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CEREBRAL ORGANOIDS AS A MODEL FOR GLIOBLASTOMA MULTIFORME

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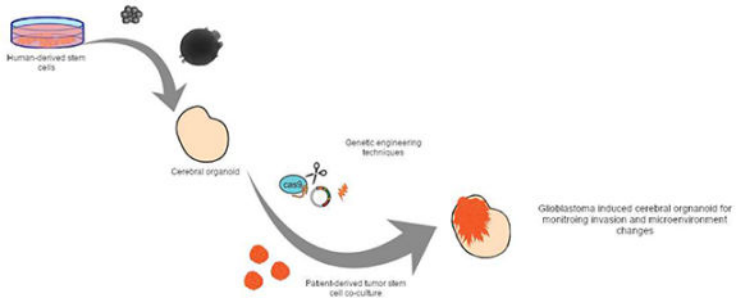
Abstract

Glioblastoma multiforme (GBM) is a highly lethal and elusive cancer. While many *in vitro* and *in vivo* models have been developed to recapitulate the factors that contribute to its invasive behavior, they suffer from drawbacks related to genetic variability, expense and scope. Technologies utilizing human pluripotent stem cells can now generate organoids which can recapitulate the relative complexity the cytoarchitecture and microenvironment of human brain tissue. In conjunction with protocols which effectively induce GBM tumors within these "cerebral organoids", such approaches represent an unprecedented model to investigate GBM invasion and its effect on the brain ECM. This review focuses on methods of brain organoid development, protocols for inducing GBM, the relevant findings on invasion and microenvironmental changes, and discusses their limitations and potential future direction.

Graphical Abstract



Keywords

cerebral organoid; brain organoid; glioblastoma; glioma; glioblastoma invasion

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Declaration of interests

'The authors declare no conflict of interest.

1 INTRODUCTION

1.1 Background

Glioblastoma multiform (GBM) is a particularly malignant brain cancer which carries a dismal prognosis with a median survival rate of 5% over five years [1]. This survival rate has remained unchanged for nearly 30 years despite aggressive chemo and radiation therapy, as GBM is highly invasive, making it very difficult to fully excise during treatment [2–5]. Additionally, tumors are complex systems which develop emergent properties that are specific to their host [6–8]. Therefore, there exists a need for a platform to study the cell dynamics of GBM so that characteristics of the disease may be uncovered for development of more effective treatments.

A variety of such platforms have been developed for preclinical modeling of GBM. Animal models such as patient-derived tumor xenografts and genetically engineered mouse models succeed in investigation of global gene expression patterns, mutational status, drug responsiveness, and tumor architecture [9–12]. However, the immunocompromised animal models are time consuming, expensive, and suffer from sample size limitations, and inherent genetic variability. *In vitro* bioengineered 3D disease models for GBM have been developed. Such models utilize synthetic and naturally-derived biomaterials to recapitulate critical features of the tumor microenvironment such as mechanics, chemistry, and topography in a tunable and repeatable manner [13]. These models are successful in modeling certain niches of cerebral tissue but fail to capture macro-level cytoarchitecture and heterogeneity of the human brain. In order to address larger scale invasion and drug responsiveness of GBM, a different approach to 3D *in vitro* modeling is needed which can imitate the human extracellular matrix and hierarchical organization of human cells in the brain. Recent development in cerebral organoids is starting to address this challenge.

