Organoids in Pediatric Brain Tumor Precision Medicine

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Malignant brain neoplasms are a heterogeneous group of tumors, including glioma, ependymoma, embryonal tumors, and many other (rare) entities and subentities, affecting patients from birth to adulthood. Organoids emerged as three-dimensional (3D) cell culture systems for modeling healthy and diseased tissues. These organoids potentially model development, diseases, and drug responses [13]. They are self-organizing three-dimensional structures that closely mimic an organ or tissue at a morphological, cellular, and functional level.

organoids | precision medicine | pediatric | Medulloblastoma | pediatric glioma | ETMR | ATRT | pediatric brain tumors

1. Introduction

Malignant brain neoplasms are a heterogeneous group of tumors, including glioma, ependymoma, embryonal tumors, and many other (rare) entities and subentities, affecting patients from birth to adulthood. Despite intensive treatment protocols, including surgery, chemotherapy, and radiotherapy, the prognosis for many high-grade brain tumor patients remains poor [1][2][3][4][5][6]. Although extensive research in this field has resulted in a detailed molecular classification of brain tumors and led to various new insights into their biology, numerous recent clinical trials have failed to significantly improve the prognosis for these patients, especially those suffering a relapse [7]. Overall, there is a noticeable gap between recent preclinical achievements and the clinical improvements in patient outcomes. This dichotomy may stem from current preclinical studies frequently being conducted in two-dimensional (2D) cell culture, which neither sufficiently recapitulates inter- nor intratumoral heterogeneity nor the cellular diversity of the tumor microenvironment (TME) [8]. Importantly, heterogeneous in vivo-like tumor cell populations respond differently to drug treatment than 2D in vitro entities, and intratumoral diversity results in a higher risk of treatment resistance and tumor recurrence [9][10]. Furthermore, the interplay of tumor cells with their TME, including endothelial cells, immune cells, and neuronal cells, affects the treatment response [11][12]. Therefore, it has become paramount to establish models which include these key characteristics and allow tumor–TME interactions, thus potentially generating more accurate predictions of tumor biology, drug efficacy, and immune response.

In the past few years, organoids emerged as three-dimensional (3D) cell culture systems for modeling healthy and diseased tissues. These organoids potentially model development, diseases, and drug responses [13]. They are self-organizing three-dimensional structures that closely mimic an organ or tissue at a morphological, cellular, and functional level. They can be divided into two major groups: organoids that mirror healthy tissue, including brain

organoids, and those that simulate diseased tissue, including tumor organoids [13]. Initial organoid models utilized the intrinsic self-patterning abilities of human pluripotent stem cells in appropriate conditions to generate small aggregates with optic cups [14] or even tissues representing a wide gamut of brain regions, forming the so-called cerebral organoids [15]. Later on, other groups investigated organoids resembling specific brain regions, including the forebrain, the midbrain, the hypothalamus, or the cerebellum [16][17][18]. Pioneering the use of organoids for tumor research, Sato et al. generated 3D in vitro models from primary colon carcinoma samples [19]. They were followed by other groups who developed tumor organoids from various entities, including prostatic, pancreatic, and liver cancers, as well as glioblastoma [20][21][22][23].

These tumor organoids can then be used for high-throughput drug and toxicity screenings to uncover new personalized therapeutics [13][24][25][26]. Additionally, healthy tissue 3D cell culture systems, such as organoids or organ-on-a-chip models, can be further used to test the side-effects of drugs. These technologies may identify drugs with a high efficacy against the tumor and a low burden of side effects on healthy tissues [24][27][28]. Recapitulating parental tumors, cancer organoids have proven to be capable of predicting cancer treatment efficiency in vivo [23][29][30], thus ringing in the era of organoid-based in vitro anti-cancer drug tests.

2. Organoids Are Superior to Prior 2D In Vitro Models in Recapitulating the Primary Tumor Characteristics

Despite making progress on exploring the mechanisms leading to tumor initiation by identifying, for example, (I) the mutational burden of tumors, (II) malignancies' cells of origin, and (III) the impact of the TME on tumor cells [31][32][33][34], many key scientific questions to finally improve brain tumor patients' survival remain unanswered. This lack of fundamental insight may partly stem from in vitro models insufficiently recapitulating the core characteristics of the primary tumors.

