REVIEW ARTICLE

Preclinical Experimental Models for Human Glioma

Article history:
Received: 06-09-2023
Revised: 29-12-2023
Accepted: 29-12-2023

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Abstract: Gliomas are one of the most common incurable brain tumors in adults with poor prognosis. Attempts at modeling human gliomas over the past decades have not only improved our knowledge of glioma biology but also boosted the development of therapeutic strategies. Despite great endeavors, gliomas are not responsive to the current tumor treatments, such as radiotherapy, chemotherapy, and immunotherapy due to their high inter- and intra-heterogenic tumor microenvironment (TME) and immune suppressive landscape. Therefore, it is significant to utilize suitable models to investigate the tumorigenesis, progression, and invasion of gliomas and evaluate potential therapies. Ideally, glioma models should fully recapitulate the genetic alterations and histological characteristics of the parental tumor, as well as reproduce the interactions between the tumor and its TME. In this review, we will discuss and compare the pros and cons of the current glioma models including traditional mouse models, established cell lines, newly 3D-cultured organoids, and 3D bioprinting glioma models in glioma pathogenesis research and therapy evaluation.

Keywords: Glioma; Glioblastoma; Experimental Models.

1. INTRODUCTION

Gliomas are the most malignant primary brain tumors that originate from neuroglial stem or progenitor cells (Weller *et al.*, 2015). Based on their histological appearances and molecular features, gliomas are diagnosed and categorized into different groups, which mainly comprise astrocytomas-with glioblastoma (GBM) being one of them-, oligodendrogliomas, and ependymomas. Depending on their degree of malignancy, gliomas are also classified as low-grade gliomas (LGGs, WHO grade I or II) and high-grade gliomas (HGGs, WHO grade III or IV) (Boccellato & Rehm, 2022; Chen *et al.*, 2017; Louis *et al.*, 2021). The WHO grade IV form of gliomas, known as GBM, has a very dismal five-year survival rate of just 6.8% (Ostrom *et al.*, 2021).

Gliomas, especially GBMs, exhibit high heterogeneity in both molecular and histological aspects. On the aspect of molecular level, a key genetic mutation is on isocitrate dehydrogenase (IDH), which frequently occurs in LGGs and secondary GBM (Cohen *et al.*, 2013). Other (epi)genetic mutations include the dysfunction of tumor suppressor genes (CDKN2A, TP53, PTEN, NF1, and RB1) and the amplification of oncogenes (EGFR, PI3K, CDK4, and PDGFRA) (Parsons *et al.*, 2008; Verhaak *et al.*, 2010). Hallmarks in histological aspects mainly involve proliferative microvasculature and pseudopalisading necrosis (Markwell *et al.*, 2022). In addition, studies focused on the TME have demonstrated that gliomas, particularly HGGs, are

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immunologically "cold". The microenvironment of GBM is notably populated by glioma-associated macrophages which display an immunosuppressive phenotype and induce the dysfunction of lymphocytes, promoting tumor progression and invasion (Chen & Hambardzumyan, 2018; Ma *et al.*, 2018; Wei *et al.*, 2020).

Given the complex heterogeneity and molecular alterations found in human gliomas, a proper preclinical model will be critical for the in-depth study of glioma biology and validations for potential drug targets. An ideal model should completely recapitulate the characteristics of the parental tumor and show resemblant therapeutic responses of human gliomas. In this review, we will discuss and summarize current experimental models for glioma research from traditionally used cell lines and mouse models to the newly *in vitro* 3D-cultured models.

2. ESTABLISHED CELL LINES AND CELL LINE-DERIVED XENOGRAFTS/ALLOGRAFTS

Cancer cell lines, generally generated from human or animals, carry specific genetic and morphologic characteristics of certain tumors, making them popular tools in cancer research. Conducting experiments on *in vitro* cultures is usually the first preclinical phase before clinical trials with a greater probability of success. In terms of gliomas, most of the cell lines are derived from human specimens of GBM or chemically induced murine anaplastic astrocytomas. Only a few lines from oligodendrogliomas and ependymomas have been reported since they are difficult to generate and maintain. Both human and murine cell lines possess certain prominent features of human glioma, either genetically or histologically (Table 1).

However, traditional 2D-cultured cell lines are too simple to reflect the heterogeneity of human gliomas and lack interactions with immune cells. By implanting commercial cell lines intracranially into mice has proven an excellent *in vivo* glioma model. Cell line-derived glioma mouse models can be classified as either xenografts or allografts. The former refers to the implantation of human cells like U87, U251, and HOG into immunocompromised mice which generally involve immunodeficient mouse strains such as nude mice, severe combined immunodeficient (SCID) mice, non-obese diabetic (NOD)/SCID mice, and NOD/SCID/interleukin IL-2 receptor γ_{null} (NSG) mice (Jin *et al.*, 2021). The latter indicates the implantation of murine cells into their syngeneic mice i.e., SMA-560 in VM/DK mice, GL-261 and CT-2A in C57BL/6J mice, which are immunocompetent (Letchuman *et al.*, 2022).

