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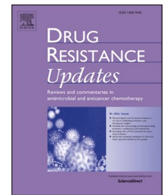

In vitro biomimetic models for glioblastoma-a promising tool for drug response studies

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A B S T R A C T

The poor response of glioblastoma to current treatment protocols is a consequence of its intrinsic drug resistance. Resistance to chemotherapy is primarily associated with considerable cellular heterogeneity, and plasticity of glioblastoma cells, alterations in gene expression, presence of specific tumor microenvironment conditions and blood-brain barrier. In an attempt to successfully overcome chemoresistance and better understand the biological behavior of glioblastoma, numerous tri-dimensional (3D) biomimetic models were developed in the past decade. These novel advanced models are able to better recapitulate the spatial organization of glioblastoma in a real time, therefore providing more realistic and reliable evidence to the response of glioblastoma to therapy. Moreover, these models enable the fine-tuning of different tumor microenvironment conditions and facilitate studies on the effects of the tumor microenvironment on glioblastoma chemoresistance.

This review outlines current knowledge on the essence of glioblastoma chemoresistance and describes the progress achieved by 3D biomimetic models. Moreover, comprehensive literature assessment regarding the influence of 3D culturing and microenvironment mimicking on glioblastoma gene expression and biological behavior is also provided. The contribution of the blood-brain barrier as well as the blood-tumor barrier to glioblastoma chemoresistance is also reviewed from the perspective of 3D biomimetic models. Finally, the role of mathematical models in predicting 3D glioblastoma behavior and drug response is elaborated. In the future, technological innovations along with mathematical simulations should create reliable 3D biomimetic systems for glioblastoma research that should facilitate the identification and possibly application in preclinical drug testing and precision medicine.

1. Introduction

Glioma is the most common and aggressive type of malignant brain tumors. High-grade gliomas, including WHO grade III (anaplastic astrocytoma and oligodendroglioma) and WHO grade IV (glioblastoma) gliomas, are hardest to treat and have the worst survival prognosis of all brain tumors (Louis et al., 2016; Moore and Kim, 2010; Ostrom et al., 2019; Wesseling and Capper, 2018). Among high-grade gliomas, glioblastoma multiforme (GBM) is the most frequently diagnosed type that commonly occurs between the fifth and the seventh decade of life (Moore and Kim, 2010; Ostrom et al., 2019; Wen et al., 2020). GBM is also the most aggressive brain tumor type with only 5-year survival rate of 6.8 % which renders it one of the cancers with the worst prognosis (Ostrom et al., 2019). The aggressiveness of this tumor is even more distressing given that it does not metastasize to other organs, like other solid tumors, but remains a rather brain-localized primary tumor which kills the patient.

The standard clinical protocol for GBM treatment includes surgical

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resection and radiation with concomitant and adjuvant chemotherapy (Stupp protocol). The only approved chemotherapeutic drug currently used in Stupp protocol is temozolomide (TMZ) that improves patient survival by only 2.5 months compared to radiotherapy alone (Stupp et al., 2005). Multiple new targeted therapies have been tested but also failed to prolong patients' survival (Mrugala, 2013; Wen et al., 2020). The latest FDA approved electrical device that generates tumor treating fields (Optune/NOVOTTF-100A System) improved patients' quality of life, but could not extend their survival beyond 3 months (Mrugala et al., 2017).

The evident stagnation in GBM treatment and the implementation of new therapeutic strategies is due to a number of factors including: (i) specific, hardly reachable, tumor location in the brain, (ii) highly invasive potential, (iii) presence of the blood-brain barrier (BBB), and (iv) considerable cellular heterogeneity and plasticity. All these factors contribute to prominent intrinsic, as well as acquired, resistance to both radiotherapy and chemotherapy. GBMs are generally large tumors, mainly located deep within the white matter of the supratentorial part of the brain. It commonly spreads into cortex, deep nuclei or even to contralateral hemisphere which is seen in images as a characteristic "butterfly" shape (Tataranu et al., 2018). Apart from the delicate position within the brain, GBMs are also extremely diffuse and highly infiltrative tumors which make them even more difficult to remove, in spite of improvements in neurosurgery procedures. GBM invasiveness is reflected by the infiltration of tumor cells into the surrounding brain parenchyma. Unlike other tumors that disseminate through the bloodstream and lymphatic system, GBM cells spread along white matter tracts and basal lamina of blood vessels (Bernstein and Woodard, 1995; Liu et al., 2019; Mair et al., 2018). Moreover, glioma cells' ability to undergo intravasation into the blood or lymphatic vessels has been rarely evidenced (Krol et al., 2018) and therefore distant GBM metastases are very rare (Hamilton et al., 2014). Although GBM remains localized within the brain, tumor cells invade different brain sections, affecting normal functions of the central nervous system. Hence, this life-threatening brain tumor is extremely difficult to completely resect. In addition, GBM cells show a dichotomous "go or grow" behavior. Depending on the interaction with stromal cells, communication with the extracellular matrix (ECM) and surrounding extracellular factors, such as oxygen level and nutrients availability, GBM cells can switch between a migratory and proliferative state (Giese and Westphal, 1996; Hatzikirou et al., 2012; Oliveira et al., 2017). This dichotomous phenotype has important implications for the efficiency of chemotherapy and acquisition of chemoresistance (Kathagen-Buhmann et al., 2016; Tiek et al., 2018).

