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Three-dimensional culture models to study glioblastoma — current trends and future perspectives

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Abstract

Glioblastoma (GBM) is the most prevalent form of primary malignant brain tumor in adults and remains almost invariably lethal owing to its aggressive and invasive nature. There have only been marginal improvements in its bleak survival rate of 12–15 months over the last four decades. The lack of pre-clinical models that efficiently recapitulate tumor biology and the tumor microenvironment is also in part responsible for the slow phase of translational GBM research. Emerging three-dimensional (3D) organoids and cell culture systems offer new and innovative possibilities for GBM modelling. These 3D models find their application to engineer the disease, screen drugs, establishing live biobank, and explore personalized therapy. Furthermore, these models can also be genetically modified by using the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology, which would allow one to study the specific role of key genes associated with gliomagenesis. Establishment of a coculture system with GBM cells to understand its invasive behavior is yet another major application of this model. Despite these merits, the organoid models also have certain limitations, including the absence of immune responses and vascular systems. In recent years, major progress has been made in the development and refinement of 3D models of GBM. In this review, we intend to highlight these recent advances and the potential future implications of this rapidly evolving field, which should facilitate a better understanding of GBM biology.

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Glioblastoma, 3D culture, Organoid, CRISPR, Human pluripotent stem cells (hPSCs).

Introduction

Glioblastoma (GBM) is the most aggressive cancer type of the brain and accounts for 14.6% of all primary brain and other central nervous system (CNS) tumors and 57.3% of all gliomas in adults [1]. GBM is almost always a fatal disease mostly owing to its highly invasive growth pattern and vast intertumoral and intratumoral heterogeneity [2]. Despite the treatment methods involving surgery in combination with postoperative chemotherapy and radiotherapy, as well as targeted treatment and immunotherapy approaches, the overall prognosis associated with GBM remains poor with a median survival of 15 months and 5-year survival of ~5% after initial diagnosis [3,4].

GBM has long been thought to arise from glial cells of the CNS. However, more recent findings indicate that GBM arises from neural stem/progenitor cells residing within the subventricular zone of the brain rather than matured glial cells [5,6]. Multiple studies have also suggested the presence of a small subpopulation of tumor-initiating and tumor-propagating cells with properties very close to the neural stem cells called glioblastoma stem cells (GSCs) [7–9]. GSCs have been demonstrated to be inherently resistant to conventional therapies through multiple mechanisms, including but not limited to increased DNA damage repair [9]. One of the classic hallmarks of GBM is extensive infiltration into the surrounding brain parenchyma [10]. GBM cells invade mostly along the pre-existing structures such as blood vessels, white matter tracts, and the subarachnoid space [11]. The infiltration of GBM cells into the healthy brain tissue is the outcome of complex

interactions between tumor cells and the surrounding components of the microenvironment composed of microglia, macrophages, astrocytes, oligodendrocytes, neurons, glial and neuronal progenitors, pericytes, endothelial cells, and extracellular matrix (ECM) [12]. Taken together, these multiple layers of heterogeneity present in GBM represented by the stem, progenitor, and differentiated cells and the plethora of cells that make up the GBM microenvironment makes *in vitro* modeling of GBM even more challenging.

The lack of suitable *in vitro* models that depict the complexity of the human brain is a major research hurdle in the fields of neuro-oncology and neurology alike. Thus, it is crucial to develop innovative models that efficiently recapitulate the complex phenotype of GBM. In this review, we discuss the potential implications of the popular three-dimensional (3D) models used to study GBM and their relevance and future perspectives as a preclinical model to study gliomagenesis, tumor invasion, and to screen and validate drugs.

Two-dimensional and three-dimensional cell line models of glioblastoma

For a long period of time, GBM cells cultured in serum-containing media and maintained as a monolayer (two-dimensional [2D]) have been used as a research tool to study GBM biology. The cell lines namely U87, U251, and T98G have been the most extensively used [13]. These 2D models have certain major limitations as the serum-containing medium in which these cells are cultured causes alterations at the genomic and transcriptomic level and can also deplete the stem cell compartment by inducing differentiation [14]. Furthermore, the tumors generated by implanting these cell lines in immunocompromised mice fail to recapitulate many of the classical GBM phenotypes such as diffuse infiltration, microvascular proliferation, and necrosis [14].