The advantages of cell line-derived models include low cost, high predictability, fast throughput, and reliable progression of tumors (Jin *et al.*, 2021; Kijima & Kanemura, 2017). Due to the commercially available stable cell lines and highly reproducible implantation methods, a large number of experimental models can be generated in the short term (Hicks *et al.*, 2021; Zalles & Towner, 2021). Moreover, these transplant mouse models can preserve glioma-associated genetic profiles and largely recapitulate the microenvironment and histopathology of primary tumors, benefiting studies of glioma biology and potential treatments. However, xenografts lack a competent immune system, and the immune systems and TME of mouse allografts differ from their human counterparts in many ways. More significantly, after being cultured in serum-containing media for a long time, cell lines may have undergone clonal selection and accumulated genetic and phenotypic variations, making it difficult to recreate the heterogeneity and intricate genetic and phenotypic characteristics of human gliomas in xenografts/allografts (Daniel *et al.*, 2009; Huszthy *et al.*, 2012).

2.1. Human cell lines and cell line-derived xenografts

Human GBM cell lines including U251, U87, LN229, LN18, and T98G, among which U87 and U251 have been broadly used in preclinical research since generated from patients with GBM in the 1960s. There are only a few commercial LGG cell lines, with the HOG cell line being commonly used, which was established from a human oligodendroglioma specimen (Tang *et al.*, 2023). These adherent cells are frequently used in various aspects of tumor progression and specific signaling pathways such as metabolic reprogramming, angiogenesis, apoptosis, and autophagy signaling, as well as potential treatments targeting these pathways (Kleihues, 2010). However, these cell lines usually undergo extended culture and hundreds of times passages and hence their original genetic profile may have changed.

Cell line	Origin	Characteristics	Advantages	Disadvantages
U87	Human astrocytoma	• Well-established and widely used • Methylated MGMT and mutated hTERT, ATRX and PTEN • Capable of forming GSCs *in vitro*	• Genetically akin to human GBM • Can be used in pre-clinical research on GSCs and anti-angiogenesis therapies	• Issues of authenticity • Different histology with human GBM • Sensitive to TMZ and radiotherapy
U251	Human astrocytoma	• Well-established and widely used • Methylated MGMT and mutated PTEN, hTERT and p53 • Capable of forming GSCs *in vitro*	• Recapitulating human astrocytoma histology • Genetically akin to human GBM • Can be used in pre-clinical research on GSCs	• Phenotypic changes caused by long-term culture • Different invasive patterns with human GBM • Sensitive to TMZ and radiotherapy
HOG	Human oligodendroglioma	• Modestly used • Expressing CNPase and the 15-kDa form of MBP • Limited glutamine requirement	• A useful LGG model • Can be used in pre-clinical research on oligodendroglioma	• Lacking GSC characteristics • Limited applications in glioma research
GL261	Carcinogen-induced murine glioblastoma	• Well-established and widely used • Mutated K-Ras and p53 • Immune-suppressive phenotype but with high expression of MHC I • Capable of forming GSCs *in vitro*	• Recapitulating human ependymoblastoma histology • Can be used in pre-clinical research on immunotherapies and GSCs	• Highly tumorigenic and immunogenic • Phenotypic changes caused by long-term culture
CT-2A	Carcinogen-induced murine glioblastoma	• Modestly used • Deficient PTEN and TSC2 • Expressing high levels of complex gangliosides with low distribution of GM3 • Immune-suppressive phenotype • Highly proliferative • Capable of forming GSCs *in vitro*	• Resistant to TMZ and radiotherapy • Can be used in pre-clinical research on immunotherapies and GSCs	• Highly tumorigenic and immunogenic • Tumor mutational burden not well characterized • Different invasion patterns with human GBM
SMA-560	Spontaneously murine astrocytoma	• Modestly used • Expressing high levels of GFAP and GS • Secreting TGFβ with low MHC I and MHC II expression • Capable of forming GSCs *in vitro*	• Recapitulating human anaplastic astrocytoma • Can be used in pre-clinical research on immunotherapies and GSCs	• More tumorigenic and immunogenic than human GBM • Different invasion patterns with human GBM • Not commercially available and limited applications in GBM research

Table 1. Comparison of human and syngeneic cell lines.