For decades, brain tumor research has been based on 2D and 3D cell cultures in mono-layers and spheroids, respectively. Traditional 2D in vitro cultures of tumor models rely on cell propagation in standard petri dishes. These 2D cell culture models undergo clonal selection for fast-growing and cell-culture-compatible cell populations, thereby losing cellular diversity and often resulting in a homogeneous cell population that are no longer recapitulating the tumors' original heterogeneity [8][35][36]. Due to their mono-layer arrangement, these cell cultures are adapted to conditions of 20% oxygen, which exceeds the usual oxygen level of about 5% in in vivo tumors [8][35]. Spheroids consist of mostly uniform aggregates of a mixture of desired and relevant cell types for a given disease model assembled in an essentially random three-dimensional arrangement. On the other hand, organoids self-arrange their cell types into micro-moieties that more closely approximate organ tissue structure and function. With the advent of these self-organizing organoid tissues, a plethora of more complex three-dimensional model systems have addressed brain tumors in recent years, including patient-derived tumor organoids (PDO), patient-derived explants (PDEs), tumor-brain organoids (TBOs), neoplastic cerebral organoids (neoCORs), and, lastly, bioprinted tumor models (**Figure 1**). These organoid models can be divided into two major groups: (I) tumoroids starting only from a tumor tissue, including PDOs and PDEs, and (II) organoids composed of a tumor and a non-tumor compartment, including TBOs and neoCORs.

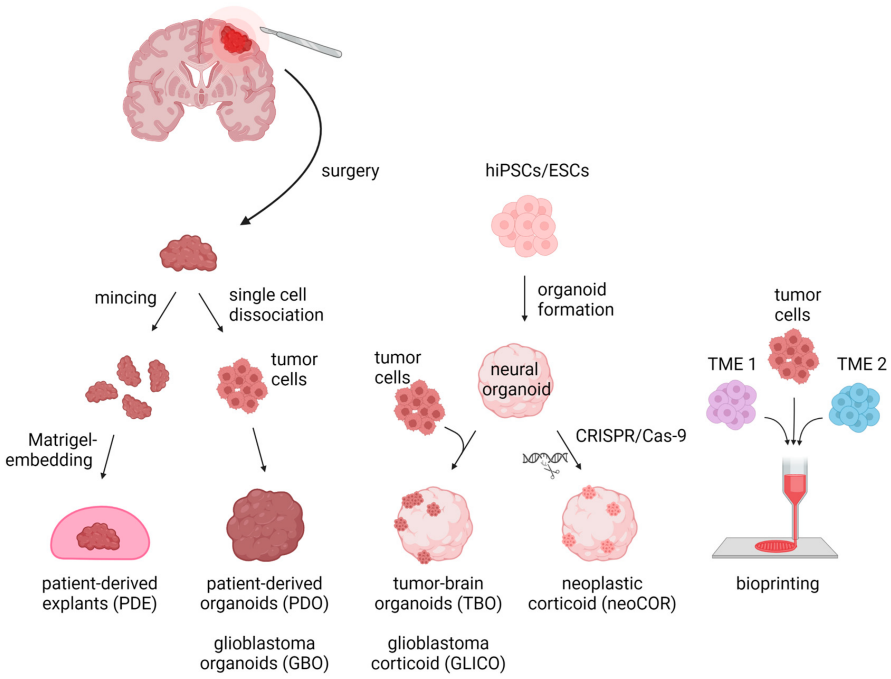

Figure 1. Three-dimensional in vitro tumor models can be derived from either primary patient materials or generated de novo from pluripotent stem cells. PDEs and PDOs are derived from patients' resected tumor tissue, which is minced and Matrigel-embedded for PDE generation or is single-cell dissociated followed by re-aggregation for PDOs/GBOs. TBOs/GLICOs and neoCORs are generated by seeding hiPSCs/ESCs for organoid generation, followed by co-culture with tumor cells for TBOs/GLICOs or CRISPR-Cas9-based gene editing for neoCORs. To create 3D models via bioprinting, tumor cells are loaded together with TME cells into a bioink and spatially printed. Abbreviations: PDE: patient-derived explant; PDO: patient-derived tumor organoid; GBO; glioblastoma organoid; TBO: tumor-brain organoid; GLICO: glioblastoma corticoid; neoCOR: neoplastic corticoid; 3D: three-dimensional; hiPSC: human induced pluripotent stem cell; ESC: embryonic stem cell; TME1/2: tumor microenvironment cell type 1 or 2. Created with BioRender.com (accessed on 7 November 2022).