Although many GBM characteristics contribute to the resistance to current therapeutic regimens, limited and varied drug delivery across the BBB can be considered a considerable reason for the slow progress in the development of more effective therapies (Agarwal et al., 2011; Haumann et al., 2020; Khaddour et al., 2020). The structure of the BBB comprises of physical, transport and biochemical barriers. Brain capillary endothelial cells prevent paracellular diffusion due to their tight junctions. Therefore, molecules from the bloodstream can pass the BBB only through luminal and abluminal plasma membranes of endothelial cells (Dyrna et al., 2013). Many anticancer drugs that do not readily cross lipid bilayers cannot pass this physical barrier. On the other hand, lipophilic drug diffusion is prevented by transmembrane efflux transporters that constitute a drug extrusion barrier on the endothelial cells. These transporters including P-glycoprotein (P-gp; ABCB1), multidrug resistance associated protein 1 (MRP1/ABCC1) and breast cancer resistance protein (BCRP/ABCG2) actively expel drugs into the capillaries (Begley, 2004; Li et al., 2016b; Wang et al., 2021). Among them, P-gp is the most abundant (Bicker et al., 2014; Zhang et al., 2021). Thus, the BBB limits the penetration into the brain of more than 98 % of small-molecule drugs, including for example paclitaxel, doxorubicin, methotrexate and vincristine (He et al., 2018).

Drug resistance due to cellular heterogeneity and plasticity is mainly attributed to a small subpopulation of cells with stem cell-like properties commonly designated as "glioma stem cells" (GSC) (Sharifzad et al., 2019). These cells possess self-renewal capacity *in vitro* and *in vivo* and the ability to form tumors upon intracranial implantation in immunodeficient mice (Singh et al., 2004). These cells express a number of stem cell specific markers including for example Nestin, SOX2, ID1, CD15, and CD44 that maintain stem-like properties and have the ability to differentiate into multiple linages of neuronal and non-neural cells (Aum et al., 2014). For example, as observed by live-imaging, glioblastoma stem cells may differentiate into endothelial cells and contribute to angiogenesis in GBM (Mei et al., 2017). On the other hand, differentiated glioblastoma cells can switch to a stem-like phenotype by reprogramming the expression of major neuro-developmental transcription factors, such as POU3F2 (BRN2), SOX2, SALL2 and OLIG2 (Suvà et al., 2014). Both acquisition and loss of stemness contribute to considerable plasticity of glioblastomas (Dirkse et al., 2019; Kondo, 2021). This diversity of cells gives rise to complex and pronounced intratumoral heterogeneity involved in chemoresistance, making glioblastoma a very difficult to treat malignancy.

Resistance to chemotherapy is mediated by multiple signaling pathways that are activated through different factors, either intracellular or originating from the tumor microenvironment (TME) (Assaraf et al., 2019; Gacche and Assaraf, 2018; Gonen and Assaraf, 2012; Jiang et al., 2020; Leonetti et al., 2019; Lepeltier et al., 2020; Li et al., 2016b; Mosca et al., 2021; Niewerth et al., 2015; Wijdeven et al., 2016; Zhitomirsky and Assaraf, 2016). Increasing evidence emphasizes the role of the TME in glioblastoma pathogenesis and response to therapy (DeCordova et al., 2020; Dirkse et al., 2019; Perrin et al., 2019; Son et al., 2017). However, classical 2D, monolayer cultures on plastic or glass surface do not mimic complex structure of brain tissue and glioblastoma cell behavior (Gómez-Oliva et al., 2021; Luo and Weiss, 2020; Pine et al., 2020). On the other hand, animal studies with human xenografts and orthotropic models do not adequately reproduce the disease status present in GBM patients (Gómez-Oliva et al., 2021; Kijima and Kanemura, 2017). Therefore, recent studies are focused on developing tridimensional (3D) *in vitro* cultures to study glioblastoma pathogenesis and response to therapy more realistically considering all the effects of cell surrounding.

In this review we summarize and discuss mechanisms involved in glioblastoma chemoresistance and recent progress in the development of 3D glioblastoma models to study their response to chemotherapy.