Ever growing volume of evidence indicates the presence of a subpopulation of cells with stem cell properties called GSCs within GBM [7,8,15]. These GSCs are established to be more resistant to therapy and contribute toward the overall drug/radio-resistance machinery of GBM [9,16,17]. GSCs from both mouse and human GBM samples could be readily cultured as 3D spheroids, by using the same conditions used in culturing the neural stem cells as neurospheres (Figure 1A) [18–20]. Cells under varying stages of differentiation are evident in a horizontal cross-section of the glioblastoma stem cell spheroids (Figure 1B).

Orthotopic implantation of these GSCs generated secondary tumors, showing similar histological features, genomics, and phenotypic properties of the corresponding primary tumor [7,21]. GSCs can also be cultured as a monolayer over laminin-coated flasks (Figure 1C). Cells grown this way on implantation generated tumors with similar phenotypes as those of the tumors originating from GSC spheroids [22]. However, these culture systems come with limitations as they fail to capture the complex interaction between the tumor cells with the native non-neoplastic cells. Collectively, GBM offers a reliable cancer type to study cancer stem cell biology and in the design of novel therapeutics.

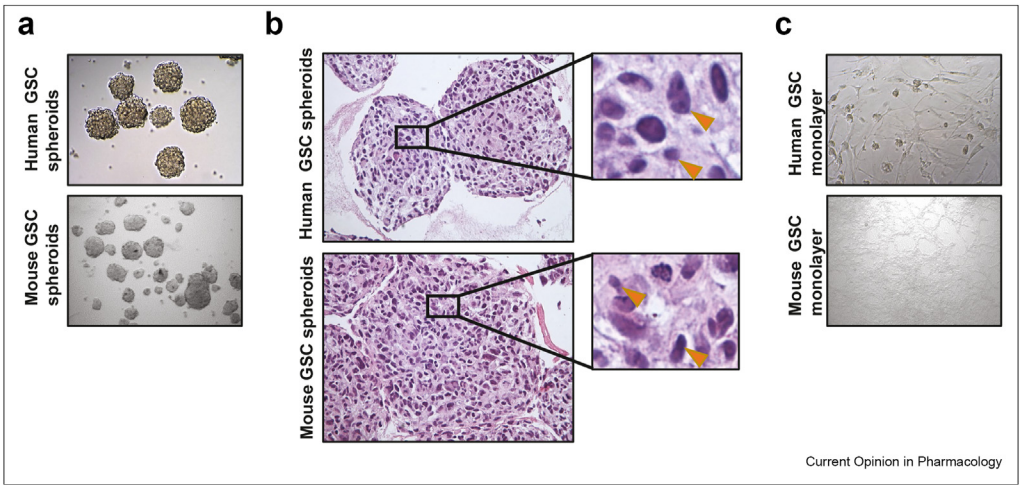
Cerebral organoids

Recently, 3D organoids have been developed that could efficiently capture the phenotypic and molecular heterogeneity found in various organs [23]. Organoid models have also been developed for several cancer types such as pancreatic, prostate, liver, breast, bladder, ovarian, and gastrointestinal cancers [24–30]. Lancaster and Knoblich [31,32] in their pioneering work have developed a protocol to generate cerebral organoids (COs) from human pluripotent stem cells. These COs closely mimic the endogenous developmental program of the brain and could serve as excellent models to study early events of brain development [33–35]. Apart from this, COs also find their application in the modeling of neurological conditions such as microcephaly (Lancaster 2014) and neurological malignancies such as GBM and primitive neuroectodermal tumors [36,37].

The study by Bian et al. [36] used transposon-and clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9-mediated mutagenesis to introduce oncogenic mutations in COs. They identified mutation combinations that result in GBM-like and CNS primitive neuroectodermal tumor-like neoplasm. These neoplastic versions of COs, named neoplastic cerebral organoids, were demonstrated to be suitable to study multiple aspects of tumor biology such as invasiveness, drug response, and so on. In yet another similar study by Ogawa et al. [37], a cancer model of gliomas was developed in human COs by using the CRISPR/Cas9 technology to target an $HRas^{G12V}$-IRES-tdTomato construct by homologous recombination into the TP53 locus. Such an approach induced a neoplastic transformation of the cells in the COs, and these transformed cell gain invasive properties and destroyed the surrounding organoid structures [37]. Orthotopic implantation of these transformed cells into an immunocompromised mouse produced a highly invasive tumor with a mesenchymal signature. Generation of such invasive mesenchymal tumors paves the way towards elucidating the molecular mediators of mesenchymal transition and associated tumor invasion.

