doxorubicin)	Image analysis	Yes
Linkous et al., 2019	Cerebral organoid glioma (GLICO)	Cerebral organoid	> 28	2 mm	hiPSC (H6) and hESCs (H1 and H9)	Gravity	3	6	2 (temozolomide and bis-chloroethylnitrosourea	Image analysis	NA
Krieger et al., 2020	NA	Cerebral organoid	32	500–900 µm	hIPSC (409b2)	Gravity	1	4	None	Immunofluorescence Genomics	NA

Table 1. Summary of GB-3DBrain models

maturation (e.g., approximately 40% myelination and spontaneous electrical activity). Structural organization into brain regions, however, could not be found.

INTRODUCING GLIOBLASTOMA CELLS INTO 3D MODELS

Mouse models can be categorized according to GB cell source. Understanding them can help us to grasp possible advantages and limitations of GB-3DBrain model because they are very similar in some cases. Mouse models for GB can be classified into two categories: genetically engineered and xenograft models (Kijima and Kanemura, 2017). In genetically engineered mouse models, the GB tumor is induced by mutations. One advantage of these models is the ability to study a specific mutation. The complex genetic heterogeneity of the GB tumor, however, is lacking. Xenografts, on the other hand, represent better tumor microenvironments, but complicate the model, making it more difficult to reproduce and draw conclusions. Xenograft models can be separated into two types, depending on whether they are cell-line- or patient-derived. Cell-line xenografts are easy to maintain in culture, although some evidence indicates that the lines can present different mRNA expression and epigenomic levels (Shen et al., 2019) and their limited genetic stability could generate artifacts (Stoczynska-Fidelus et al., 2014). Patient-derived xenografts better reflect the true biological nature of GB and retain the heterogeneous histological and genetic features of human GBs (Zeng et al., 2020). Obtaining these cells, however, is difficult. In addition, GB mouse models can be orthotopic (the GB tumor is incorporated into the relevant organ of tumor origin) or heterotopic (when the tumor is engrafted at another location—for example, under the skin, such as in the standard subcutaneous models), which is easily monitored. It is obvious that heterotopic models do not represent the complexity of the tumor microenvironment that plays such a key role in GB progression.

Two different approaches have been used to introduce GB into 3D cell models (Table 1). GB cells (directly from biopsies or patient-derived glioma cells) are added into the 3D structures. This primarily utilizes gravity approaches (Linkous et al., 2019; Krieger et al., 2020; Nayernia et al., 2013; Ogawa et al., 2018), allowing GB to attach cells to the surface of the organoid (Table 1). This could initially be viewed as an unfavorable approach, because contact between the GB with microenvironment cells will not be ideal. However, Krieger and collaborators, by using a combination of live stained (or fixed) cerebral organoids embedded in Matrigel, together with clearing tissue technique and confocal imaging, were able to show tumor invasion after only 2 days of culture (Krieger et al., 2020). Tumoral cells added to the surface of the cerebral organoids were able to invade deeply into the tissue, exceeding 100 μm in the majority of organoids, with some cells detected at 300 μm from the organoid surface. The authors also observed differences in invasiveness (Krieger et al., 2020), depending on the line used. The developmental stage chosen for incorporating cells into cerebral organoids varies between models (see Table 1). Plummer and collaborators, for example, added the GB cells to single-cell neural progenitor cells (NPCs) at the moment of the aggregation to form the 3D structure that will be further differentiated with the GB (Plummer et al., 2019) (Table 1). In this way, GB cells can be seen in the center of the 3D model from the beginning, facilitating the interaction of tumor cells with the microenvironment.