2. Intracellular factors contributing to glioblastoma chemoresistance

The majority of common chemotherapeutics, as well as radiotherapy, inflict severe DNA damage, predominantly DNA double strand brakes (DSB). (Borrego-Soto et al., 2015; de Almeida et al., 2021; Huang and Zhou, 2020; Woods and Turchi, 2013). It was shown that glioblastoma cells, particularly their CD133+ stem-like subpopulation, have enhanced activation of DNA damage checkpoint proteins (ATM, Rad17, Chk1 and Chk2) and subsequent DNA repair upon treatment, therefore causing therapy failure (Ali et al., 2020; Bao et al., 2006a; Bighetti-Trevisan et al., 2019). However, resistance to TMZ, as an alkylating agent, is mediated by another set of DNA repair proteins, involving increased expression of MGMT (Sun et al., 2013), and deficiency in components of base excision and mismatch repair systems (Cahill et al., 2007; Felsberg et al., 2011; Montaldi and Sakamoto-Hojo, 2013).

Apart from causing direct DNA damage, chemotherapy also generates reactive oxygen species (ROS) that cause additional DNA breaks and damage of important cellular proteins and lipids (Cui et al., 2018a; Yang et al., 2018). Unfortunately, glioblastoma cells have developed the ability to resist such therapeutic assault by lowering ROS production and increasing expression of components of their scavenging system including superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), glutathione reductase (GR), Solute Carrier Family 7 Member 11

(SLC7A11) (Lee et al., 2004; Oliva et al., 2011; Polewski et al., 2016).

Normally, cells damaged upon chemotherapy would undergo autophagy (to self-repair) and/or apoptosis (to self-destruct) (Hou et al., 2020; Knizhnik et al., 2013; Pawlowska et al., 2018; Russo and Russo, 2018). However, glioblastoma cells have developed additional defense mechanisms against therapy based on these two processes. They have enhanced pro-survival autophagy pathways (increased expression of VPS34, Beclin1, ATG5, LC3, BNIP3) and developed the ability to evade apoptosis (Chen et al., 2015; Hu et al., 2012b; Ruano et al., 2008), contributing to further tumor mass growth despite significant cellular impairments. High grade glioma stem cells were shown to have increased expression of anti-apoptotic proteins (Fanfone et al., 2020; Jin et al., 2008), while pro-apoptotic molecules were downregulated (Capper et al., 2009; Daniele et al., 2018). Such alterations in apoptotic machinery eventually contribute to glioma resistance to therapy (Ma et al., 2002; Trejo-Solís et al., 2018; Valdés-Rives et al., 2017).

In the very essence of the abovementioned mechanisms of chemoresistance is the deregulation of numerous signaling pathways. The most commonly altered signaling molecules in chemoresistance are involved in maintaining stem-like phenotype (Notch and Wnt/β-catenin pathways) and have pro-survival and anti-apoptotic effect (PI3K/Akt/mTOR and RAS/MAPK pathways) (Hombach-Klonisch et al., 2018; Valdés--Rives et al., 2017; Yamada et al., 2011). In addition, ATP-binding cassette (ABC) transporter superfamily is another class of molecules particularly important for the anti-glioma chemotherapy resistance. Glioma cells have predominantly overexpressed multidrug resistance-associated proteins (MRPs) conferring intrinsic chemoresistance (Calatozzolo et al., 2005). High MRP1 and BCRP expression is characteristic of glioma stem-like cell population and high grade gliomas (Calatozzolo et al., 2005; Garrido et al., 2014; Jin et al., 2008, 2009). P-gp, as the best characterized ABC transporter, is also reported to be expressed to some extent in gliomas but its role in chemoresistance is still controversial. Its presence, together with that of BCRP, in the BBB appears to be the most responsible for reduced drug efficacy in glioblastoma (de Trizio et al., 2020; Declèves et al., 2006).

3. Microenvironment factors contributing to glioblastoma chemoresistance

Sensitivity to anti-glioma therapy also considerably depends on different microenvironment factors, primarily hypoxia. Severe hypoxia is a major characteristic of high grade gliomas. It causes formation of pseudopalisade structures with necrotic areas, thrombotic vessels and a characteristic rim of highly migratory cells that are moving away from hypoxic regions (Brat et al., 2004). In response to low oxygen levels, glioma cells undergo phenotypic and genetic changes that allow them to survive and even proliferate in a hypoxic environment. Hypoxic conditions predominantly promote stem-like properties of both stem and non-stem glioma cells by stimulating their self-renewal and neurosphere formation (Colwell et al., 2017; Heddleston et al., 2009; Li et al., 2009). This hypoxia-induced response is driven by changes in the expression of hypoxia-induced factors (HIF1α and HIF2α), various downstream pro-survival signaling molecules (PI3K-Akt or ERK1/2 pathways) and numerous stemness markers (CD133, CXCR4, CD44, A2B5, OCT4, NANOG and c-MYC) (Heddleston et al., 2009; Li et al., 2009; Soeda et al., 2009). Such stemness-like phenotype, promoted by hypoxia, additionally contributes to therapy resistance (Kolenda et al., 2011; Raz et al., 2014). Moreover, hypoxia can have direct negative effect on anti-glioma therapy efficacy, by eliminating free radicals or slowing down tumor cell proliferation = as well as indirect effect mainly through HIF-1α activation. Specifically, increased HIF activity up-regulates the expression of MDR efflux transporters of the ABC superfamily which mediate chemoresistance (Chou et al., 2012; Uribe et al., 2017). Moreover, HIF-1α affects glioma sensitivity to therapy by regulating autophagy/apoptosis, metabolism, proliferation, and the tumor vasculature (Amberger-Murphy, 2009; Huang et al., 2019). Sanzey and colleagues showed that severe hypoxia strongly upregulated the expression of glycolysis-related genes in patient-derived GBM cells that resulted in increased glycolytic activity and promoted tumor invasiveness (Sanzey et al., 2015).