1.2 Development of cerebral organoids

1.2.1 hPSC-derived neural cell aggregates—Complex arrangements of brain cells in 3D have been generated using human pluripotent stem cells (hPSCs). The most common protocol for generating 3D human brain tissue from hPSCs is the serum-free floating culture of embryoid body (EB)-like aggregates with quick aggregations (SFEBq). This protocol involves EB-like formation by reaggregation of dissociated hPSCs [14, 15]. Subsequent differentiation occurs into several polarized neuroepithelial rosettes which are structurally similar to cortical neuroepithelia found *in vivo* that generate layer-specific cortical neurons [15]. The resulting cortical progenitor cells are heterogeneous with respect to their rostral-caudal characteristics [16]. Further studies incorporated components of the extracellular matrix (ECM) either by dissolving it in medium [17, 18], replating the aggregates on ECM-coated dishes [19], or by embedding the ECM-like structures into Matrigel [20]. This improved polarization of the neural progenitor sheet, which supported development of elongated neural epithelia. This elongation caused rosettes to more closely resemble the ventricle structure found in native brain tissue. Furthermore, ECM incorporation brought on the appearance of neuronal “layers” (Fig. 1), in which earlier-born neurons were situated below later-born neurons, which is what occurs in embryonic human brain development [17, 18, 20].

1.2.2 Cerebral organoids—Building upon the methods above, Lancaster et al. developed a protocol which focused on optimization of growth conditions and providing a necessary environment for intrinsic cues to influence development of aggregated EBs. To accomplish this, EBs were taken from suspension culture and embedded in Matrigel droplets, then transferred to a spinning bioreactor. This led to the generation of tissue with a variety of brain regional identities and was termed cerebral organoids or “mini brains”. They contained distinct and interdependent brain regions in a single organoid including dorsal cortex, ventral forebrain, retina, hippocampus, choroid plexus, and midbrain-hindbrain boundary patterned in a manner like that of the developing human brain. Furthermore, they exhibited sophisticated development phenomena, with interneurons being produced in the ventral forebrain and migrating to the dorsal cortex [20]. Subsequent studies were conducted to determine similarities in the development of cerebral organoids *in vitro* to that of the human brain *in vivo* using neocortical culture microarrays, [19, 21], RNA-seq [22] and single cell RNA-seq [23] whereby transcriptomes were compared to gene expression in the developing brain. Findings showed that methods of producing cerebral organoids *in vitro* recapitulated *in vivo* brain development up to 8–10 weeks of gestation [21, 24] (Fig. 2).

2 RECENT IMPROVEMENTS TO CEREBRAL ORGANOIDS

2.1 Vascularization of cerebral organoids

Diffusion of nutrients to cells at the interior of cerebral organoids becomes a major issue as organoids grow beyond a certain size without the formation of a vascular network [25]. The center of the organoid may become necrotic, as cells more than 200–400 μm from the cell surface do not receive enough oxygen and nutrients by diffusion alone [26]. Additionally, vascularization of brain tissue is a crucial factor in brain tissue development [27], and the natural stem cell niche for glioblastoma stem cells (GSCs) is the brain perivascular microenvironment [28]. Therefore, in order to properly recapitulate brain tissue for the purpose of modeling disease, vascularization of cerebral organoids is necessary.

Recently, several methods to induce vascularization on cerebral organoids *in vitro* have been developed. One approach is to include human pluripotent stem cells genetically modified to express ETV2 in the cell population used to form the cerebral organoids. This results in formation of vascular-like structures in the resulting organoids over time [25]. Another method is to introduce VEGF during EB formation [29]. Yet another method is to embed human endothelial cells (hECs) in Matrigel to which the early stage organoids are added. This results in self-assembly of the hECs into capillaries at the periphery of the organoid and invasion of a vascular network over time [26]. In all cases, significant vascular network formation is appreciated at the periphery of the organoid, with lesser vascular network formation toward the center. Enhanced vascular development was verified by transcriptome analysis, showing upregulation of genes relevant to vasculogenesis [29]. Functionality of blood vessels was examined by testing perfusability of the vascular structures using dextran. Therefore, *in vitro* techniques promote the formation of a functional vascular network, with the stipulation being that it fails to fully penetrate throughout the whole cerebral organoid.