U87

The U87 line, established from a grade III astrocytoma-glioblastoma in a 44-year-old woman is the most ubiquitously used human GBM cell line (J. Ponten & Macintyre, 1968). Genetically, this cell line shows certain similarities to human GBM. U87 cells have mutations in hTERT, ATRX, and PTEN, carry no p53 or IDH1 mutations, and have a methylated MGMT status (Haddad *et al.*, 2021; Patil *et al.*, 2015). These cells also carry mutations in genes controlling the cell cycle, causing deletions in $p14^{ARF}$/p16 regulatory subunits of cyclin-dependent kinases (Ishii *et al.*, 1999; Schulz *et al.*, 2022). In addition, this cell line contains a fraction of stem-like cells, possessing the ability of self-renewal and the formation of secondary tumor spheres (Yu *et al.*, 2008), which could be useful for the study of glioma stem cells (GSCs).

Although the U87 line is widely used in thousands of publications, its authenticity is still controversial. Researchers by using DNA fingerprinting found that the widely used U87 cell line from the American Type Culture Collection (ATCC) was not identical to the initial one (Allen *et al.*, 2016). The current U87 line does have GBM features whereas it deviates from its origin. Besides, U87 cells are sensitive to radiotherapy and temozolomide (TMZ) treatments in cell culture, which also differ from human GBM (Akbarnejad *et al.*, 2017; Ryu *et al.*, 2012; Wachsberger *et al.*, 2007; Wang *et al.*, 2014). Histologically, U87 orthotopic xenografts have been demonstrated to be very unlike human GBM, presenting expansile growth, lack of glial fibrillary acid protein (GFAP) expression (an astrocyte marker), functional p53 expression, and inconsistent hypoxia-inducible factor 1α (HIF-1α) expression. U87 tumors are highly vascularized but rarely necrotic in their core without pseudopalisading patterns (Radaelli *et al.*, 2009; Schulz *et al.*, 2022).

U251

The U251 line was isolated from a 75-year-old male patient with GBM (Jan Ponten, 1975). Genetically, the U251 cell line covers a broad spectrum of genetic variability, including mutant PTEN, upregulation of PI3K and Akt, non-functional p53, $p14^{ARF}$/p16 deletion, and a methylated MGMT status (Ishii *et al.*, 1999; Schulz *et al.*, 2022). Similar to U87, U251 cells also contain a subset of cells expressing CD133, a marker for GSCs, capable of forming neuro-spheres. These $CD133^{+}$ cells can serve as a useful model for GSCs studying and drug screening (Qiang *et al.*, 2009). Histologically, U251 orthotopic tumors resemble most of the key salient features of GBM, such as positive staining for vimentin and GFAP, expansile invasion into surrounding brain parenchyma, microvascular proliferation with hemorrhage as well as pseudopalisading necrosis (Candolfi *et al.*, 2007; Jacobs *et al.*, 2011). The presence of active caspase-3 signaling and HIF-1α expression along pseudopalisades may indicate the intricate network of apoptosis, hypoxia, angiogenesis, pseudopalisading necrosis, and neoplastic invasion (Radaelli *et al.*, 2009).

However, unlike the invasive pattern observed in human GBM specimens, tumor cells migrating into the white matter tracks were not detected in the U251 model (Candolfi *et al.*, 2007). Additionally, U251 is known to be responsive to TMZ and radiation treatments both *in vivo* and *in vitro* (Haddad *et al.*, 2021). Similar to U87, long-term subclones of U251 accumulated genetic aberrations resulting in a variety of phenotypic changes compared with the original U251 line (Torsvik *et al.*, 2014).

HOG

The HOG glioma line was established from a human oligodendroglioma specimen two decades ago (Post & Dawson, 1992). HOG cells do not express astrocyte marker GFAP, but express oligodendrocyte markers including CNPase protein and the 15-kDa form of myelin basic protein (MBP), a marker for immature oligodendrocytes (Nistér & Westermark, 1994). Key applications of the HOG cell line are based on cell assays specifically on cellular metabolism. HOG cells were found to have minimal levels of glutamine synthetase activity and exhibit limited glutamine requirements for survival and growth (Chiu *et al.*, 2018). An analysis of the cellular metabolome of HOG cells demonstrated that IDH1 and IDH2 mutants change the levels of amino acids, glutathione metabolites, tricarboxylic acid (TCA) cycle intermediates, and choline derivatives (Reitman *et al.*, 2011).

However, HOG cells are IDH wild-type and lack expression of GSCs-related genes like CD133, SOX2, nestin, and Olig2 (Long *et al.*, 2013). This genetic profile may hamper the applications of HOG cells in the study of oligodendroglioma. Moreover, unlike cell lines derived from GBM, HOG cells are rarely used in mouse xenograft models. Huang and

colleagues evaluated the anti-glioma effects of Caesalpin A *in vivo* by utilizing a nude mouse model that subcutaneously bore HOG IDH1-mutant tumors (Huang *et al.*, 2021).

