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An overview of glioblastoma multiforme *in vitro* experimental models

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Abstract

Glioblastoma multiforme (GBM) is the most common primary brain tumor, characterized by a remarkable inner complexity and inter-tumor variability. Moreover, it is very aggressive and resistant to conventional treatments, so that it rapidly relapse. Therefore, there is an immediate need for experimental strategies to enhance our comprehension of GBM, aiming to mitigate its economic and social impact. Here, we described different *in vivo* and *in vitro* strategies currently used for the study of GBM. First, we gave a brief and general overview of the classical *in vivo* models, including xenograft mouse and zebrafish models and canine models, offering a wide range of advantages but also presenting a series of strong limitations. Thus, we described *in vitro* models, starting from more traditional 2D culture models, comparing different approaches and critically exposing the advantages and disadvantages of using one or the other methods. We also briefly described GBM 2D culture systems that allow recreating multiple cell-cell and cell-extracellular matrix contacts but still do not reflect the complexity of *in vivo* tumors. We finally described the intricacies of the more novel 3D *in vitro* models, e.g., spheroids and organoids. These sophisticated models have demonstrated exceptional suitability across a wide spectrum of applications in cancer research, ranging from fundamental scientific inquiries to applications in translational research. Their adaptability and three-dimensional architecture render them invaluable tools, offering new insights and paving the way for advancements in both basic and applied research.

Introduction

Glioblastoma multiforme (GBM) is a type of malignant brain tumor that arises from the glial cells in the brain, primarily astrocytes, but can also appear in the brain stem, cerebellum, and spinal cord. It is the most common and aggressive form of primary brain tumor in adults, accounting for approximately 45% of all tumors, and it is characterized by rapid growth and the ability to infiltrate surrounding brain tissue, making it difficult to treat and often associated with a poor prognosis.[1]

The fifth edition of the World Health Organization (WHO) Classification of Tumors of the Central Nervous System (CNS), published in 2021, introduced several changes compared to the previous editions. Thus, to date, GBMs are classified as "glioblastoma, isocitrate dehydrogenase (IDH)-wildtype" tumors within this system, by taking into account their histological and molecular characteristics.[2] In IDH-wildtype GBM, there are no mutations in the IDH genes (IDH1 or IDH2) and it is classified as Grade IV due to its highly aggressive and invasive nature. Moreover, the diagnosis of GBM is made by the finding of a glial neoplasm with a diffusely infiltrating appearance, presenting at least one of the following features: microvascular proliferation, necrosis, telomerase reverse transcriptase (TERT) gene promoter mutation, epidermal growth factor receptor (EGFR) gene amplification or simultaneous gain of an additional chromosome 7 and complete deletion of chromosome 10.[3]

GBMs may be classified as primary or secondary. Primary GBM, also known as *de novo* GBM, is the most common form and represents the majority of cases. It occurs without a prior history of lower-grade gliomas or other brain tumors. Secondary GBM arises from the transformation of a pre-existing lower-grade glioma, such as an astrocytoma or oligodendroglioma. This transformation involves the progression of a lower-grade tumor to a higher-grade, more aggressive GBM.[4]

A further, less frequent entity without an official WHO 2021 nomenclature is the "GBM NOS" which stands for "GBM Not Otherwise Specified". This term is used in pathology and medical documentation to describe a tumor that does not fit into the more specific subcategories or molecular classifications within the GBM spectrum. It is a general designation for GBMs that do not have unique molecular or histological features that would classify them as primary or secondary GBMs or into GBM, IDH-wildtype. GBM NOS is essentially a diagnosis of exclusion, indicating that the tumor shares the aggressive characteristics of GBM, but lacks specific molecular or histological markers that would categorize it differently. It is still considered a high-grade and highly malignant brain tumor.[2]

Symptoms of GBM vary depending on its size, location, and growth rate and include headaches, nausea, vomiting, vision problems, confusion, and changes in mood and personality. The diagnosis relies on a combination of neurological examinations and radio-diagnostic tests. However, despite

advancements in the current standard of care, the prognosis for GBM remains poor, typically resulting in a survival period of 14-15 months post-diagnosis. Notably, elderly patients tend to experience a notable worsening, with an average survival of fewer than 8.5 months from the point of diagnosis.[5]