Apart from modulating glioma cell phenotype and treatment response, hypoxia affects other microenvironment factors which additionally contribute to therapy resistance (Gacche and Assaraf, 2018). Due to the hypoxic conditions, tumor cells switch to anaerobic metabolism causing acidification of the TME. This acidic stress promotes and maintains glioma stem-like phenotype (Hjelmeland et al., 2011). Specifically, increased expression of HIF-1α, in response to hypoxia, enhances survival of nearby endothelial cells and induces the expression of vascular endothelial growth factor (VEGF) that further stimulates formation of new blood vessels (angiogenesis) (Bao et al., 2006b; Ezhilarasan et al., 2007; Tamura et al., 2020; Zhang et al., 2015). Reciprocally, endothelial cells induce glioma stem cell state and stimulate sphere formation *via* Notch signaling (Xu et al., 2015; Zhu et al., 2012). They also release different factors, such as bFGF or nitric oxide, which additionally favor glioma plasticity and induction of stem-like phenotype (Charles et al., 2010; Fessler et al., 2015).

In addition to interaction with surrounding cells and different factors they secrete, the ECM is another important component of the perivascular niche that determines glioblastoma cell behavior and response to therapy. The composition of brain ECM is quite unique and distinct from other tissues. Its abundant components are glycosaminoglycans, predominantly hyaluronic acid, and proteoglycans, such as heparan sulfate, while the presence of fibrous glycoproteins collagen, laminin and fibronectin is scarce and mainly restricted to blood vessels basement membrane (Belousov et al., 2019). In glioblastoma, most of these components are overexpressed and contribute to GBM invasion (Virga et al., 2017), as well as acquisition of stem-like phenotype and chemoresistance (Farace et al., 2015; Hu et al., 2012a). Moreover, changes in the ECM composition modulate its physicomechanical properties, in the first place stiffness and rigidity, which additionally contribute to increased invasiveness and chemoresistance (Coppola et al., 2017; Erickson et al., 2018; Ulrich et al., 2009).

4. *In vitro* 3D models of glioblastoma

Different glioblastoma 3D cell culture models were developed with the intention to recreate a TME and mimic interactions between tumor cells, different cellular components and ECM (Fig. 1). The aim is to create biomimetic systems that are user-friendly, cost-effective and compatible with downstream analysis, to finally obtain a reliable GBM model which could be used for therapy response studies in personal medicine.

4.1. Types of 3D GBM cell cultures

One of the highly biomimetic GBM models, used for drug testing, is growing a tumor tissue explant in a collagen-coated Petri dish. It conserves a real structure of the tumor with all components of its microenvironment (Freeman and Hoffman, 1986). A similar model, which includes growing GBM cells on an organotypic brain slice, enables investigation of tumor invasion in healthy brain tissue (Jung et al., 2001). Disadvantages of these models are low reproducibility and difficulties with the preservation of the tissue.

Therefore, more reproducible models of 3D cell culture were introduced and widely used in GBM research. Depending on the cell culture environment, we can distinguish between scaffold-free and scaffold-based cultures (Saji Joseph et al., 2019).

4.1.1. Scaffold-free models

Scaffold-free cultures are spherical multicellular aggregates that well represent different biochemical gradients, as the diffusion within them is limited. Larger aggregates develop gradients of oxygen, nutrients,