Linkous et al. [38] in their recent study demonstrated another promising 3D model system called cerebral organoid glioma (GLICO). The GLICO model enabled

Figure 1



Spheroids and monolayer culture models of glioblastoma stem cells (GSCs) **(a)** The bright field image of GSCs maintained as spheroids generated from human and mouse GBM samples **(b)** Hematoxylin and eosin (H&E) staining on sections of mouse and human GSC spheroids showing heterogeneous population of cells (indicated with arrows) **(c)** The bright field image of the monolayer generated from mouse and human GSCs.

retroengineering of patient-specific GBMs using patient-derived glioblastoma stem cells (GSCs) and COs. The GSCs could deeply invade the COs and proliferate within the host tissue, forming tumors closely resembling patient GBM. Thus, the GLICO model provides a system for modeling primary human GBM *ex vivo* and provides a promising platform for high-throughput drug screening.

Glioblastoma organoids from patient tumors

It has been increasingly appreciated that the heterogeneity existing at the molecular level among tumors and within tumors [39,40] is likely connected to poor outcomes of numerous clinical trials [41]. Generation of GBM organoids that would at least in part preserve the tumor heterogeneity is crucial for timely empirical testing of personalized treatment strategies for GBM. Fadi et al. [42] in their recent work have put forward a protocol to generate a robust model of GBM organoids (GBOs) from primary GBM samples. These GBOs are generated without mechanical or enzymatic dissociation of the resected tumor tissue. Furthermore, GBOs are maintained in a fully defined medium devoid of serum and supplements such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) or ECM that may contribute to further clonal selection. Comprehensive histological, molecular, and genomic analysis performed on established live biobank of GBOs showed that GBOs recapitulate intertumoral and intratumoral heterogeneity and retain many key features of their corresponding parental tumor [42]. On implantation into the mouse brain, these GBOs generated aggressive tumors with an invasive phenotype that maintained key driver mutation expression. These GBOs were also tested effective for predicting response to standard of care therapy as well as targeted treatments, including drugs from clinical trials and chimeric antigen receptor T cell immunotherapy on a clinically relevant timescale [42].

Yet another recent study by Yi et al. [43] has shown that bioprinted reconstituted GBM tumors consisting of patient-derived tumor cells, endothelial cells, and decellularzed ECM from brain tissue in a compartmentalized cancer-stroma concentric-ring structure that sustains a radial oxygen gradient recapitulates many of the structural, biochemical, and biophysical properties of the parental tumor. This patient-specific GBM on a chip model showed resistance to the standard chemo-radiation approach used in a clinical setting and hence might be useful for the identification of effective treatment for patients with GBM resistant to the standard first-line treatment.

The fetal brain aggregate to model glioblastoma invasion

An organ culture system to grow the fetal rat brain samples was developed around 35 years back [44]. These 3D aggregates called the fetal brain aggregates (FBAs) imitated several morphological aspects of the developing brain. FBAs served as efficient models to validate invasion of GBM cells in a coculture model established between GBM cell lines and the FBAs. The invasion pattern of the tumor cells into the FBA resembled the pattern seen in an *in vivo* setting [44]. In another study, the administration of the tyrosine kinase inhibitor was found to reduce invasion of GBM