In contrast to traditional mono-layer and spheroid cultures, organoids across all models can preserve intra- and intertumoral heterogeneity [23][29][37] and establish diffusion-limited oxygen gradients within the organoids similar to that in early primary tumors, which may aid in maintaining a diverse tumor cell pool [23][38].

This might be a significant advantage in drug screens, as intratumoral heterogeneity plays an important role in intrinsic and acquired therapy resistance [39]. Two-dimensional cell lines, spheroids, and organoids respond differently to treatment, with organoids more accurately recapitulating the biological response of the parenteral tumor [8][40][41][42]. These findings are driving the hopes for organoid-based drug screens for improved clinical relevance [23][29][40][43].

3. Current Limitations of Organoids

While 3D model systems have improved over recent years, they still face several limitations. To properly mimic a tumor in vitro, current organoid models are missing vascularization, TME cells (immune cells and neuronal TME), and protocols resulting in more reproducible and scalable organoid generation (**Figure 2**).

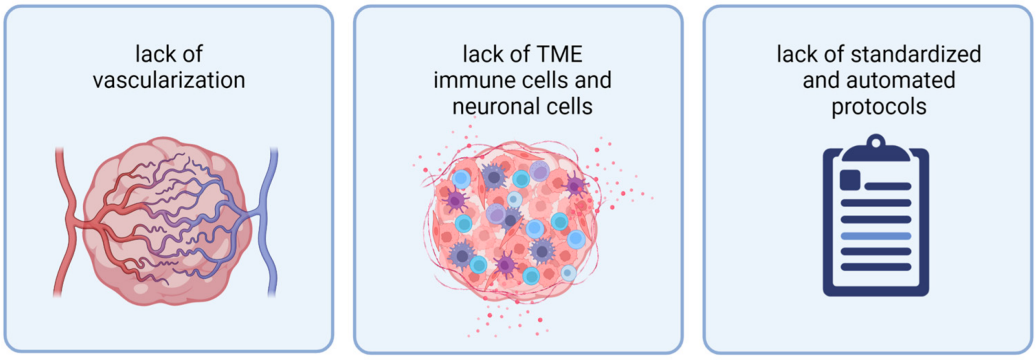

Figure 2. Current organoids face three limitations: from left to right: lack of vascularization, lack of TME immune cells and neuronal cells, and lack of standardized and automated protocols for organoid generation. Abbreviations: TME: tumor microenvironment. Created with BioRender.com (accessed on 7 November 2022).

In current brain tumor organoid models, the lack of vascularization impairs their growth, further tumor development, and long-term culture [44]. In brain tumors, the vasculature and blood–brain barrier (BBB) play an essential role in metastasis and selective drug delivery [45]. Despite recent achievements in modeling a BBB in cerebral organoids, vasculature has yet to be introduced into 3D brain tumor models, and this challenge is a very active field of research [46][47][48].

In recent years, several groups have started elucidating the essential role of the TME in brain tumor progression, immune escape, and chemoresistance [11][12][49][50][51]. Therefore, in vitro models should ideally recapitulate the rich gamut of cells, extracellular matrix, and signaling of the TME. However, current organoid models only host single TME cell populations. Primary tissue-derived tumor organoids can retain tumor-resident immune cells for a short time but progressively lose them [29][37]. Recent insights, especially into the importance of neuronal activity and neuron–glioma interactions in glioma proliferation, highlight the need for a neuronal TME in brain tumor models [51]. In TBOs and neoCORs, which are based on cerebral organoids, tumor cells interact with their neural surrounding [40][52][53][54].

Despite their advantages as the next-generation in vitro models, both TBOs and neoCORs are derived from cerebral organoids and, thus, share their challenges. They are difficult to standardize, with a high variance from one sample to the next, likely due to their reliance on self-organization. Currently, the field needs comprehensive strategies to incorporate key cell types, including microglia and near-native levels of astrocytes. These organoids self-arrest at the fetal levels of cellular maturity, peaking at the equivalent of weeks 17–24 of pregnancy [55][56]. Many researchers believe that further maturation requires a functional, perfusable vascular bed and a blood–brain barrier, which has not been demonstrated yet [57]. In this manner, organoids mimic early embryonal brain development (and thus a basic TME) and not mature brain tissue [58].