Treatment depends on several factors and includes surgery to remove the tumor, as well as radiotherapy and chemotherapy to target residual cells. Thus, GBM is very difficult to treat, as many drugs cannot cross the blood-brain barrier, the tumor cells are very resistant to conventional therapies, and the brain, susceptible to damage by conventional therapy, has a very limited ability to repair itself. To date, the drugs capable of crossing the blood-brain barrier that can be used in therapy are: Temozolomide, Lomustine, Irinotecan, and Cisplatin.[6]

Classical *in vivo* approaches in the study of GBM

Classical *in vivo* models employed for the study of GBM encompass various strategies that attempt to recapitulate the complexity of this aggressive brain tumor. Xenograft models, involving the transplantation of either cultured isolated cells or tumor tissue into immunocompromised mice, have been extensively utilized for their ability to mimic the tumor microenvironment.[7] These models provide valuable insights into tumor growth, invasion, and response to therapies.

Genetically modified mouse models of GBM engineered to harbor mutations found in human GBM, offer the advantage of studying the role of specific genetic alterations in tumor initiation and progression.[8] Additionally, canine models of spontaneous gliomas share similarities with human GBM, providing a unique opportunity to study the disease in a larger, more physiologically relevant context.[9]

A final note regarding the use of xenograft tumor models is the zebrafish model. Studies for the creation of zebrafish GBM models are becoming of increasing interest in the field of oncology research, and various experiments have been carried out using different immortalized and primary cell lines.[10] This model, thanks to its biological characteristics and easy manipulation, appears to be particularly useful for the study of various pathologies,[11] especially those that affect the nervous system,[12,13] allowing the combination of *in vitro* experimentation with *in vivo* experimentation for the study of GBM.

However, these classical *in vivo* models have inherent limitations.[14] Xenograft models may not fully capture the intricate interactions within the tumor microenvironment, given the absence of a functional immune system in immunocompromised mice. Genetically modified mouse models may oversimplify the genetic landscape of human GBM, lacking the full heterogeneity observed in patient tumors.[14] Canine models, while offering a closer approximation to human disease, also

exhibit variations that necessitate careful interpretation of results.[14] Similar limitations in the use of zebrafish as a GBM model might be encountered due to the lack of some critical oncogenic factors that are not expressed in zebrafish and epigenetic or metabolic alterations related to GBM.[15] Figure 1 summarizes the classical and novel experimental models to study GBM biology, highlighting the pros and cons of each approach.

Collectively, these models provide essential tools for GBM research, yet their limitations underscore the ongoing need for more sophisticated and contextually relevant model systems.

In vitro approaches for the study of GBM

Primary and immortalized GBM cell lines: pros and cons

Cell culture is one of the most common *in vitro* techniques used in cancer research, including GBM research, as it allows the study of cancer cell biology in an environment with controlled variables and parameters. Moreover, this technique is relatively low-cost and provides an unlimited supply of cells with fewer ethical concerns compared to the use of biological materials derived from animals and humans.[16] However, the tumor microenvironment is a complex and continuously evolving system, as it consists of different cell types, both resident and migrating/infiltrating cells (tumor cells, immune cells, stromal cells), as well as blood vessels, secreted factors, and extracellular matrix, which actively contribute to tumor growth, progression and spreading.[17]

Therefore, to have a reliable *in vitro* 2D model, it is necessary to select an adequate cell line and to set accurately the most appropriate culture conditions according to the experimental questions and aims.

As for other cancer types, also for the study of GBM, two different types of cell lines are used: primary cells, directly isolated from the human brain, and transformed/immortalized cell lines, generated either naturally or by genetic manipulation. Both have their advantages and disadvantages.

Among the most commonly used human GBM immortalized cell lines there are U-87MG, U-251MG, T98G, A-172, and LN-229, which display different morphologies (epithelial-like, fibroblast-like), as reported by the American Type Culture Collection (ATCC), and derive from gliomas of different grades (Table 1).

The main advantage of these well-characterized and commercially available cell lines is that they can proliferate indefinitely and can be easily maintained and manipulated in a serum-containing medium.[23] Therefore, they are usually used for large-scale studies, such as the screening of novel anti-cancer drug candidates and strategies, to rapidly obtain preliminary results and undertake further investigations.[24,25] Moreover, they show enrichment of GBM stem cells (GSCs) when

cultured as neuro-spheres in a serum-containing medium allowing the study of cancer stem-cell subpopulations.[26,27] However, they also have many drawbacks. First, they were established a long time ago, so it is difficult to trace their origin and to assess their authenticity.[18] Another important issue is that these cell lines underwent numerous *in vitro* passages, and successive cell passages selected cells with the highest proliferative rate, reducing the genetic heterogeneity, a distinctive hallmark of GBM responsible for therapy resistance and tumor recurrence.[28] In addition, successive and prolonged cell passaging may induce genetic drift (Wright effect), and a substantial accumulation of chromosomal aberrations, resulting in phenotypic/morphologic alterations, raising the differences between these cell lines and the native tumors and making them poorly representative of *in vivo* human gliomas.[19,28]