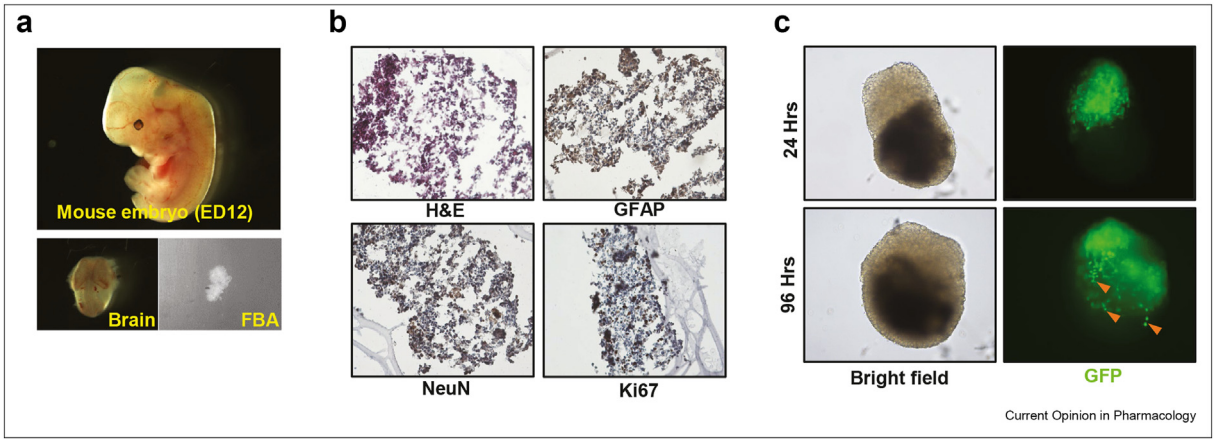
cells into the FBAs. We have established FBAs cultured in our laboratory from the mouse fetal brain and have been able to maintain these aggregates for more than six-month period in good condition with media change once a week (Figure 2). Similarly, FBAs have also been developed from human fetal brain samples by using a slightly different protocol [45]. One major advantage of FBAs is that these models are easy to generate, cost-effective, and can be maintained in culture for a long period of time. At the same time, several cell types and architecture of the developing brain are well-preserved in the FBAs. Most importantly, this model can serve as an excellent platform to delineate GBM invasion *ex vivo* and could be used to unravel the mechanisms that drive GBM invasion into the normal brain.

Gene editing in organoids

To gain the full potential of organoids, gene alterations have to be applied to study gene functions in a disease setting. The discovery of the CRISPR/Cas9 system to engineer specific gene alterations has permitted a great improvement of disease modeling, including organoids [46]. By designing specific single guide RNA (sgRNA)s to the target gene, this method allows introductions or deletions of base pairs in the coding sequence, which often results in a loss of function mutation [46]. This method can be applied to 2D or 3D systems to induce mutations in target genes. However, the method is harped by the delivery of the sgRNAs and Cas9 proteins for introduction of the DNA break. Often, CRISPR editing in organoids generates a pool of wild-type and mutant cells, which complicates the interpretations of the results. Different methods for delivering of sgRNA's and Cas9 protein applies, including virus-based delivery and electroporation of synthetic modified sgRNA's [47]. Moreover, antibiotic selection can be used to eliminate wild-type cells, especially when lentivirus has been used. We have taken advance of fetal cells from Cas9-EGFP^{LSL} mice to generate 3D FBAs. By using Adeno-associated viruses (AAV) particles for delivery of sgRNA and activation of Cas9 and EGFP expression by Cre recombinase (Cre) recombination, transduced cells will express green fluorescent protein [48]. The green fluorescent protein allows sorting of the cells from the FBAs that are fully transduced by the AAV particles (Figure 3). One issue by CRISPR-induced mutations is the lack of unified mutation profiles and few cells, where the target genes have not been mutated. Therefore, the mutation profile should be addressed for each experiment to assess the overall mutation frequencies [49,50]. CRISPR/Cas9 technology is still a new method, and rapid progression is made for delivery and precision of the method to increase efficiency and minimize the off-target effect.