3. Organoid Models in Pediatric Brain Tumors

Pediatric brain tumors are much rarer than brain tumors in adults but belong to the most frequent tumor entities in children. Besides pediatric glioma and ependymoma, children are affected by embryonal tumors, including medulloblastoma, atypical teratoid and rhabdoid tumor (ATRT), and embryonal tumor with multilayered rosettes (ETMR), most of which are associated with a poor prognosis [59]. Despite recent achievements in establishing organoid models for adult brain tumors, such as glioblastoma or LGG [29][40][54][60], a similar body of work for pediatric brain tumors is still missing (**Table 1**). Unfortunately, the results gathered from in vitro tumor models cannot necessarily be transferred from adult to pediatric entities as tumor location and molecular characteristics of the tumor and the developmental state of the brain, as a key player of the TME, differ. Recent studies have shown that drugs that work perfectly in adults may cause major side effects on the developing brain of children [61]. Nevertheless, drugs are primarily established for adults and then transferred to pediatric patients [61]. Using 3D cell culture techniques might help to overcome this problem and directly establish drugs for this age group. Thus, one major challenge is the development of individual model systems for each pediatric brain tumor entity and subentity. In the last few years, some groups started testing PDO or neoCOR models for some of these entities [52][62][63][64]. Due to the scarcity of publications, the next section will summarize recent achievements by entity and not by model.

Table 1. Established three-dimensional brain tumor models.

Reference	Entity	Model-Type	Method
Hubert et al., 2016 [23]	glioblastoma	GBO	Tumor cells embedded in Matrigel
Jacob et al., 2020 [29]	glioblastoma	GBO	Tumor pieces on an orbital shaker
Loong et al., 2020 [65]	glioblastoma	GBO	Tumor cells embedded in Matrigel
Chen et al., 2022 [43]	glioblastoma	GBO	Tumor pieces on an orbital shaker
LeBlanc et al., 2022 [37]	glioblastoma	PDE	Tumor pieces in Matrigel
da Silva et al., 2018 [66]	glioblastoma	GLICO	Murine brain organoids, GBM cells
Linkous et al., 2019 [40]	glioblastoma	GLICO	Brain organoids + GBM cells
Krieger et al., 2020 [54]	glioblastoma	GLICO	Brain organoids + GBM cells
Gorancia-Buzhala et al., 2020 [67]	glioblastoma	GLICO	Brain organoids + GBM cells
Azzarelli et al., 2021 [68]	glioblastoma	GLICO	Brain organoids + GBM cells

Reference	Entity	Model-Type	Method
Ogawa et al., 2018 [69]	glioblastoma	neoCOR	HRas, TP53 mutations
Bian et al., 2018 [53]	glioblastoma	neoCOR	Several different mutations in combination and alone as PTEN, Myc, and EGFR
Yi et al., 2019 [70]	glioblastoma	bioprinting	GBM cells + endothelial cells + HUVECs
Heinrich et al., 2019 [71]	glioblastoma	bioprinting	GBM cells + macrophages
Tang et al., 2020 [72]	glioblastoma	bioprinting	GBM cells + neuronal progenitor cells + astrocytes + macrophages
Abdullah et al., 2022 [60]	LGG	PDO	Tumor pieces on an orbital shaker; 5% O_2
Sundar et al., 2022 [62]	pediatric HGG	PDO	Tumor cells embedded in Matrigel
Frisira et al., 2019 [64]	medulloblastoma	PDO	Tumor cells embedded in Matrigel
Ballabio et al., 2020 [63]	medulloblastoma	neoCOR	Different mutations, e.g., Otx-2 or c-Myc
Parisian et al., 2020 [52]	ATRT	neoCOR	SMARCB1-KD [73]

different. Recently, Sundar et al. generated organoids from pediatric HGG patients [62]. Here, organoids were formed by embedding single cells into Matrigel, followed by a shaking culture [62]. Distinct proliferative phenotypes were observed in the organoids pre- and post-treatment and evaluated via immunohistochemistry microarrays of the organoids. By testing the effects of the clinical standard of care (temozolomide and radiotherapy) on the proliferation of glioma sphere cultures and organoids, Sundar et al. found the organoids were resistant to this therapy, while the glioma spheroids stayed sensitive [62].