2.2. Syngeneic murine cell lines and cell line-derived allografts

Murine GBM cell lines such as GL261, CT-2A, and SMA-560 have been popularized in glioma research. GL261 and CT-2A were generated from murine glioma induced by carcinogens, while SMA-560 was spontaneously derived. As these cells are orthotopically implanted in a syngeneic mouse, one can carry out GBM tumor immunology research and test immunotherapeutic compounds *in vivo* with an immunocompetent system (Kijima & Kanemura, 2017; Ren *et al.*, 2023). Nevertheless, these murine models cannot model the actual human GBM microenvironment as the mouse immune system and TME is different from their human counterparts. A big disadvantage of the chemically induced murine cell lines is that they carry higher mutational burdens than human GBM. As a result, these species and immunogenic discrepancies between syngeneic murine models and human GBM may lead to the inconsistency between positive preclinical results and negative outcomes in human clinical trials (Letchuman *et al.*, 2022; Oh *et al.*, 2014).

GL261

The GL261 tumor was first generated in the 1930s, from chemically induced C57BL/6J mice via injecting 20-methylcholanthrene into their brains (Seligman *et al.*, 1939). Through serial *in vivo* passages-transplanting tumor fragments subcutaneously and intracranially into C57BL/6J mice, a stable GL261 cell line was established in the mid-1990s, facilitating future *in vitro* assays and *in situ* primary brain tumor studies in the syngeneic mice (Ausman *et al.*, 1970; Szatmari *et al.*, 2006). Genetically, GL261 cells carry mutations in the K-Ras oncogene and p53 tumor suppressor gene, resulting in high expression of c-myc (Oh *et al.*, 2014). Given that GL261 cells carry wild-type IDH1, the introduction of the R132H mutation has been utilized to develop the IDH1-mutated intracranial glioma model, supporting translational research of immunological targeting of the mutation of IDH1 in glioma patients (Pellegatta *et al.*, 2015). Histologically, GL261 tumors mimic human ependymoblastomas well. The H&E sections of GL261 tumors show that there are multiple necrotic areas throughout the tumors, with densely packed cells lining these areas in a pseudopalisading pattern (Kleihues, 2010). Several studies demonstrated that murine GL261 glioma recapitulates its human counterpart in a variety of ways, but most significantly in its features of invasiveness and angiogenesis (Ausman *et al.*, 1970; Zagzag *et al.*, 2000). GL261 tumors also show an immune-suppressive phenotype with large numbers of exhausted T cells and macrophages (Khalsa *et al.*, 2020). Besides, similar to human GBM cell lines, GL261 cells contain a population expressing CD133 and nestin when grown in serum-free media. These $CD133^+$ GL261 cells have elevated tumorigenicity and a stem-like phenotype, which facilitates their applications in GSC research (Wu *et al.*, 2008).

Although the GL261 cell line has been widely used in preclinical studies, its disadvantages should also be taken into account. Concerning immunological features, GL261 may not accurately reflect the immunogenicity of human GBM. GL261 cells express high levels of major histocompatibility complex MHC I, while the expressions of MHC II and T cell activation co-stimulatory molecules B7-1 and B7-2 are relatively low, indicating that GL261 cells are sensitive to MHC I-dependent $CD8^+$ cytotoxic T cells (Szatmari *et al.*, 2006). Moreover, unlike human GBM, GL261 cells bear a higher tumor mutational load and numerous predicted neoepitopes (Johanns *et al.*, 2016). This may render the inconsistency of the immunotherapy outcomes between preclinical research and clinical trials. Last, like many other cell lines, it is also noteworthy that the GL261 cell line may differ between labs under a long-term culture.

CT-2A

Like GL261, CT-2A tumor was generated from 20-methylcholanthrene intracranially injected C57BL/6J mice as well (Zimmerman & Arnold, 1941). The CT-2A cell line was obtained from the flank-growing tumors which were maintained by subcutaneous transplants over many generations (Seyfried *et al.*, 1992). Genetically, similar to human astrocytomas, the CT-2A cell line is p53 wild-type with deficient PTEN and tuberous sclerosis complex 2 (TSC2) expression (Marsh *et al.*, 2008). Remarkably, CT-2A express high levels of the structurally more complex gangliosides and low levels of

the anti-angiogenesis monosialoganglioside GM3, suggesting the angiogenic features of the CT-2A tumors (Abate *et al.*, 2006; Seyfried *et al.*, 1992; Seyfried & Mukherjee, 2010). Histologically, the CT-2A experimental tumors possess features akin to human astrocytomas, including high mitotic index, elevated cellular density, angiogenesis, hemorrhage, and pseudopalisading necrosis. (Martínez-Murillo & Martínez, 2007; Mukherjee *et al.*, 2004). Additionally, the immunogenomic landscape of CT-2A also shows some characteristics in accordance with human GBM, such as decreased αPD-L1 sensitivity, abundant infiltrating macrophages, and hypofunctional T cells (Liu *et al.*, 2020). Researchers found low enrichment of genes related to immune response pathways compared with GL261 tumors (Khalsa *et al.*, 2020). In terms of stemness, CT-2A neurospheres can be formed in serum-free media. Of note, CT-2A cells and neurospheres are highly proliferative and aggressive, which may offer potential utilization in the research of GSCs in an immunocompetent environment (Binello *et al.*, 2012).