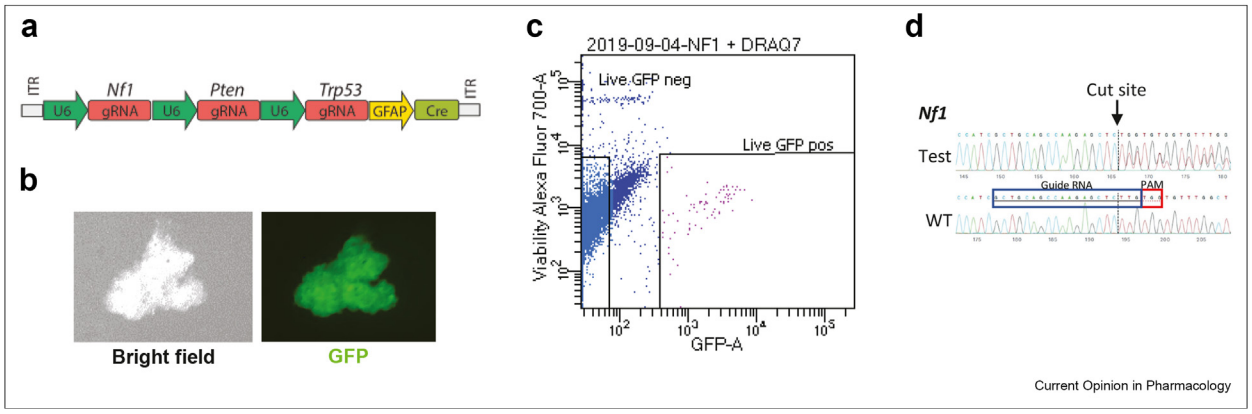
CRISPR technology can also be applied to generate specific gene insertion by homology-directed repair of the DNA break. To do this, a homology-directed repair templet has to be present in the cells, and this method has still very low efficiency [51]. Instead of CRISPR to generate gain of function mutations in organoids, lentivirus can be applied. Lentivirus is integrated into the genome of the host cell and will be replicated during cell division. Lentivirus can contain a DNA fragment up to 10.000 bp, which allows expression from a specific promotor and expression of an antibiotic-resistant gene, such as Puromycin N-acetyltransferase (*PAC*) gene, which conferred resistance to puromycin [52]. One problem with the use of lentivirus is the integration site in the host cell genome, which is random. Therefore,

Figure 2



Fetal brain aggregates (FBAs) generation and application **(a)** The mouse embryo at embryonic day 12 along with the harvested brain and FBA at 10 days in culture **(b)** HE staining along with immunohistochemical staining of FBA showing the expression of astrocytic marker Glial fibrillary acidic protein (GFAP), neuronal marker (NeuN), and proliferation marker (Ki67) **(c)** The spheroid confrontation assay established between GSC spheroids (in green) and FBA showing diffuse infiltration of GSCs (indicated with arrows) into the FBA at 96 hrs. H&E, hematoxylin and eosin, GSC, glioblastoma stem cell.

Figure 3



Use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 technology to engineer the FBAs **(a)** Construct expressing Cre containing Single guide RNA (sgRNA)s against Transfromation related protein 53 (Trp53), Phosphatase and tensin homolog (Pten), and Neurofibromatosis type 1 (Nf1) for Adeno-associated virus (AAV) particles **(b)** FBAs showing GFP expression 10 days after infection with the AAVs **(c)** Fluorescence Activated Cell Sorting (FACS) sorting of the GFP-positive cells from FBAs **(d)** Sequencing results from sorted GFP-positive cells. FBA, fetal brain aggregate.

multiple copies can be inserted, and this can disrupt expression of random genes and result in nondesired phenotypes [53]. A more site-specific system, such as CRISPR, will gain more robust models in organoids, but here, a strong need for improvement of the delivery and efficiency is needed. Overall, 2D cultures are more suitable for gene alterations owing to delivery of the vector system, but the 3D structure of organoids opens ways for additional scientific questions to be addressed that are not possible in a 2D setting.

Conclusion

The current 3D models used in studying glioma biology have offered an excellent opportunity to visualize multiple aspects of glioma pathogenesis closer than ever before. The numerous cell types present in the organoid models once could partly mimic the complex microenvironment associated with GBM in a dish. These models also give deeper insights into the mechanism of tumor invasion, and the heterogeneity evident at the levels of the differentiation scale also makes them a great tool to study GSC biology. They also provide advanced systems for drug screening and the nomination of new targets for therapeutic efforts. Adopting strategies such as barcoding or fluorescent labeling of specific cell types and establishing a coculture system with endothelial cells, microglial, and so on with the organoid models would give deeper insights into the complex interaction and networking between different cell types within GBM. Lack of immune cells and vasculature are two of the most widely discussed limitations of the organoid models, and these pitfalls are partially circumvented in two recent studies from Mansour et al. [54] and Daviaud et al. [55]. Overall, the rapid refinement of these model systems over time can potentially bring about major improvements in the management of patients with GBM.

Conflict of interest statement

Nothing declared.

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