Another means of vascularization is to graft organoids into immunodeficient mouse models. For organoids with no treatment to promote vascularization *in vitro*, it has been shown that

for cerebral organoids grown for 40–50 days, murine blood vessels begin to invade 7–10 days post implantation. Immunostaining for human-specific CD31 markers did not coincide with the newly formed vascular network, indicating that it was host-derived. However, unlike *in vitro* vascularization efforts, the vascular network was not confined to the periphery, but rather permeated the entire organoid [30, 31]. Similar results were seen for organoids with *in vitro* vascularization treatment prior to implantation *in vivo*. In the case of *in vitro* vascularization by ETV2 expression, vasculature formed prior to implantation connected to mouse vasculature [25]. For organoids that had undergone Matrigel-based coating with ECs to induce *in vitro* vascularization, organoids were grafted after 20 days of growth rather than the 40–50 days of growth required of control organoids. By 2 weeks *in vivo*, the organoid showed robust vascularization in several regions throughout the whole organoid [26]. Therefore, vascularization of cerebral organoids can be achieved both *in vitro* and *in vivo*, with a combination of both approaches being the most efficient and effective means of generating an organoid with a robust and functional vascular network.

Holistically, the *in vitro* methods developed by Lancaster et al. and others have presented a method of recapitulating brain tissue with a higher degree of structural patterning than is achievable by other 3D disease models for glioblastoma multiform thus far. This opens the door to studies of GBM dynamics with a greater scope in terms of migration.

3 CEREBRAL ORGANOIDS FOR MODELING GBM

3.1 Induction of GBM in cerebral organoids

The first step in modeling GBM in cerebral organoids is to induce the formation of a tumor. One method for accomplishing this is to co-culture cerebral organoids with fluorescent-labeled patient-derived glioblastoma stem cells (GSCs) [32]. Tumor induction success is determined qualitatively by daily monitoring using immunofluorescence microscopy. The advantage of this method is the resulting tumors have genetic identities specific to the patient from which the GSCs were excised. It also lends itself well to studying invasion behavior of GBM (section 3.2). This patient specificity is a disadvantage in some contexts. It does not offer control over oncogenic mutations which lead to incipient cancer development, as tumor formation is initiated by mature glioblastoma stem cells. Therefore, it does not recapture the earliest stages of tumorigenesis. An alternative method of inducing GBM in cerebral organoids is by using genetic engineering techniques. This generally involves the design of one or several plasmids containing elements to facilitate oncogenic gene expression with a CRISPR-Cas9 system. One approach is to target a region within the human TP53 domain with a donor plasmid containing an expression cassette for an oncogenic cytomegalovirus promotor-driven HRas with fluorescent marker that will disrupt the open reading frame of TP53 by homologous recombination [33]. The main advantage of this approach is the ability to select genes to generate a specific initial oncogenic genotype. Organoids generated by this method more suitable for observing the early stages of glioblastoma initiation and tumorigenesis. Plasmids are delivered by electroporation either after the organoid has fully formed, or before embedding the embryoid bodies in Matrigel [34] (methods of GBM inductions in cerebral organoids summarized in Fig. 3). In either case, organoids are cultured

for several weeks post transduction, then observed for tumor development using immunofluorescent microscopy (Fig. 4A).

Regardless the of technique used, tumor growth is typically quantified by immunofluorescent staining of cell proliferation markers. Proliferation markers tend to be co-localized with fluorescently labeled GSCs, or cells containing the fluorescent marker which indicates they have been successfully transduced to an oncogenic genotype [32, 34], with 18–20% of oncogenic cells within the cerebral organoids being positive for these markers. Additionally, transcriptome analysis is used to verify that the gene expression of cancerous cells in the cerebral organoids is like that of patient-derived GBM cells. GBM is divided into four gene expression-based molecular subtypes: Proneural, Neural, Classical, and Mesenchymal. These classifications principally arise from aberrations and gene expression of EGFR, NF1, and PDGFRA/IDH1 [35]. The transcriptome of cells positive for the transduction success marker tended to express genes that were upregulated in established GBM subtypes, specifically the mesenchymal subtype, while non-transduced cells had a transcriptome which resembled that of neural stem cells [33]. More comprehensive RNAseq studies will be worthwhile to ensure that the overall cancer phenotype can be fully recapitulated by validating that the organoid model can be used to generate cells of all four subtypes.