The use of primary cell lines, directly obtained from patients' fresh tumor samples, is becoming more frequent, replacing the use of immortalized cell lines. Primary cell lines represent a more valuable preclinical model as they maintain the heterogenicity typical of GBMs, thanks to the presence of a rich GSCs subpopulation.[29] Moreover, they resemble both the genotype and the phenotype of parental tumors and allow us to obtain more representative, relevant, and reliable data.[30] However, the culture conditions may alter their behavior, genotype, and phenotype and reduce the fraction of GSCs. GCSs play a key role in GBM initiation, maintenance, invasion, immune evasion, and recurrence, thanks to their high ability of self-renewal and differentiation. Therefore, they represent a crucial target for therapeutic interventions and treatments.[31] However, GCSs tend to disappear in cell culture after prolonged serum exposure, as they begin to differentiate into more committed cells, losing many of the primary tumor characteristics. To avoid this issue, GBM-derived primary cells can be grown in a serum-free medium supplemented with basic fibroblast growth factor (bFGF) and/or epidermal growth factor (EGF), plus additional supplements such as N2 or B27. These culture conditions seem to preserve the same proliferation capacity, migration/invasion features, genetic aberrations, and gene expression profiles of the tumors of origin.[32] Nevertheless, it is estimated that around 20 different GBM primary culture conditions exist. Therefore, there is no consensus on the ideal condition to grow these primary tumors.[33]

Another important issue, intrinsic in all primary cell lines, is that they require more time to grow than immortalized cells and have a limited growth potential even under optimal growth conditions, undergoing senescence or death. It was demonstrated that after 20-30 passages *in vitro* GBM primary cell lines undergo significant genomic and transcriptional changes that compromise their value as reliable models for the identification of biomarkers and the development of therapeutic strategies.[34]

In summary, GBM 2D *in vitro* models show a variety of advantages and disadvantages, being more appropriate to perform specific experiments and less for other assays (Table 2). However, cell culture always fails to reflect the complexity of the GBM *in vivo* since it does not allow multiple cell-cell contacts or cell-extracellular matrix contacts. To recreate the tumor microenvironment, various 2D culture systems, using flasks coated with polymers that reduce the stiffness of their plastic surfaces, were developed. For instance, Matrigel is a natural hydrogel derived from the extracellular matrix of mouse sarcoma tumors, containing different components such as laminin and collagen IV, and has been widely used for GBM 2D culture since it allows multiple cell-to-cell interactions and is particularly suitable for the study of cancer cells invasiveness and migration. However, its composition does not reflect that of the extracellular matrix (ECM) surrounding GBMs and may introduce some variables across experiments.[35,36]
Therefore, it is necessary to introduce further improvements and refinements of the *in vitro* techniques to overcome these issues and recreate more reliable models.

3D models for the study of GBM

3D models mimic the complexity and heterogeneity of the tumor, as they allow to recreate the cell-cell and cell-ECM interactions present *in vivo*. Figure 1 summarizes classical and novel experimental models to study GBM biology, highlighting the pros and cons of each approach.
In spheroids, cells grow as suspended spheres or in a matrix (such as Matrigel) in an appropriate culture medium (Figure 2). Both "non-scaffold" and "scaffold" methods can be used to generate spheroids. In non-scaffold methods, plates coated with matrix or hydrophobic polymers are used to counteract cell attachment. Two other non-scaffold methods are the Hanging Drop method, where spheroids grow in a suspended drop of the medium, and the use of an agitation device, a bioreactor spinner flask.[37] In scaffold methods, cells are cultured in the presence of a hydrogel-based support and polymeric materials, natural or synthetic, animal or plant origin. The scaffold constitutes a three-dimensional polymer matrix that mimics the tumor microenvironment.[37] Spheroids, like the solid tumor *in vivo*, are composed of several layers. They have an outer layer of proliferating cells that are accessible to nutrients and oxygen, an intermediate layer composed of senescent cells, and finally a necrotic core. Furthermore, spheroids mimic the presence of an organized ECM composed of fibronectin, laminin, collagen, and glycosaminoglycans.[37] GBM spheroids derived from human tissue were used in xenografts in nude mice to analyze the early stages of tumor development *in vivo* when there is not yet the appearance of symptoms, and thus study the molecular mechanisms of the earlier stages.[38]