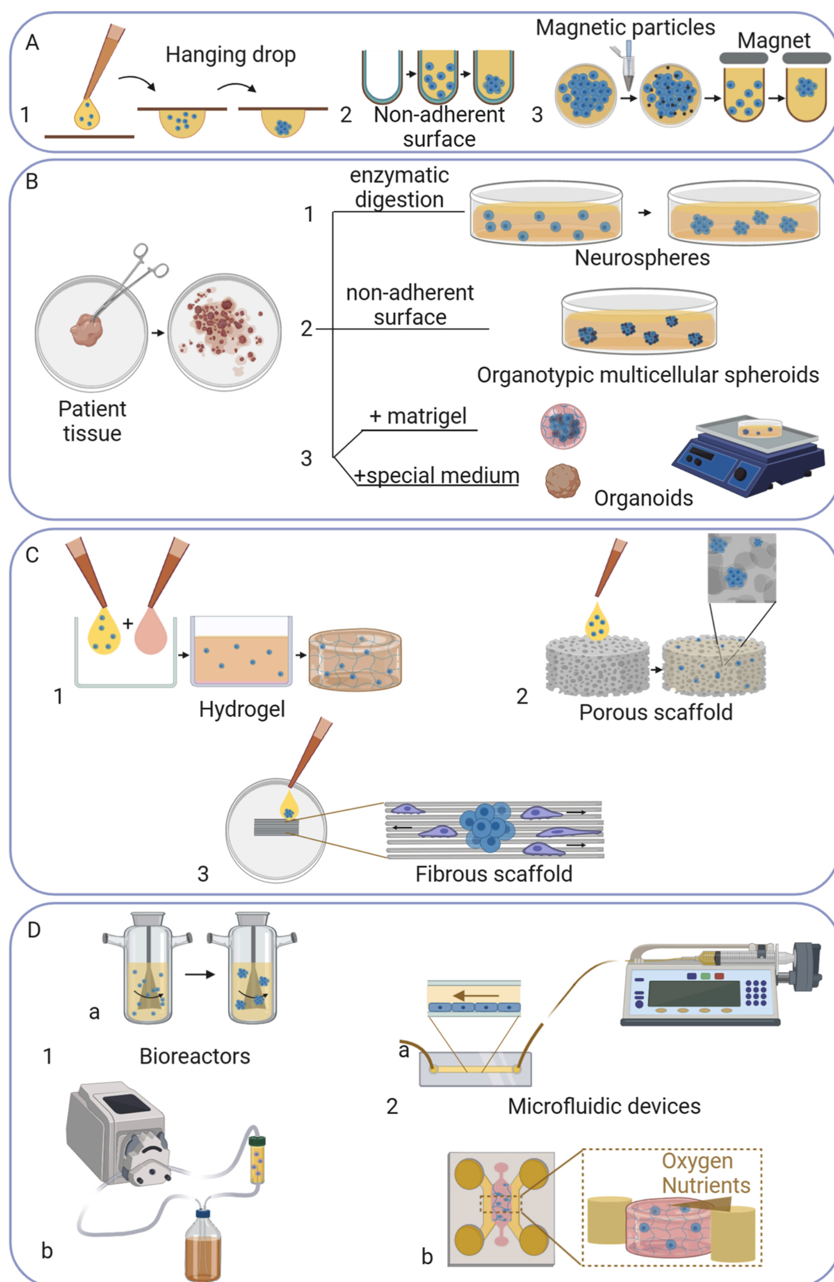

Fig. 1. 3D GBM cell culture models: (A) Multicellular tumor spheroid (MCTS) formation: (1) Hanging drop method – Growing cells in a small volume of medium on a lid of a Petri dish permits the cells to fall to the tip of a drop, aggregate and form MCTS; (2) Non-adherent surface method – Seeding cells on a surface that is not suitable for cell attachment, leads to cell accumulation on the bottom of the well and formation of MCTS; (3) Magnetic levitation – Incorporation of magnetic nanoparticles within the cells allows magnetic-induced cell floating and formation of MCTS on the liquid-air interface; (B) Cultures made from patient samples: (1) Neurospheres – Primary cells obtained by enzymatic dissociation of tumor tissue are grown in medium without serum and supplemented with growth factors, which promote sphere formation; (2) Organotypic multicellular spheroids – Fragments of fresh tissue, grown under non-adherent conditions, round up and form spheroids; (3) Organoids – Primary cells or tumor tissue fragments can form complex 3D structures, when embedded in matrigel or grown in special medium on orbital shaker; (C) Scaffold-based models: (1) Hydrogels – Cell suspension can be mixed with liquid polymer precursor which after cross-linking converts to solid scaffold with encapsulated cells; (2) Porous scaffolds – Cells seeded on the top of the solid scaffold enter within the scaffold and form 3D structures; (3) Fibrous scaffolds – Organized scaffold that mimics GBM invasion pathways; (D) Cultures with medium flow: (1) Bioreactors: (a) Stirring bioreactor – Constant agitation of cells prevents cell attachment to the surface and promotes spheroid formation; (b) Perfusion bioreactor – Scaffold based culture connected to peristaltic pump permits the simulation of physiological mass transport; (2) Microfluidic devices: (a) Simple microfluidic device connected to syringe pump for investigation of sheer stress effects; (b) Gradient microdevice – Hydrogel with embedded cells is filled within the central chamber, while lateral channels are perfused with medium, that way permitting formation of different gradients. Created with BioRender.com.

growth factors, signaling molecules and molecular waste and can simulate drug penetration within a solid tumor. Scaffold-free models include spheroids and organoids.

4.1.1.1. Spheroids. Spheroids are the most frequently used 3D models. They successfully mimic cell-to-cell interactions. Limited diffusion within spheroids causes formation of different cellular zones: proliferating zone on the surface (sufficient level of oxygen, nutrients and signals), necrotic zone in the central part of the spheroid (lack of oxygen and nutrients, higher concentration of the waste) and quiescent zone between these two zones (Bell et al., 2001; Nath and Devi, 2016).