3.2 Investigating tumor progression and invasion in cerebral organoids

One hallmark of GBM is its highly aggressive infiltration of brain tissue. It follows that invasion of GBM is a major topic of investigative interest, and cerebral organoids with induced tumorigenesis present an excellent model to study it. In glioblastoma cerebral organoids generated from co-culture with GSCs, glioma cells infiltrated brain tissue in a similar pattern to that seen in surgical and autopsy specimens from GBM patients (Fig 4B). Additionally, different patient derived GSC lines generated unique migration patterns and degrees of tumor cell invasion and proliferation (Fig 4C). This reflects the heterogeneity of invasive phenotypes that are clinically observed in GBM patients and shows that this vital characteristic is captured *in vitro* using organoids [32]. In a similar study, da Silvia et al. co-cultured patient-derived GBM spheroids with early stage mouse embryonic stem cell-derived cerebral organoids. GBM spheroids spontaneously experienced attachment with the organoids, followed by fusion and invasion into the cerebral tissue. Compared to a control neuroprogenitor cell line, GBM cells displayed significantly greater migration capacity, larger compartment area, and more effective infiltration to inner layers of the organoid. Immunohistochemical analysis showed that GBM cells expressed more stemness markers than the neuroprogenitor counterparts [36].

In another study, organoids were stereotaxically injected in the hippocampus of immunocompromised mice. Upon the death of the animal, sections of the hippocampal tissue were taken. H&E staining demonstrated microscopic invasiveness and nuclear pleomorphism as previously described in clinical cases of human GBM. As with human GBM, tumors tended to spread along blood vessels, presented with hypercellular phenotype with large-nucleated giant cells, multinucleated cells, and other unique characteristics of later-stage GBM development [33]. Therefore, it has been shown that cerebral organoids

closely recapitulate *in vivo* invasion dynamics and glioma cell phenotype, which make them an excellent platform for future studies on the underlying mechanisms and subsequent treatment of this issue.

3.3 GBM tumor organoids

Cerebral organoid culture protocols have been adapted to generate sophisticated GBM tumor organoids. By replacing pluripotent stem cells with patient-derived glioblastoma stem cells, tumor organoids have been developed which recapitulate tumor cytoarchitecture rather than that of a brain [37]. This platform for studying GBM is comparable to the tumor-induced cerebral organoids discussed in section 3.1, with some advantages and some caveats. Given that tumor organoids only contain cancerous tissue, their main limitation is that *in vivo* studies are necessary to investigate their interaction with healthy brain tissue. In those studies, applications are comparable to those of tumor-induced cerebral organoids. For example, tumor invasion resembles that which occurs historically in clinical cases of GBM. One advantage of tumor organoids is their ability to effectively recapitulate certain aspects of the tumor microenvironment more effectively than tumor-induced cerebral organoids. Specifically, they have been shown to generate gradients of stem cells density and hypoxia (Fig 4D&E). This ability of tumor organoids was used to show that GSCs near the surface of organoids divide and die frequently, while GSCs in hypoxic areas are quiescent. Therefore, while tumor-induced cerebral organoids are beneficial for their ability to capture interaction of tumors with healthy brain tissue *in vitro*, tumor organoids appear to be the most effective platform moving forward for *in vivo* studies.

4 OUTLOOK OF CEREBRAL ORGANOIDS - GBM MODEL

4.1 Current limitations of cerebral organoids for GBM modeling

Though cerebral organoids have many advantages, there exist limitations which make them a sub-optimal preclinical model in their current state. One major limitation is cerebral organoids contain multiple and variable sizes and numbers of ventricular zone-like regions, which is detrimental to reproducibility and quantification of cytoarchitecture [16]. Additionally, the lack of normally present embryonic surroundings (specifically lack of body axes) means that although organoids develop discrete brain regions, they do not organize themselves in the same pattern as present *in vivo* [38]. Another current shortcoming is the lack of immune response, which is an aspect of particular importance to modeling glioblastoma.