In recent years, there has been an increase in the use of brain organoids (Figure 1). In 2016, the first GBM organoid (GBO) was created, which, unlike spheroids, has a more heterogeneous cell population. The created GBOs from primary cultures derived from patients or directly from patient samples by finely mincing and encapsulating GBM tissues in Matrigel for a long time. The organoids showed cell morphology as *in vivo*, with the hypoxic gradients and radiation resistance typical of cancer.[39] Subsequently, Jacob *et al*. optimized the time required to establish GBOs and developed a protocol for their cryopreservation. Crucially, they noted that these cell lines retained consistent characteristics, such as the ability to invade surrounding healthy tissue, mirroring the features of the patient samples from which they were derived. This is particularly valuable for studying a tumor-like GBM, known for its significant heterogeneity across key characteristics.[40]
The approach of 3D cultures from patient biopsies appears to be a first step for personalized tumor therapy, which is necessary for GBM that shows considerable mutations and heterogeneous characteristics from patient to patient. A study confirmed the technical feasibility of using GBOs derived from a patient's tumor tissue after surgery for drug sensitivity testing for the selection of subsequent personalized treatments.[41]
A new and growing field in cancer research is the combination of 3D models and microfluidic technologies (Figure 1). Thus, microfluidic technology is being used to create models of GBM-on-chip with the advantage of a system in the dynamic circulation of the cell culture medium, a condition that mimics blood flow and overcomes the limitations of cell culture in static as GBM tumors are characterized by a constantly changing microenvironment. This model was used to assess both the invasiveness of GBM and the response to drugs or drug combinations.[42]
Another model in the literature for studying GBM is the use of organotypic cultures meaning brain slices into which GBM cells are transplanted. They are mainly used to study the phenomena of tumor invasiveness and progression inside a realistic brain microenvironment.
However, the limitation of the use of organotypic cultures is the lack of interactions with blood flow factors or non-reproduction of hypoxic conditions.[14]
Finally, bioprinting, which is an advanced technique involving layer-by-layer deposition of bio-ink-containing living cells to create three-dimensional structures that promote cell growth, differentiation, and proliferation, has also been used to create study models for GBM. The creation of an accurate and representative GBM model is a complex but valuable tool to better understand the disease in its heterogeneity (Figure 1).[43]
In summary, GBM 3D cultures can have a variety of applications including basic and translational research, allowing the study of tumor molecular mechanisms and characteristics, but also the

identification of specific genetic and epigenetic mutations for drug screening directed at cancer stem cells that are responsible for cancer recurrence.

Conclusions

In conclusion, *in vitro* 3D systems represent the most valuable model for the study of GBM, allowing it to encompass the limitations of both more traditional 2D *in vitro*, and classical *in vivo* models, and offering several notable advantages, as supported by the scientific literature.

Classical *in vivo* xenograft models offered the possibility to understand the genetics of various GBMs and to understand the pivotal role played in tumor initiation and progression. Moreover, spontaneously arising GBM canine models were primarily used for preclinical tests. However, animal models fail to reflect human biology.

Traditional 2D cell cultures, while having been widely used in the past for the understanding of GBM biology, lack the complexity of the tumor microenvironment, which plays a pivotal role in GBM pathogenesis. By contrast, 3D models, such as spheroids and organoids, better replicate the intricate interactions between tumor cells, stromal elements, and the extracellular matrix found *in vivo*.

Firstly, 3D models, including spheroids and organoids, allow for the maintenance of the heterogeneous nature of GBMs, as seen in their representation of cancer stem cell subpopulations, which are critical for tumor progression and recurrence. These models retain the genetic and phenotypic features of the parental tumors, rendering them more representative and reliable platforms for studying GBM biology and developing therapeutic strategies.

Secondly, 3D cultures provide a more accurate depiction of the tumor's complex architecture, including the presence of proliferative cells, senescent cells, and necrotic cores, akin to the *in vivo* scenario. This structural resemblance, along with the ability to mimic the extracellular matrix composition, enables researchers to study cancer cell invasiveness and migration more effectively. Additionally, 3D models offer the advantage of preserving hypoxic gradients and radiation resistance observed in GBM.