While CT-2A tumors are highly proliferative, they exhibit a distinct border with surrounding brain parenchyma. This low-invasion pattern is not consistent with human GBM (Martínez-Murillo & Martínez, 2007; Shelton *et al.*, 2010). In addition, due to the fact that CT-2A tumors were caused by carcinogens, this cell line also carries greater tumorigenesis and immunogenesis mutational burdens than primary human GBM (Liu *et al.*, 2020). Compared to GL261, the CT-2A model is relatively less used in GBM research. Although described as radio- and chemo-resistance by a few research, more specific effects and mechanisms need to be elucidated (Oh *et al.*, 2014; Riva *et al.*, 2021).

SMA-560

Unlike GL261 and CT-2A, the SMA-560 model was generated from the inbred VM/DK mouse strain in which astrocytomas occurred spontaneously (Fraser, 1971). The stable tumorigenic cell line was established through culturing tumors that were transplanted subcutaneously into syngeneic mice in the 1980s (Pilkington *et al.*, 1983; Serano *et al.*, 1980). Histologically, SMA-560 mirrors human anaplastic astrocytomas well, expressing high levels of astrocyte marker GFAP and glutamine synthetase (GS) and minimal levels of S-100 protein. These tumors exhibit such a solitary invasive pattern that nearly all the tumors are confined in the white matter and tend to spread along white matter tracts (Fraser, 1971; Serano *et al.*, 1980). Interestingly, SMA-560 secretes the immunosuppressive cytokines TGF-β and exhibits low expression of MHC I and MHC II, indicating the indispensable application of SMA-560 for studies on the impact of immunosuppression on GBM immunotherapies (Sampson *et al.*, 1997). Furthermore, the serum-free media supports SMA-560 sphere formation. The *in situ* VM/DK mice that bear SMA-560 spheres show higher expression of the vascularization marker CD31 and tend to have shorter median survival days compared to the SMA-560 non-sphere model (Ahmad *et al.*, 2014).

However, genetically, this cell line has also been demonstrated to be more tumorigenic and immunogenic, as it carries more genetic mutations than human GBM (Johanns *et al.*, 2016). Recently, Silginer and her colleagues found that SMA-560 expresses the hepatocyte growth factor (HGF) and its receptor MET both *in vivo* and *in vitro*, and synergistic suppression of tumor growth by MET inhibition and irradiation was observed (Silginer *et al.*, 2023). Despite its potential use in investigations of GBM immunotherapies, the SMA-560 cell line has not been widely used, most likely because it is not commercially available (Oh *et al.*, 2014).

3. PATIENT-DERIVED XENOGRAFTS

Similar to cell line-derived xenografts, by implanting fresh human glioma cells or tissues into immunocompromised mice, patient-derived xenografts (PDXs) are generated. Due to the potential influence of culture conditions on human cell line phenotype and heterogeneity, PDXs tend to gain more popularity in preclinical research. Freshly isolated human glioma specimens in immunodeficient mice can recapitulate the most original salient features of primary tumors without any *in vitro* artificial selections (Liu *et al.*, 2023; Vaubel *et al.*, 2020). Immunocompromised mouse strains like nude mice, NOD/SCID mice, and NSG mice have been widely used to generate human glioma PDX models (Shi *et al.*, 2022; Stringer *et al.*, 2019; Wang *et al.*, 2017). Moreover, the introduction of humanized mice has been reported to increase the PDX success rate. Humanized mice are created through genetic approaches or by transplanting human hematopoietic stem cells into severely immunodeficient mice (Bosenberg et al, 2023). These mice can somewhat imitate the human immune system but fall short of

a fully developed and functional human immune system (Okada *et al.*, 2019).

Since PDX models preserve the (epi)genetic characteristics and heterogeneity of the primary tumor in glioma patients, they provide the most pertinent *in vivo* cancer model for precision therapy. More significantly, the establishment of humanized PDXs made it possible to simulate the interactions between gliomas and human immune systems. Nevertheless, there are multiple shortcomings that need to be considered. Unlike xenografts from cell lines, PDXs are costly, time-consuming, and technically challenging, with the success rate varying from 10% to 90% (Jung *et al.*, 2018; Yoshida, 2020). Another obvious limitation of PDXs is the utilization of immune-deficient mice and non-fully-developed humanized mice, which means that PDXs cannot model the accurate immunological TME of glioma in patients. Besides, PDX models may experience mouse-specific tumor evolution and exhibit different genetic and histological characteristics with tumors acquired from patients (Ben-David *et al.*, 2017). For example, a study found that glioma orthotopic PDXs show absent PDGFRA amplification and lack proliferative microvasculature and necrosis (Vaubel *et al.*, 2020).