4.2 Future directions of GBM modeling using cerebral organoids

Though the vascularized cerebral organoids platform has been developed (see section 2.1), there are currently no studies which use it to investigate GBM invasion. Glioblastoma stem cells are known to occupy a perivascular niche [28, 39], and an increasing body of evidence indicates that glioma stem cells are largely responsible for GBM invasion [40–43]. Additionally, there is strong evidence for preferential GBM invasion along existing blood vessels [44]. Therefore, vascularized cerebral organoids are a logical future direction for investigating invasion dynamics of GBM. Studies of invasion using vascularized glioblastoma cerebral organoids will open the prospect of isolating cells at the invasive front

and subsequent characterization by RNA seq to uncover the mechanisms underlying aggressive migration and develop treatments to mitigate the invasiveness of GBM. Additionally, cerebral organoids induced with GBM, especially those generated from patient-derived cells, present an effective platform for drug screening as it allows one to study the effects of certain treatments *ex vivo*. Future studies will focus on developing protocols to quickly generate patient-specific organoids so they can be used to test the efficacy of existing clinical treatments before using them on a patient in a medium to high throughput manner. Other studies will use cerebral organoids as an alternative to xenografts and other 3D tissue models to trail novel GBM treatments [32]. Finally, introduction of immune cells (microglia) improve cerebral organoids' ability to recapitulate brain tissue response to GBM invasion.

5 CONCLUSION

This review presented the lead up to, and current state of brain organoid research with special attention paid to its utility in studying glioblastoma multiforme. It has also addressed current limitations of the technology, and likely future directions of the field. Without question, cerebral organoids show potential to bridge the gap between low-scope 3D tissue disease models and highly variable and expensive xenografts. Additionally, they show great promise as a high throughput platform for drug discovery and patient-specific drug screening. Recent advancements such as vascularization leave cerebral organoids on the cusp of a boom in terms of modeling GBM invasion. Additional improvements to protocols and techniques related to the generation of GBM induced cerebral organoids will allow researchers to more effectively investigate the mechanisms behind the elusive behavior of GBM, providing a path toward more effective treatment of such a grave disease.

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Highlights

- Cerebral organoids present promising model for glioblastoma
- Organoid vascularization is a major improvement made recently
- Glioblastoma can be induced in brain organoids by a variety of methods
- Glioblastoma invasion dynamics recapitulated in cerebral organoids