3.2. Medulloblastoma

Medulloblastoma is the most frequent malignant brain tumor of the cerebellum in children [74] and is divided into four consensus molecular subgroups: WNT, SHH, Group 3, and Group 4 [75]. Despite recent progress in the establishment of in vitro and in vivo medulloblastoma models, current models mainly cover SHH and Group 3 medulloblastoma and models for WNT and Group 4 are clearly underrepresented [76][77]. Therefore, a majority of patient tumors are not represented in current preclinical studies and the resulting information might only be relevant for a small group of patients [77].

Ivanov et al. pioneered a co-culture model of neuronal stem cells with tumor cells in spheroids to mimic tumor–host interactions for a cytotoxicity screen [42]. They generated medulloblastoma-neuronal stem cell (NSC) spheroids by seeding the same amount of medulloblastoma cells and NSCs, both labeled with distinct fluorescent dyes, followed by seven days of culture. For the cytotoxicity screen, spheroids were treated with different concentrations of Etoposide on day 3. Due to the presence of both tumor and healthy stem cells in the spheroid, they were able to

simultaneously assess the toxicity in both compartments by dissociation, followed by flow cytometry on day 7 [42]. Later, other groups used organoids to model medulloblastoma in vitro, using either organoids derived from patient cells [64] or neoplastic cerebellar organoids [63]. Medulloblastoma organoids can be derived from single cells and similarly cultured in Matrigel as glioblastoma organoids [23][64]. Ballabio et al. used another approach: they first pre-differentiated cerebellar organoids until day 35, when all progenitors were present, and this was followed by transfection of the intact organoid with several potential oncogenic mutations derived from a patient-specific screening [63]. This approach proved the ability of Oct-2 and c-Myc mutations to elicit a medulloblastoma-like phenotype in the organoids [63]. These pioneering studies display an important starting point of 3D medulloblastoma modeling which might help establish model systems that can represent the whole molecular diversity of medulloblastoma, via direct patient-derived organoids, or help establish organoid modeling for all entities in future studies.

3.3. Atypical Teratoid Rhabdoid Tumors

ATRTs, belonging to the group of embryonal brain tumors, are much less prevalent than medulloblastoma. ATRTs are characterized by the loss of the SMARCB1 or SMARCA4 gene, which encodes for a subunit of the SWI/SNF chromatin-remodeling complex [52][78]. Through the inactivation of SMARCB1 with CRISPR-Cas9 in neoCORs, Parisian et al. uncovered the effects of its knockdown (KD) on neuronal development [52]. The impact of SMARCB1 KD on the cells depended on the organoids' developmental stage. Interestingly, the KD blocked differentiation termination only in neuronal progenitor cells, while mature neuroblasts stayed unaffected, and human-induced pluripotent stem cells (hiPSCs) died [52]. This might indicate that malignancy initiation is only possible in a specific developmental window. In this model, only early neuronal progenitor cells had the capacity to transform into tumor cells [52], which hinted at the progenitor cells being the potential cells of origin for this tumor entity. This finding in organoids recapitulates current findings in mice, in which only defined cells of origin and differentiation states give rise to ATRT development when Smarcb1 or Smarca4 is lost [31][79][80][81].

3.4. Conclusions for 3D Models of Pediatric Brain Tumors

In summary, few organoid models have been established for pediatric brain tumors, and published work only exists for a subset of the overall tumor entities [52][62][63][64]. This may be partially due to the overall rarity of these diseases, and, thereby, limited sample availability. Examples of organoid models in pediatric brain tumors have shown that the same techniques used for adult glioblastoma can be transferred to pediatric brain tumors, opening similar opportunities for a wide range of potential applications in cancer research and personalized medicine. Special difficulties include how to model the variety of different entities and associated microenvironments. Differences in the componence of the tumor microenvironment, especially in the immune microenvironment, need to be considered when designing and establishing a new model. For instance, the immune infiltration in embryonal tumors, such as ATRT, medulloblastoma, and ETMR, is much lower than that of gliomas [82]. Additionally, within one entity, such as medulloblastoma, the infiltrating immune cell populations and numbers are significantly differing between distinct subgroups [82]. This makes the development of 3D models, which include also immune cells, even

more challenging. Moreover, in this field, further research is needed to establish organoid models for entities that have not yet been addressed, including ependymoma and ETMR.

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