The other primary approach to generating a tumor in a 3D model is based on genome-editing techniques used in two of the published models (Table 1). By inducing specific mutations into the cerebral organoids, different authors have been able to produce a GB tumor (Bian et al., 2018b; Ogawa et al., 2018) without modification of the cerebral organoid protocol and without the need to introduce tumor cells into the organoids by gravity or other techniques. These methods allow analysis of gain- and loss-of-function mutations, individually or in combination (Bian et al., 2018b; Ogawa et al., 2018). Bian and collaborators, for example, induced 18 single-gene mutations or amplifications (using 15 of the most common clinically relevant combinations observed in brain tumors) into the organoids. Interestingly, they were able to generate GB-3DBrain models (called neoCORs by the authors) that over-express the oncogene MYC, producing a tumor with similar histopathological features, cellular identities, and transcriptome signatures as those described in patients (Bian et al., 2018a, 2018b). For this type of tumor, no adequate animal or *in vitro* model existed until the publication of this study (Bian et al., 2018b). However, the induction of tumors by mutations presents problems similar to those observed with GB in genetically engineered mouse models, i.e., tumors composed of cells with specific homogeneous genetic changes (induced by specific mutation), not completely representative of the high genomic and phenotypic GB heterogeneity (Kijima and Kanemura, 2017).

Depending on the aim of the study, all three techniques can be valuable and useful—allowing the study of specific tumors from patients or lines of interest (gravity and aggregation) on the one hand and of genes

involved in specific tumors (gene-editing techniques) on the other. Regardless of the chosen technique, the GB-3D brain model is always orthotopic.

MIMICKING THE TUMOR MICROENVIRONMENT

Brain organoids, despite being more complex and similar to human physiology, are still imperfect models for studying the GB microenvironment. For example, the maturation stage of the cells constitutes a limitation, because *in vitro* cultures are generally considered developmental models and GBM's median age of diagnosis is in the seventh decade of life. Nevertheless, the various 3D models do present different stages of maturation. Cerebral organoids, for example, recreate the spatial organization of diverse CNS structures, but cells do not reach a high level of cell maturation (Lancaster et al., 2013). BrainSpheres do not present spatial organization, but exhibit highly mature cells, such as myelinating oligodendrocytes, whereas stem cell markers vanish (Pamies et al., 2017c). Although they do not fully attain mature brain tissue, these models are clearly closer to human physiology than traditional 2D *in vitro* cultures, and numerous improvements on the differentiation protocols are reported each year. Maturation is also desirable in the brain organoid to mimic the microenvironment; the level of maturation of the GBM cells is questionable, however, with no current consensus.

Another critical factor in mimicking the tumor microenvironment is the cell type composition of the human organoids. Recent studies have shown that macrophages and oligodendrocytes are the predominant non-neoplastic cells within the tumor (Neftel et al., 2019). It is important, therefore, to pay attention to the presence of these cells, which are usually absent from *in vitro* models. Nevertheless, somo models present myelinating oligodendrocytes, and macrophages can be incorporated (Abud et al., 2017; Abreu et al., 2018; Nzou et al., 2018; Muffat et al., 2018). Alternatively, by modifying the differentiation media, it is possible (to a certain extent) to adjust the cell phenotypes present in the 3D structure (Xiang et al., 2019; Nickels et al., 2020).

ADVANTAGES AND DISADVANTAGES OF 3D MODELS FOR THE STUDY OF GLIOBLASTOMA

Complexity brings potentially better models of human physiology and pathology, but it can also bring disadvantages and limitations. Complexity may increase the price of the cell system, reduce reproducibility, and make the experimental design difficult, but it also has major advantages (Figure 2):