Depending on the cell line type, spheroid complexity and the method for its formation, one can distinguish between different types of glioblastoma spheroid cultures: multicellular tumor spheroids (MCTS), neurospheres, tissue-derived tumor spheres and organotypic multicellular spheroids (Weiswald et al., 2015). Growing tumor cell lines under non-adherent conditions leads to cell aggregation and formation of spherical multicellular structures. here are different ways for the MCTS formation: non-adherent surface method, hanging drop method, suspension culture and magnetic levitation (Hoarau-Véchot et al., 2018). It is also possible to create spheroids within scaffolds and microfluidic devices, which will be discussed later.

Neurospheres (also called tumor spheres and gliomaspheres) are a special type of spheroid cultures generated from patient-derived primary GBM cells. Primary cells from GBM patients, obtained by dissociation of tumor tissue, are normally grown under suspension culture conditions, in serum-free medium, supplemented with B27, bFGF and EGF (Gómez-Oliva et al., 2021; Lee et al., 2006; Lenting et al., 2017). This enables spontaneous formation of neurospheres. Glioblastoma stem-like cells are a predominant component of the neurosphere, while the rest of cell subtypes are being lost (Lee et al., 2006; Xiao et al., 2017).

Fragments of fresh tumor tissue from patients can be grown *in vitro* in agar coated flasks, without prior dissociation. Differing from tumor tissue explant mentioned above, anti-adherence agar coating allows the

tissue to round up and form organotypic multicellular spheroids. This model properly represents the tumor. It conserves the TME with all cell types and ECM and better preserves the *in vivo* phenotype. On the other hand, its reproducibility is low and different results can be obtained, as they depend on the fraction of tumor cells that is successfully grown (Bjerkvig et al., 1990; Christensen et al., 2010; De Witt Hamer et al., 2009; Mahesparan et al., 1999).

4.1.1.2. Organoid culture. Organoids are complex 3D structures that better represent the heterogeneous structure of the tissue (Andreatta et al., 2020; Gómez-Oliva et al., 2021; Klein et al., 2020; Zhang et al., 2020). They are usually obtained by growing primary stem cells or pluripotent stem cells, which are able to differentiate in various cell subtypes and to self-organize, creating specific tissue structures. A growth medium containing specific factors or ECM components are needed to promote cell differentiation and organization (Lancaster and Knoblich, 2014). The first glioblastoma organoids were formed by embedding tumor cells in matrigel and growing them in a stem cell medium on orbital shaker (Hubert et al., 2016). The disadvantages of these organoid cultures are the long formation time, lack of vascularization and the absence of complete maturation of the reproduced organ (Lancaster and Knoblich, 2014; Saji Joseph et al., 2019). Recently, Jacob et al., developed a new method for organoid formation (Jacob et al., 2020). Patient derived tumor tissue is cut into small pieces and grown on an orbital shaker in serum-free medium without exogenous addition of growth factors and ECM, hence preventing cell selection and allowing conservation of molecular signatures of parental tumor and different cellular components of TME for prolonged time. Limitations of this model are decreased rate of organoid formation from IDH-1 mutant and recurrent tumors as well as decrease of vasculature and immune cells after long culture (Jacob et al., 2020).

4.1.2. Scaffold-based models

Scaffold-based models comprise of different components of GBM microenvironment in an *in vitro* system. They are based on diverse biocompatible materials that give support to cells and mimic biochemical and mechanical properties of ECM. Within scaffolds we can study cell growth, invasion, cellular interactions with its microenvironment and the effects of potential therapy.

Scaffolds can be made from natural and synthetic materials (Hoarau-Véchot et al., 2018; Lv et al., 2017; Saji Joseph et al., 2019). Natural material-based scaffolds consist of ECM-derived biomolecules, such as hyaluronic acid (HA) (Ananthanarayanan et al., 2011), collagen (Cheng et al., 2015; Rao et al., 2013a; Ulrich et al., 2010), fibrinogen (Bayat et al., 2018), basement membrane extracts (Ahmed et al., 2018; Musah-Eroje and Watson, 2019) and even decellularized patient tissue (Koh et al., 2018). Cells grown in these scaffolds are able to receive transduction signals and to respond to changes in the microenvironment. The disadvantage of these materials is that, as they originate from mammalian organisms, they can contain pathogens, variations in soluble factors and protein concentrations, so the results obtained in such scaffolds can vary. In order to overcome these problems, non-mammalian polymers are used (alginate and chitosan) (Benson et al., 2014; Kievit et al., 2010; Zustiak et al., 2016), as they are also biocompatible with GBM cells, but are non-immunogenic. Also, different synthetic polymers are being developed. These polymers are inert, their properties can be highly controlled and they give reproducible results. As they do not have cellular adhesion sites, bioactive proteins can be attached, such as RGD functionalized proteins (with the adhesive peptide of tri-amino acid sequence, arginine-glycine-aspartate), in order to enable cellular adhesion or biodegradation (Pedron et al., 2013; Wang et al., 2014). Some of the synthetic polymers used are polystyrene (Gomez-Roman et al., 2016), polydimethylsiloxane (PDMS) (Rao et al., 2013b), polylactic acid (Ma et al., 2018), poly(ethylene-glycol) (PEG) (Li et al., 2016a; Wang et al., 2014, 2017a), polycaprolacton (PCL) (Martínez-Ramos and Lebourg, 2015).