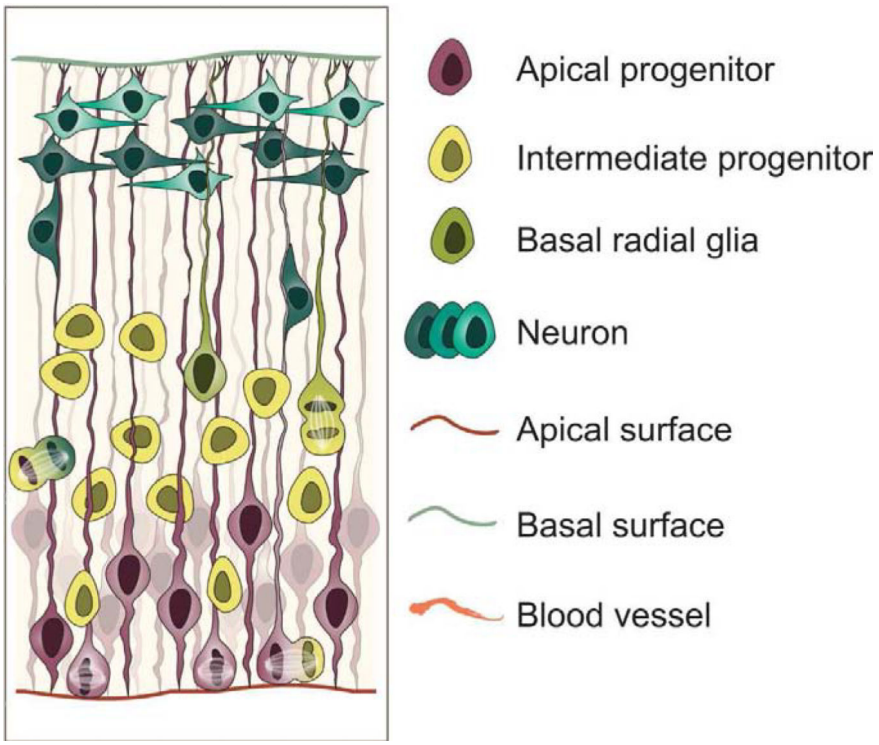

Figure 1:
Layering structure of 3D brain organoid generated by serum-free floating culture of embryoid body-like aggregates with quick aggregations followed by incorporation of ECM elements. Neuronal layers appear in which earlier-born neurons were situated below later-born neurons. Legend presents cell types and other elements found in the tissue. Adapted from Kelava et al., 2016 [38].

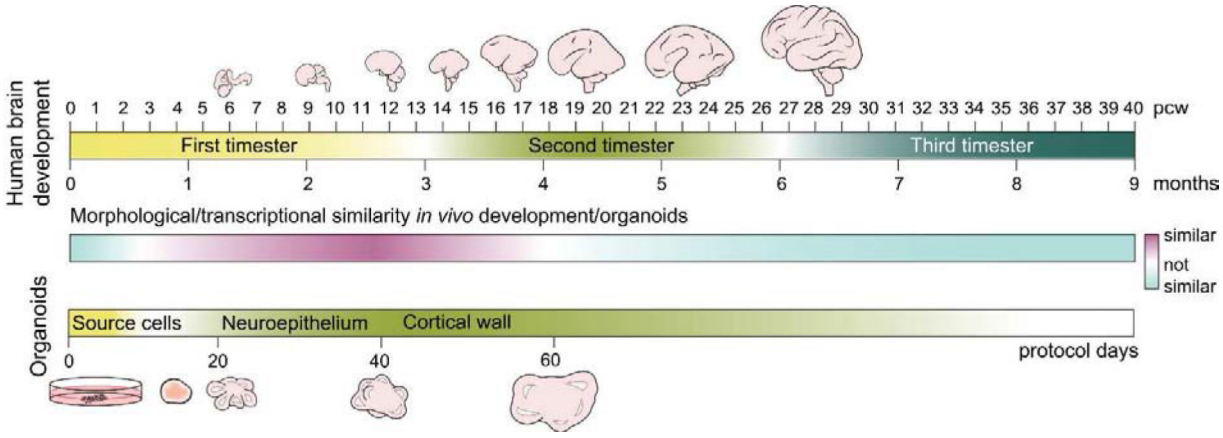

Figure 2:

Morphological similarity of cerebral organoids grown *in vitro* (bottom) compared to *in vivo* development of the human brain during gestation (top). The top bar depicts the general progression of morphological changes to the developing brain *in vivo*. The middle bar is a qualitative representation of degree to which organoid development *in* vitro is like the native brain development *in* vivo. The bottom bar and accompanying graphics give an overview of the cerebral organoid protocol and development *in vitro*. Adapted from Kelava et al., 2016 [38].