Furthermore, the recent advent of GBM organoids derived from patient biopsies represents a promising step toward personalized tumor therapy. These organoids closely mirror the characteristics of the patient's tumor, allowing for drug sensitivity testing and the selection of tailored treatment options, addressing the significant interpatient heterogeneity seen in GBM. Therefore, the development and improvement of these novel techniques are strongly encouraged, especially in theranostic applications and drug screening assays, to obtain rapid and robust results. In this regard, a novel emerging approach aims to isolate extracellular vesicles (EVs), which are

released by almost all human cells and were found in nearly all biological fluids[44], from GBM 3D models and use them as nanovesicles for delivering anti-cancer agents directly into the tumoral mass allowing the development of personalized therapy. These models hold significant promise for both basic and translational research aimed at improving outcomes for GBM patients.

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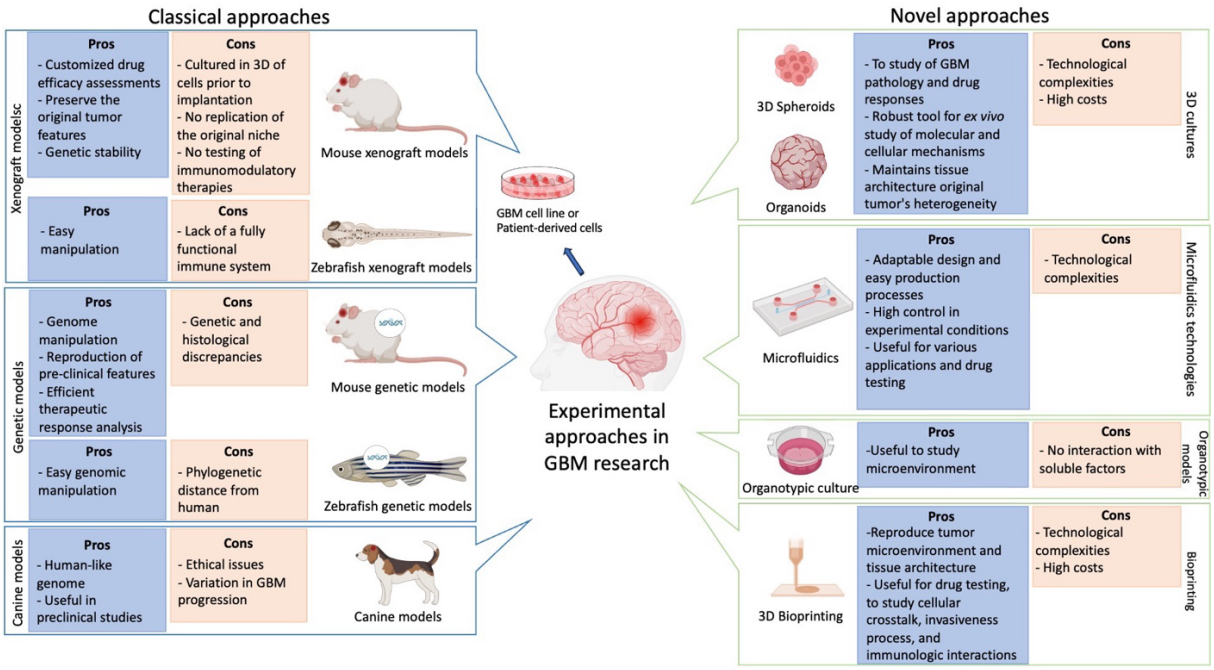

Figure 1. Comprehensive overview encompassing both traditional and innovative methodologies in Glioblastoma Multiforme (GBM) research, delineating their respective strengths and limitations.