- Healthy cells are also present in the model, so drug efficacy and drug safety can be tested concomitantly, enabling determination of adverse effects of drugs on non-tumor cells.
- Some models can be studied in a high-throughput manner and other models could be adapted to do so.
- Cells are typically more effectively matured in 3D, although no *in vitro* models fully represent human tissue maturity.
- Because tumor cells interact with the microenvironment, tumor cell-healthy cell interactions can be studied and potentially used to find new treatment targets.
- Progression and infiltration of the tumor into healthy tissue can now be studied.
- The models represent free-floating organoids under gyratory shaking, which can be transferred to new culture plates; thus, wash-out experiments for candidate drugs (mimicking pharmacokinetics) can be tested, whereas in other models, drugs will stick to cell culture plastic and can redistribute later.
- Ideally, healthy tissue and tumor would originate from the same patient. However, since derivation of an iPSC line and optimization of organoid formation from a given iPSC line takes up to 6 months each, this is very expensive and not practically feasible. As the brain tissue is immunoprivileged (i.e., free of immune cells other than microglia), the brain organoids are devoid of immune cells if no microglia are added (as in Abreu et al., 2018). Macrophage-like cells, such as microglia, do not sense a mismatch between the graft tumor and stem-cell donor cells. As any donor's iPSC-derived healthy brain tissue may be readily available when patient material arrives (under the form of NPCs or even organoids), this opens up the possibilities for personalized therapy approaches.

The limitations depend on the model used:

- Due to their complexity, the majority of these models require sophisticated and costly protocols.
- Some models present high intra-organoid neuroanatomical and cell composition variability (Lancaster and Knoblich, 2014), making their application for drug screening difficult. As GBs interact strongly with their microenvironment, variation in the proportions of the diverse cell phenotypes from batch to batch may affect these interactions and, by consequence, could affect the study's readouts. Reproducible models, therefore, are desirable.
- The large size of some 3D organoids makes their adaptation to high-throughput screening difficult.
- Lack of vasculature: Generally, 3D cultures lack a vascular system to distribute oxygen and nutrients across the tissue. That has a direct impact in the bigger organoids (>500 μm), resulting in necrotic centers that could produce artifacts when testing chemotherapies. Also, penetration of drugs into organoids becomes an issue that increases with the organoid size. In addition, vasculature is very important for GB tissue invasion (Watkins et al., 2014). Several groups have been working on the incorporation of vasculature in some 3D cultures (Cakir et al., 2019; Pham et al., 2018), as has been reviewed elsewhere (Grebenyuk and Ranga, 2019).
- The majority of these models do not present immune compartment, such as microglia, the main immune cells in the brain (Song et al., 2019). Although there is some controversy, recent research has indicated the presence of microglia in some cerebral organoids (Ormel et al., 2018), apparently not in large (physiological) numbers. Conversely, several groups are working on incorporating microglia into brain 3D cultures (Abreu et al., 2018; Song et al., 2019; Abud et al., 2017), even within the GB field (Leite et al., 2020). Immune cells, especially microglia, have shown to have a very important role in the pathology and tumor progression (Matias et al., 2018; Ghosh and Chaudhuri, 2010; Yang et al., 2010), and should be included, whenever possible, in GB research.
- There is also the possibility of finding non-CNS cells in the organoids, making interpretation of the results difficult (Quadrato et al., 2017; Ogawa et al., 2018).
- Quality assurance for organoids and stem-cell-derived models, such as Good Cell Culture Practice, is only now emerging (Pamies et al., 2017a, 2018a, 2020; Eskes et al., 2017).

Despite these limitations, it seems quite clear that these new models constitute novel tools for the study of GB. It is very important, however, to be aware of the advantages and limitations when choosing a model.