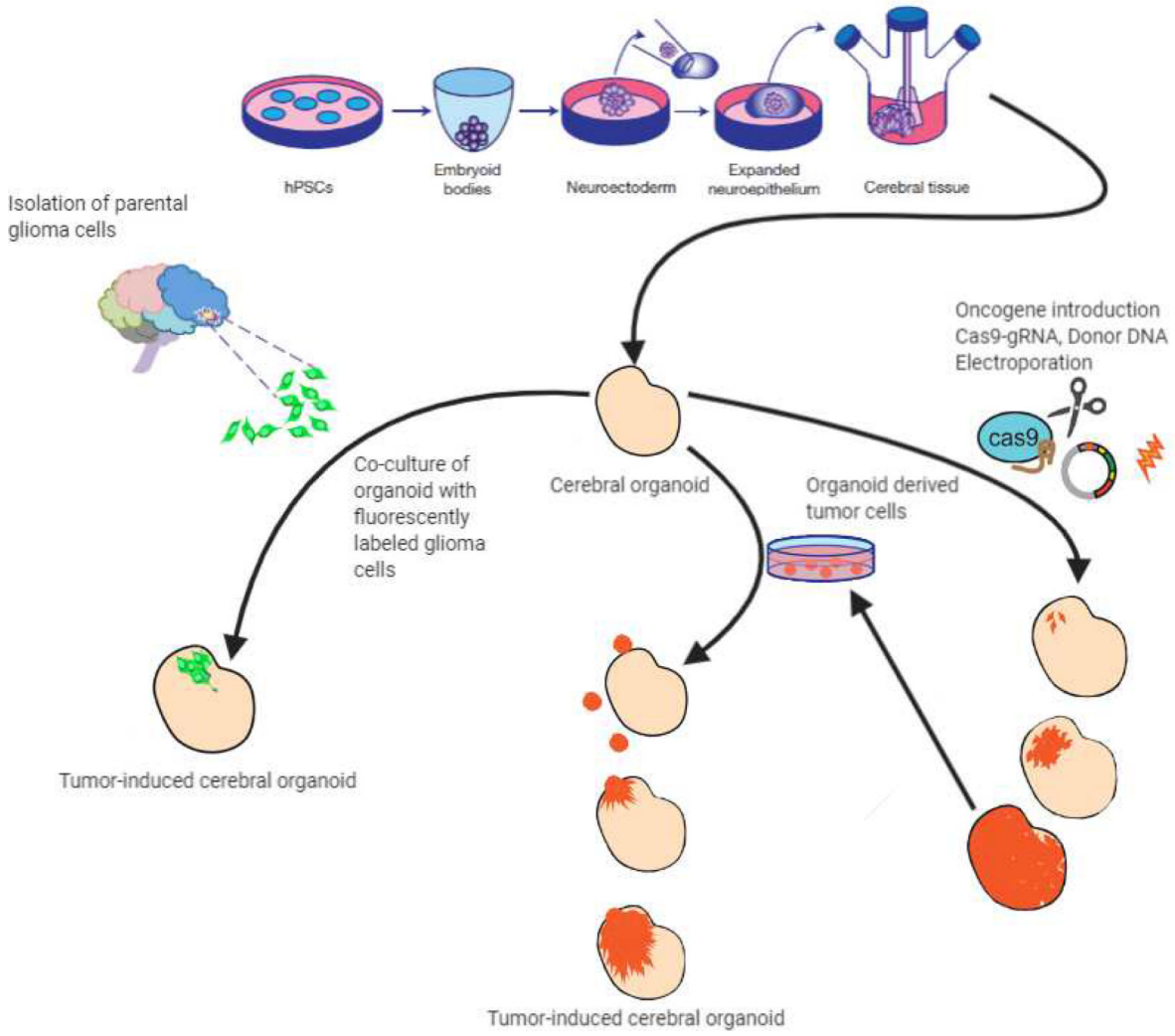

Figure 3:

Overview of the process for obtaining GBM induced cerebral organoids. The top row depicts an abbreviated process for organoid formation. Below show alternative methods of tumor induction either by co-culture with patient-derived primary tumor stem cells (left) or by genetic engineering techniques (left) adapted from Ogawa et al., Linkous et al., and Lancaster et al. [20, 32, 33]