4. GENETICALLY ENGINEERED MOUSE MODELS (GEMMs)

Although allografts and xenografts are commonly used, the tumorigenesis in these models by implanting large numbers of cells or pieces of fresh tumor tissue into mice does not resemble the pathogenesis of human gliomas, which is assumed to initiate through the variation of a single cell (Stylli *et al.*, 2015). Therefore, a new approach known as GEMMs has emerged. By manipulating the genome of mice, researchers are able to engineer a strain of mice that spontaneously develops glioma. The major strategy to generate GEMMs of cancers is to introduce specific gene alterations that activate oncogenes or inactivate tumor-suppressor genes in germline or somatic cells (Jin *et al.*, 2021). Advanced genetic engineering technologies like the Cre-LoxP system, RCAS-TVA system, CRISPR-Cas9 editing, and transposon- or viral-based integration have facilitated the precise manipulation of mutations discovered in human gliomas and genes of interest (Noorani, 2019). For instance, co-expressing oncogene H-Ras and AKT and loss of p53 through Cre-LoxP-controlled lentiviral vectors in a small number of $GFAP^+$ cells have been shown to induce human high-grade gliomas in adult immunocompetent mice (Marumoto *et al.*, 2009). Additionally, researchers have successfully developed the mutant IDH1-driven astrocytomas cooperated with clinically relevant gliomagenesis mutations and revealed that the *de novo* pyrimidine synthesis pathway is a potential therapeutic target in IDH1 mutant gliomas (Philip *et al.*, 2018; Shi *et al.*, 2022).

As the *de novo* tumor is achieved by specific genetic manipulations, GEMMs are powerful tools to investigate the genetic drivers for gliomagenesis and the underlying molecular mechanisms (Kersten *et al.*, 2017). Comparatively, unlike orthotopically transplant models, transgenic mice can prevent potential damage to the blood-brain barrier (BBB) (Haddad *et al.*, 2021). Hence, GEMMs are suitable models for studying the microenvironment of gliomas and drug distribution as they have competent immune systems and intact BBB (Hetze *et al.*, 2021; Lentin *et al.*, 2017). Despite the advantages of molecular investigations, GEMMs suffer from massive costs, long experimental periods, complex breeding schemes, and variable tumorigenesis rates (McNeill *et al.*, 2015). Moreover, GEMMs cannot model intratumoral heterogeneity, and transgenic mice may result in tumors characterized by mixed histological grades so that they fail to reflect key features seen in human gliomas (Huszthy *et al.*, 2012). It is also noteworthy that the genetic alterations may disrupt important signaling pathways and induce severe developmental defects before a desired tumor phenotype emerges (Rankin *et al.*, 2012).

5. ORGANOIDS

Although mouse models can simulate interactions between multiple cells, organs, and microenvironments, the absence of human targets may result in variations in drug effectiveness due to species differences. Additionally, the rodent brain cannot accurately replicate the GBM microenvironment found in humans (Klein *et al.*, 2020; Xu *et al.*, 2023). Consequently, scientists have redirected their focus towards organoids, the three-dimensional models that are self-assembled by tissue or induced pluripotent stem cells (iPSCs) in response to specific growth factors. These structures exhibit certain structural and functional similarities to the original organs and can be consistently expanded within an *in vitro* 3D-cultured system to mimic the developmental and functional characteristics of human

organs (Clevers, 2016). Generally, there are three commonly employed methods for constructing GBOs: (1) Organoid construction using iPSCs; (2) Organoid generation utilizing GBM cells derived from patient tumor tissues; (3) Organoids generated through co-culturing gene-edited brain organoids or brain organoids with GBM.