LACK OF BLOOD-BRAIN BARRIER IN ORGONOTYPIC CULTURES

As previously mentioned, lack of vasculature is a disadvantage of current organotypic cultures, particularly the larger ones, due to the limited diffusion of oxygen and nutrients in their centers. The lack of blood vessels is even more important when developing GB models, as angiogenesis, *in vivo*, takes place in GB to facilitate tumor growth, and tumor progression (Das and Marsden, 2013). In addition, represents an important target of chemotherapeutics. The blood-brain barrier (BBB) regulates and protects the microenvironment of the brain by selectively controlling the movements of molecules, cells, and ions between the blood and brain parenchyma to maintain brain homeostasis. This barrier has been considered the bottleneck of neurotherapeutics, as the majority of large-molecule (~100%) and small-molecule drugs (>98%) are unable to cross it (Pardridge, 2005). The capability of new drugs to cross the BBB is crucial for developing targeted treatments for brain diseases, making incorporation of a BBB compartment into the 3D brain models essential for identification of adequate treatments. Until now, no organotypic iPSC-derived brain model containing both a functional BBB and the brain tissue has been found. BBB models can come in many forms (Bagchi et al., 2019; Wilhelm et al., 2011), however, from the more commonly used transwell plates to on-a-chip technologies (Kilic et al., 2016; Jiang et al., 2019) and BBB spheroids (Cho et al., 2017). These models could be applied for drug development studies if used in tandem with GB-3DBrain. In addition, advances in computer science regularly provide new tools for predicting the BBB permeability of compounds (Carpenter et al., 2014; Liu et al., 2004; Fan et al., 2010; Roy et al., 2019).

APPLICATIONS OF GLIOBLASTOMA-BRAIN ORGANOTYPIC CULTURES

Organotypic cultures have shown diverse applications in GB research. They can be used to better understand tumor pathology and progression. Likous and collaborators found, after neuropathological

evaluation of tumor-bearing organoids, a hypercellular bulk tumor with an infiltrating edge of GB stem cells, demonstrating tumor invasion (Linkous et al., 2019,). They also observed differing tumor progression between lines. Moreover, several authors were able to observe networks of gap-junction-mediated interconnecting microtubes (described by Osswald et al., 2015), multiple desmosome-mediated connections, cytoplasmatic fusion (Linkous et al., 2019), and formation of tumor microtube networks (Krieger et al., 2020). In all of these, infiltration of the GB tumor cells was observed, showing, in some cases, GB-induced necrosis in cerebral organoids (Linkous et al., 2019) and increases in proliferation markers due to tumor cells (Linkous et al., 2019; Ogawa et al., 2018). In addition, some results indicate that the microenvironment created by the 3D organotypic models is important for maintaining and enhancing viability and growth of GB (Linkous et al., 2019).

The models described here are also novel tools for studying tumor-microenvironment interactions. Thanks to molecular biology, especially genomics technologies, interactions between the tumor and cells in the surrounding 3DBrain models are relatively easy to study. Several publications employed transcriptomics to examine these interactions (Bian et al., 2018a; Linkous et al., 2019; Krieger et al., 2020). Krieger and collaborators were able to identify genes upregulated in GB-3D cultures derived from all the patients included in the study—genes that were confirmed to be related to growth regulation, neuronal migration, extracellular secretion, and stimulus response (Krieger et al., 2020). Moreover, Bian and collaborators compared the expression levels of invasion-related genes in their neoCORs (GB-3DBrain model) with control organoids using RNA sequencing and fluorescence-activated cell sorter analysis. They found that genes encoding transcription factors related to epithelial-mesenchymal transition, migration-related receptors, extracellular matrix molecules, and proteases were upregulated, whereas many genes involved in the inhibition of tumor invasion were downregulated (Bian et al., 2018b).