Depending on the 3D structure of the scaffold they can be divided into hydrogels, fibrous scaffolds and porous scaffolds (Cha and Kim, 2017; Saji Joseph et al., 2019). Hydrogels are microporous polymer networks that have high water absorption capacity. Physical or chemical cross-linking of liquid precursors leads to the creation of solid scaffolds. This permits the encapsulation of the cells within the scaffold at the beginning of the fabrication process. As hydrogels are rich in water, the transport of oxygen, nutrients and growth factors is possible. Depending on their composition, hydrogels can have similar biophysical and biochemical characteristics to ECM, hence presenting a more realistic *in vitro* model and the most frequently used one (Caliari and Burdick, 2016; Xiao et al., 2017).

Fibrous scaffolds mimic fibrous structures of white matter tract or blood vessels, which serve as invasion routes for GBM cells (Cha and Kim, 2017). They are made by electrospinning of synthetic polymer solutions, such as PCL (Johnson et al., 2009; Unal et al., 2020), polystyrene (Sharma et al., 2013), polyacrylonitrile (Saleh et al., 2019) or PDMS (Rao et al., 2013b). Cells are seeded on the top of the scaffold, they attach on the surface of the material and their migration along scaffold can be followed.

Porous scaffolds are solid scaffolds, composed of interconnected pore network. They provide a physical support to cells and permit formation of 3D structures. Cells are seeded on the top of the scaffold and they enter it passively or by migration, attach to the walls, proliferate, cluster and form spheroids. Different techniques can be used for scaffold fabrication. Some of them are freeze drying, micro molding, gas foaming, solvent casting/particulate leaching and bioprinting, as the most advanced method (Lv et al., 2017; Saji Joseph et al., 2019).

3D bioprinting is a manufacturing process that enables creation of tissues and organs using different hydrogel-based biomaterials (bioinks) and cells. The fabrication process requires digital design of the desired structure and segmentation of the image, in order to enable printing of successive layers of material and cells and formation of 3D systems (Shafiee and Atala, 2016). This process is highly controlled and facilitates precise distribution of different cell types and ECM, thereby mimicking TME with its cellular, biochemical and biophysical components. Using 3D bioprinting, one is able to obtain complex, highly reproducible 3D cell cultures. However, depending on the parameters of the printing processes, it can provoke cell death or changes of phenotype; thus, it is important to optimize the conditions (Hoarau-Véchot et al., 2018; Zhang et al., 2016). Recently, more bioprinted models of GBM are being developed, as the technique has great potential for GBM studies (Dai et al., 2016; Heinrich et al., 2019; Hermida et al., 2020; Lee et al., 2019; Maloney et al., 2020; Smits et al., 2020; Tang et al., 2020; Wang et al., 2018b, 2020c; Yi et al., 2019). Furthermore, additional components of TME were added to GBM bioprinted models, oxygen gradients were created (Yi et al., 2019) and different cell types were included, such as macrophages (Heinrich et al., 2019; Hermida et al., 2020; Tang et al., 2020), astorocytes (Smits et al., 2020; Tang et al., 2020) and vascular endothelial cells (Yi et al., 2019).

4.1.3. 3D GBM cell cultures with media flow

In order to reconstitute real tumor conditions more faithfully, researchers have developed complex 3D cell cultures that introduce media flow through the system. Depending on the design of these perfusion 3D cultures, the presence of media flow, mimics blood flow through the vessels and/or interstitial fluid flow. Bioreactors and microfluidic devices are examples of these complex 3D cell cultures, developed also for GBM studies.

Bioreactors are closed systems in which biological and biochemical processes are under a strictly controlled environmental and operating conditions (*e.g.*, temperature, pH, pressure, shear stress, nutrient supply, oxygen and CO_2 concentration, metabolites and regulatory molecules) (Hoyle et al., 2020; Martin et al., 2004; Selden and Fuller, 2018; Wang et al., 2020a). Since bioreactors provide us with a high degree of control,

reproducibility and automation, they have found applications in various fields. Bioreactors are also designed and used for growing cancer cells and scaffold-based tumor tissues as a new 3D model of malignant neoplasms.

Bioreactors for tumor tissue engineering have to be made from bio-inert and biocompatible materials. The whole system should operate under sterile culture conditions and allow specific mass transport and nutrient supply. Also, it should be transparent to allow visualization and use of fluorescence and optical imaging of tumor tissue (Bregenzer et al., 2019; Guller et al., 2016).

Depending on the physicochemical parameters to be controlled and the desired outcome of the experiment, many different types of bioreactors have been developed and used for tumor tissue engineering. They can be grouped into the following types: bioreactors with static cultivation systems, stirring bioreactors, rotary bioreactors, hollow-fiber bioreactors and perfusion bioreactors. Detailed description of these bioreactors and their application in tumor tissue engineering have been previously reviewed (Guller et al., 2016).