In 2016, Hubert et al. utilized patient-derived GBM cells to generate glioblastoma organoids (GBOs) in Matrigel, which successfully replicated the stem cell heterogeneity and hypoxic gradient observed in human tumors. These organoids also maintained the tumorigenic and diffuse invasive phenotypes of primary tumors and were employed to investigate their interaction with GSCs in proliferative and hypoxic regions (Hubert *et al.*, 2016). Through long stable passages, these patient-derived GBOs still preserve the heterogeneity, gene expression, and mutational spectrum of parental tumor cells, allowing for the utilization to investigate the mechanisms underlying patient-specific responses to immunotherapy (Jacob, Ming *et al.*, 2020; Jacob, Salinas *et al.*, 2020). Ogawa et al. employed a combination of organoid technology and CRISPR/Cas9 technology to target oncogenes/tumor suppressors in brain organoids and observed tumor development in GBOs with pronounced aggressiveness (Ogawa *et al.*, 2018). Additionally, by co-culturing human embryonic stem cell-derived brain organoids with patient-derived GSCs, Linkous et al. established a "GLICO" model, which enables the investigation of structural and biological characteristics of patient-derived GBM within an *in vitro* brain-like microenvironment and can be readily scaled up for high-throughput drug screening (Linkous *et al.*, 2019). GBOs overcome certain limitations of traditional glioma models and exhibit comparable tissue structure and functional characteristics to primary tumors. They effectively recapitulate the high heterogeneity, aggressiveness, and drug resistance exhibited by parental tumors. Furthermore, these GBOs can be generated within a short timeframe while allowing for long-term passage and cryopreservation. Their ease of expansion makes them highly suitable for medium- to high-throughput drug screening, facilitating biobank establishment and enabling rapid identification of optimal drug combinations tailored to individual patients' needs. Therefore, GBO represents an advantageous model for evaluating therapeutic drugs targeting GBM (Jacob, Salinas, *et al.*, 2020; Rajan *et al.*, 2023; Ratliff *et al.*, 2022; Xu *et al.*, 2023; Zhang *et al.*, 2020).

Despite their widespread use in GBM research, there are still certain limitations, such as high-cost requirements for sampling immediately after tissue resection and the lack of vascular systems or transient immune components, which may hinder validation studies on immunotherapeutic effects (Jacob, Salinas, *et al.*, 2020; Linkous *et al.*, 2019; Ogawa *et al.*, 2018; Zhang *et al.*, 2020).

6. 3D BIOPRINTING GLIOMA MODELS

The application of advanced 3D bioprinting technology enables the rapid generation of diverse spatially organized cultures comprising various cell types and substrates like biological tissues, organoids, and tumor models. This cutting-edge technology holds immense potential in disease modeling, drug research, and cancer research (Heinrich, Liu, *et al.*, 2019; Matai *et al.*, 2020; Tang *et al.*, 2021; Wu *et al.*, 2023). Notably, cancer research stands to benefit significantly from bioprinting techniques as they allow for the reproduction of TME, a crucial determinant in tumorigenesis, progression dynamics, and metastatic dissemination (Bejarano *et al.*, 2021; Shukla *et al.*, 2022; Wu *et al.*, 2023).

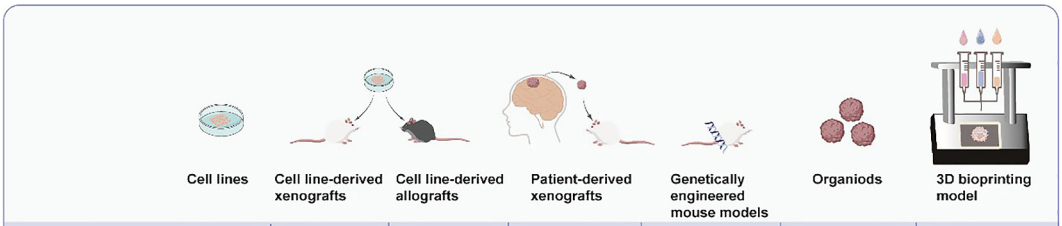
The initial utilization of 3D bioprinting technology in GBM research was printing GAF hydrogel scaffolds containing GSCs using a bio-printer to investigate key mechanisms underlying gliomagenesis and drug resistance (Dai *et al.*, 2016). GSCs represent an exclusive subset of cells within GBM that possess remarkable self-renewal capacity along with differentiation potential and are intricately associated with gliomagenesis, invasion, malignancy, and heterogeneity. Compared to traditional *in vitro* cell cultures, the bioprinting GSC model exhibits prolonged viability while also closely mimicking physiological conditions by displaying heightened expression levels of genes implicated in angiogenesis (Dai *et al.*, 2016; Suva & Tirosh, 2020). Consequently, this unique model offers unprecedented opportunities for investigations into gliomagenesis, drug resistance, and the involvement of GSCs during GBM angiogenesis (Dai *et al.*, 2016; Ruiz-Garcia *et al.*, 2020; Wang *et al.*, 2019; Wang *et al.*, 2018; Wang *et al.*, 2021). In addition, the combination of a 3D bioprinting system and light crosslinking technology allows for the selection of bioinks with diverse components to make co-culture GBM models. This approach not only recapitulates the microenvironment of GBM, its invasion of brain parenchyma, and recruitment and

polarization of glioma-associated macrophages but also exhibits characteristics of hypoxia response. It can be utilized to investigate the interaction of GBM with microenvironment and macrophages in a biomimetic 3D environment, as well as the hypoxia signal of GBM in a physiological environment (Heinrich, Bansal, *et al.*, 2019; Neufeld *et al.*, 2021; Tang *et al.*, 2020). Additionally, the multilineage model constructed with the multi-nozzle extrusion bioprinter and bioprinted human-GBM-on-a-chip maintained the primary tumor structure and glioma cells show robust metabolic activity and proliferation ability. By maintaining the microenvironment and hypoxia gradient of glioma, these models serve as valuable tools for investigating glioma TME, conducting preclinical drug sensitivity tests, and screening effective drug combinations (Hermida *et al.*, 2020; Yi *et al.*, 2019).