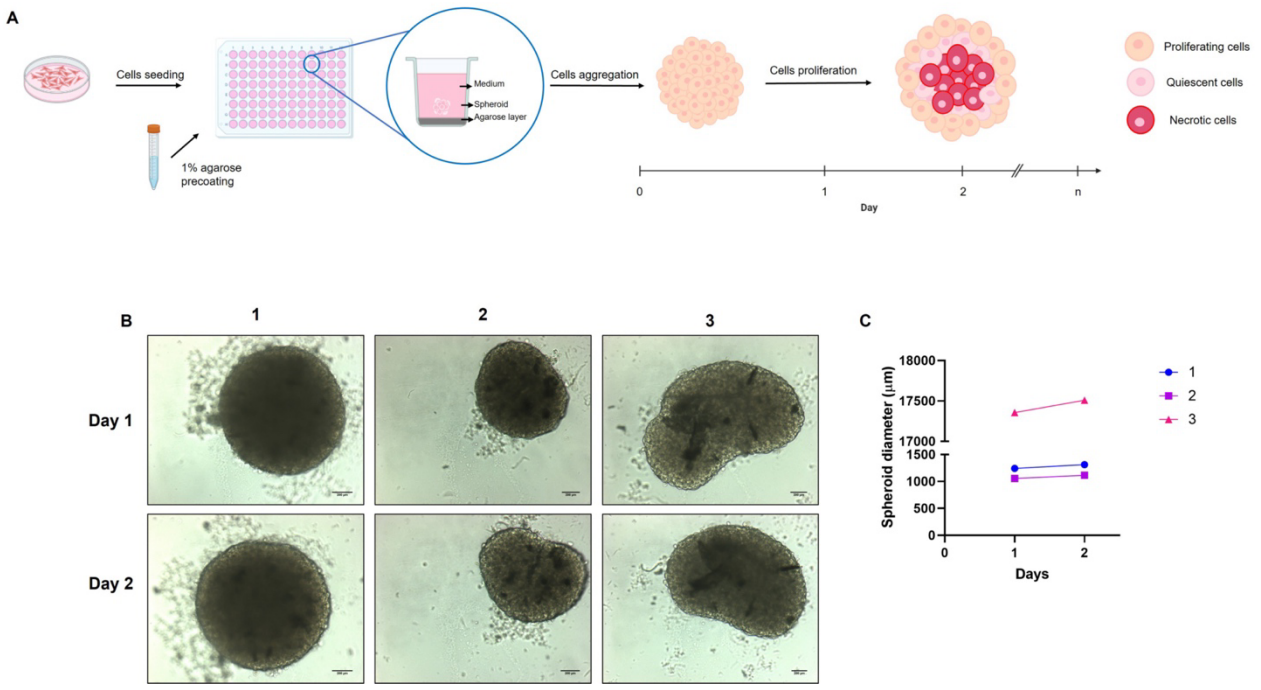

Figure 2. GBM spheroids culture and characterization. A) 3D spheroid cultures were established by seeding cells at optimized densities between 10,000–20,000 cells/well in agarose-coated 96 wells. Spheroids have been maintained in culture for different time points and morphological evaluations have been performed. B) Representative images of the GBM spheroids grown at the optimal seeding densities between days 1 and 2. The scale bar represents 200 µm. C) Spheroid diameter of cells 24- and 48-hours post-seeding of 20,000 cells/well. The diameter of each spheroid was measured using ImageJ (NIH) assuming a spherical shape.

Table 1. Characteristics of human GBM-derived cell lines.

Human gliomas immortalized cell lines	**Morphology**	**Derivation**	**Genetic Characteristics**	**Ref.**
U-87MG	Epithelial-like morphology	Derived from a GBM of unknown origin	Mutations in the PTEN gene and amplification of the EGFR gene	18

U-251MG	Fibroblast-like morphology	Derived from a GBM	Mutations in the TP53 and PTEN genes and EGFR amplification	19
T98G	Fibroblast-like appearance	Derived from a GBM	Alterations in the TP53 gene	20
A-172	Epithelial-like morphology	Derived from a GBM	Mutations in the TP53 gene and alterations in the CDKN2A/p16INK4a pathway	21
LN-229	Epithelial-like morphology	Derived from a GBM	Mutations in the PTEN gene and displays alterations in the PI3K/Akt pathway	22

Table 2. Immortalized and Primary GBM cell lines pros and cons.

Type of cell line	**Advantages**	**Disadvantages**
Immortalized	Commercially available and easily accessible Genetically and phenotypically well-characterized Can grow indefinitely Easy to maintain and manipulate Suitable for high-throughput drug screening Suitable for the study of cancer stem-cell subpopulation	Uncertain origin Sequential *in vitro* passages reduce genetic heterogenicity Sequential *in vitro* passages induce genetic mutations and chromosomal aberrations resulting in phenotypic changes Poorly representative of parental tumors
Primary	Preserve the *in vivo* intratumor heterogenicity Resemble the parental tumors both genetically and phenotypically Allow to obtain more representative and reliable data	Can be maintained for a limited number of passages before undergoing senescence or death After sequential passaging cells accumulate genomic and transcriptional changes

		GSCs tend to disappear after prolonged cell culture Different cell culture conditions exist