Drug screening is most effective when using *in vitro* methods. Because they are faster and cheaper than *in vivo* testing, they allow hundreds of chemicals to be tested for ideal drug candidates. With the advances in human complex *in vitro* models, they also have the potential for better prediction of human drug efficacy and safety. GB-3DBrain models present a very compelling platform for studying treatments for GB. In addition, they also have the capability for studying treatments related to tumor microenvironment interactions. And because they also contain healthy cells, it is possible to simultaneously determine the safety window to affect the tumor but not the healthy tissue. Some models have been used to study the effects of GB treatments (Bian et al., 2018a; Plummer et al., 2019; Linkous et al., 2019). Bian and collaborators, for example, tetsed five epidermal growth factor receptor (EGFR) inhibitors (afatinib, erlotinib, gefitinib, and two experimental drugs, canertinib and pelitinib). Despite the fact that they did not study safety or effects on healthy cells, they were able to show reduction in GB size after 40 days of afatinib and erlotinib treatment (Bian et al., 2018b). In some of the studies, even radiation was evaluated, demonstrating the flexibility of these models (Linkous et al., 2019). GB lines were more resistant to drug- and radiation-induced genotoxic stress when incorporated into a 3D organotypic culture than in traditional 2D cultures (Linkous et al., 2019). Although it is logical to posit that more human physiologically relevant models could better predict the effects of drug treatments in humans, there is no evidence to prove this point, so we should be careful with this assumption. All these results suggest that GB-3DBrain models can be used as a tool to study drug treatments and could reduce or substitute for animal testing. Another possible benefit is their capability to be adapted to high-throughput (or at least medium-throughput) screening. The adaptation of 3D cultures to high-throughput screening can be difficult, because: (1) high cell density in 3D cultures may complicate image analysis (a process that is rather simple in 2D traditional cultures); (2) organoid size (>1mm) can make the adaptation to small-well reading platforms, which are normally used in high-throughput screening, difficult (96- or 384-well plates); and (3) some organotypic cultures present lower reproducibility, an impediment for toxicology and efficacy assessments (Quadrato et al., 2017). From our understanding, only one GB-3DBrain model (BrainSpheres) has been adapted for high-throughput screening (Plummer et al., 2019), developing a highly reproducible (low variability between samples) model (Pamies et al., 2017b) suitable for toxicological assessments (Zhong et al., 2020; Leite et al., 2019; Pamies et al., 2018b). The smaller size of the spheres allows for easy adaptation to 96- or 384-well plates, enabling measurement of varying concentrations and drugs in a relatively short period of time. This aligns with a "utilitarian" approach (as complex as necessary, but not more) (Rossini and Hartung, 2012). The complexity required for GB drug testing is still an unsolved question, but these important advances bring some light to the field.

CONCLUSIONS

GB is a devastating tumor, with an incredibly low survival rate and an absence of effective treatments. Unfortunately, new drug development has a very low success rate, and novel methods for assessing efficacy and safety of potential new treatments are necessary. 3D cultures, now more technically feasible than before, promise to boost scientific relevance—but this comes with a cost in complexity. Human iPSC, when combined with new 3D culture technology, constitutes a promising tool for generating more human-physiologically relevant models in GB research. These GB-3DBrain models have shown the ability to mimic (to some extent) human tumor microenvironments and GB progression in an orthotopic approach. As GB are very dependent on their microenvironment, these models are instrumental in better understanding the disease, developing new treatments, and to find new biomarkers. In addition, their adaptability to high throughput may speed up drug screening and the treatment development. There is still a long way to go to surmount the limitations, however, the most prominent being the lack of vasculature (as the BBB is a limiting factor for drug penetration into the brain). Moreover, the models need to be more widely used to fully understand their applicability. Validation of the models and the establishment of specific biobanks, as for other types of cancer (Schutte et al., 2017), could help support applicability of the GB-3DBrain model. Academia and industry are at the forefront of developing these techniques, as Francis Collins, director of the US National Institutes of Health, said as early as 2016: "I predict that ten years from now, safety testing for newly developed drugs… will be largely carried out using human biochips… This approach… will mostly replace animal testing for drug toxicity and environmental sensing, giving results that are more accurate, at lower cost, and with higher throughput." Raising awareness of the opportunities and challenges—as in this review—will hopefully accelerate the developments of treatments for this devastating disease.

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AUTHOR CONTRIBUTIONS

D.P. has written the first draft of the manuscript. T.H. and M.-G.Z. have revised, corrected, and improved the document substantially.

DECLARATION OF INTERESTS

T.H. and D.P. are named inventors on a patent by Johns Hopkins University on the production of mini-brains (also called BrainSpheres), which is licensed to AxoSim, New Orleans, LA, USA. They consult AxoSim, and T.H. is a shareholder.

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