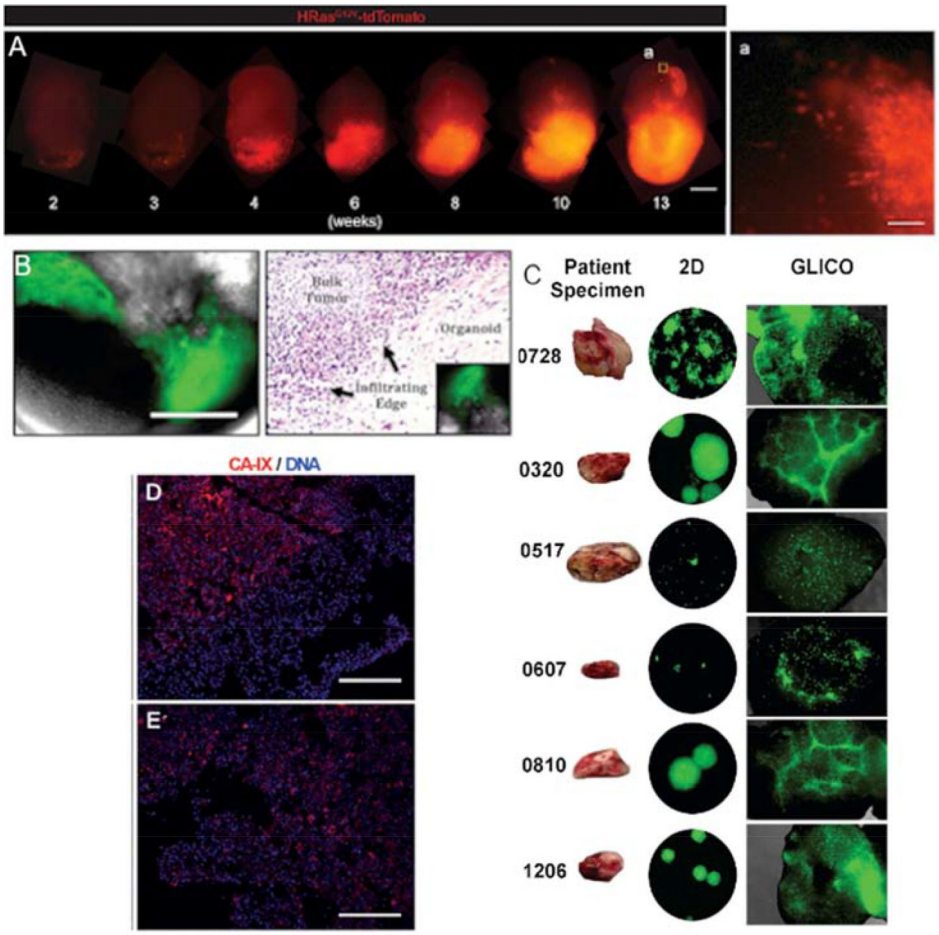

Figure 4:

Selected results from studies using cerebral organoids to model GBM. (A) Example of tumor growth in a cerebral organoid by induction using genetic engineering techniques. In this case, an HRas cassette with tdTomato fluorescent marker is used to disrupt the TP53 domain by homologous recombination facilitated by CRISPR-Cas9. Immunofluorescent images depict tumor invasion over the course of 13 weeks. Image at the right is a close-up of the invasive front. Adapted from Ogawa et al., 2018. [33] (B) Tumor formation by 1-week co-culture with GFP-expressing GSCs. Scale bare is 400 μm. Adapted from Linkous et al. [32] (C) Tumor infiltration of different patient-derived GSC lines 1 week after introduction into organoid demonstrates that invasive profiles are highly variable from patient to patient. Adapted from Linkous et al., 2019. [32] (D & E) Immunofluorescence images of CA-IX (carbonic anhydrase marker presented by cells in response to low-oxygen conditions) in cerebral organoid tissue. Adapted from Hubert et al. [37]