However, it is significant to note that the current limitations of 3D bioprinting technology, such as high costs associated with specific bioprinters and biocompatible bio-inks, challenges in scaling production capacity, and difficulties in constructing complex tissue models (Heinrich, Liu, *et al.*, 2019; Murphy *et al.*, 2020; Wu *et al.*, 2023). Nevertheless, with the innovation of technology and the development of new materials, the field of 3D bioprinting is expected to make significant progress.

7. DISCUSSION

Reliable preclinical models are crucial to investigating the mechanisms and signaling pathways of glioma initiation, invasion, and drug resistance. Consequently, they should faithfully recapitulate diverse characteristics of gliomas, including tumor behaviors, mutational spectrum, microenvironmental heterogeneity, and cell-cell interactions. Current preclinical experimental models for human glioma can be classified into: *in vitro* models such as glioma cell lines, organoids, and 3D bioprinting glioma models, and *in vivo* models including cell line-derived mouse xenografts/allografts, PDXs, and GEMMs (Fig. 1).



	Cell lines	Cell line-derived xenografts	Cell line-derived allografts	Patient-derived xenografts	Genetically engineered mouse models	Organiods	3D bioprinting model
Cost	$	$$	$$	$$$	$$$	$$$	$$$$
Time/Labor	+	+ +	+ +	+ + +	+ + +	+ + +	+ +
Immune system	No	No	Yes	No	Yes	Yes	Yes
Recapitulation of human TME	No	Yes	Yes	Good	Good	Good	Excellent
Maintenance of parental tumor characteristics	No	No	No	Good	No	Excellent	Excellent
High-throughput drug screening	Yes	No	No	No	No	Yes	Yes

Figure 1. Current preclinical experimental models for human glioma.

Cell lines are cost-effective and sustainable models for investigating specific signaling pathways that contribute to glioma progression. Whereas they lack interactions with the TME and present challenges in maintaining the genetic background of primary cells during passages (Van Meir *et al.*, 2010; Xu *et al.*, 2023). By contrast, the two novel *in vitro* 3D-cultured models, organoids

and 3D bioprinting glioma models, comprehensively recapitulate the complex TME within GBM, and afford the ability to retain the heterogeneity, gene expression, and mutational spectrum of parental glioma. Despite the expensive cost and high requirements for apparatus, these technologies are still valuable instruments for investigating the mechanisms underlying patient-specific responses to immunotherapy and highly effective tools for conducting high-throughput drug screening (Jacob, Ming *et al.*, 2020; Jacob, Salinas, *et al.*, 2020; Linkous *et al.*, 2019; Ruiz-Garcia *et al.*, 2020; Wang *et al.*, 2019; Wang *et al.*, 2018; Wang *et al.*, 2021). As the most popular *in vivo* glioma model, mouse models can provide valuable insights into glioma biology and certain molecular mechanisms that contribute to tumor progression. Cell line-derived mouse models are widely used to test the efficacy and toxicity of novel therapeutics, as they are relatively more reproducible and consume less time and labor compared to PDXs and GEMMs. Nevertheless, PDXs can offer more actual clues in human glioma TME and GEMMs enable tumorigenesis investigation on a molecular level, both of which facilitate in-depth research on human glioma (Daniel *et al.*, 2009; Klein *et al.*, 2020; Xu *et al.*, 2023).

However, it is imperative to acknowledge that no single model can comprehensively recapitulate diverse facets of gliomas, as each model has its inherent limitations. As such, selecting a suitable combination of these models based on specific research objectives would effectively enhance the fundamental understanding of glioma, facilitate the development of novel drugs, and promote translational applications (Ren *et al.*, 2023).

Acknowledgment & funding

This study was supported by STI2030-Major Project #2021ZD0201100 Task 1 #2021ZD0201101, CAMS Major Collaborative Innovation Project 2022-I2M-2-002, the National Natural Science Foundation of China grants (82002094), the CAMS Major Collaborative Innovation Project 2016-I2M-1-011 and Beijing Natural Science Foundation (7222254).

Conflict of interest disclosures

The authors declare that there are no conflicts of interest.

List of Abbreviations

Abbreviation	Definition
GBM	glioblastoma
TME	tumor microenvironment
LGG	low-grade glioma
HGG	high-grade glioma
GSC	glioma stem cell
TMZ	temozolomide
PDX	patient-derived xenograft
GEMM	genetically engineered mouse model
BBB	blood-brain barrier
GBO	glioblastoma organoid
iPSC	induced pluripotent stem cells

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