Bioreactors have been mainly used to cultivate a large amount of human GBM-derived cancer stem cells (GSCs), hence circumventing the limitations of massive cell propagation in conventional *in vitro* 2D cell cultures (Serra et al., 2012). The type of bioreactor most commonly used for expansion of CSCs of various tumors, including GBM, is the stirring bioreactor. This type of bioreactor provides dynamic mixing of the growth medium and significantly improves mass transfer between cells and the culture medium. However, these conditions place the cells under the constant influence of fluid-induced shear stress (Bregenzer et al., 2019; Guller et al., 2016). Recently, perfusion bioreactors have been used as a new strategy for improving the 3D *in vitro* models of the GBM. This type of bioreactors achieves the most accurate simulation of mass transfer in a living organism (Ahmed et al., 2019). Combining scaffold-based 3D cell culture and perfusion bioreactor has found application in producing large amounts of glioblastoma tumor-initiating cells (TICs) required for drug screening, as well as for basic cancer research (Li et al., 2016a, 2018).

Microfluidic platforms or chips are microfabricated bioreactors that enable growing cells in micrometric chambers within well controlled physiological-like conditions. They consist of one or more fluidic channels where small volumes of liquid and low number of cells can be handled (Whitesides, 2006). Depending on the characteristics of the device, we can mimic interactions between cells, between cells and ECM, as well as to mimic biophysical and biochemical characteristics of the TME, such as gradients of nutrients, oxygen and signaling molecules (Ayuso et al., 2016). As the laminar flow is present within the channels of the microdevice, there is no fluid mixing and molecules travel through the device by diffusion (Paguirigan and Beebe, 2008).

The simplest microfluidic devices consist of a simple channel that permits the 2D growth of cells. They are used to investigate flow-induced sheer stress effects, migration, nutrient gradients and drug effects (Han et al., 2016; Huang et al., 2011; Jo et al., 2018; Liu et al., 2010; Rezk et al., 2020). As mentioned above, microfluidic platforms can be used for spheroid formation. These devices are made from anti-adherent materials and their geometry promotes capturing of the cells within microwells, their aggregation and spheroid formation (Vadivelu et al., 2017). Within some devices, cells are embedded in hydrogels and thus, by providing physical and biochemical components of ECM, a more adequate biomimetic model is created (Ayuso et al., 2017, 2016; Lee et al., 2014; Qazi et al., 2011; Samiei et al., 2020).

Microfluidic devices permit a real time monitoring of cell culture. They can be used for different purposes, to investigate cell proliferation, metabolism, migration and invasion, angiogenesis, immune system function and most importantly therapy response (Andrei et al., 2019; Ayuso et al., 2017, 2016; Logun et al., 2018; Samiei et al., 2020).

4.2. 3D glioblastoma models specifically used for anti-glioblastoma drug screening

Most of the studies that examined anti-glioblastoma effects of different chemotherapeutic drugs, as well as novel compounds in a 3D setting used spheroids grown in medium as a model system. Some examples of drugs/compounds tested for their anticancer effect in gliomaspheres are listed in Table 1.

Recently, Quereda and collaborators developed a high-throughput spheroid-based proliferation assay for testing cytotoxicity of up to 3,300 compounds simultaneously on GSC cells (Quereda et al., 2018).

Although simple spheroid systems in growth media are widely used for drug screening in 3D GBM cultures they have significant limitations, primarily the simplified architecture that lacks complex TME conditions. Therefore, in several publications drug effects on glioblastoma cells were tested in different 3D scaffolds mimicking natural ECM (Table 1). In most of these studies the authors examined the invasion of 3D culture in response to various compounds, such as inflammatory cytokines IL-1β and TNFα (Sarkar and Yong, 2009), FasL neutralizing antibody (Merz et al., 2015) or drug combination of imatinib and docetaxel (Kinsella et al., 2011). However, two research groups, An et al. (2010) and Iwasaki et al. (1993), performed more comprehensive studies and investigated then effects of vorinostat (histone deacetylase inhibitor) and TNFα, respectively, on 3D cell culture growth and viability, morphological and adhesive characteristics and gene expression in addition to invasive properties.

Lee et al., constructed a type of 3D platform with alginate matrix for high-throughput drug cytotoxicity and efficacy testing on both GBM cells and normal astrocytes (Lee et al., 2017). This 3D chip with micropillars (for growing cells in alginate matrix) and microwells (containing drugs) is suitable for simultaneous screening of 72 compounds in 7 replicates (Lee et al., 2017). Recently, technological advances enabled us to perform more accurate and reliable drug testing in complex 3D cultures, such as in microfluidic devices, which more faithfully recapitulate glioblastoma TME and its conditions observed in patients. Examples of microfluidic devices specifically developed for anti-glioblastoma drug screening are summarized in Table 1. Fan and colleagues developed microfuidics-based brain cancer chip for high-throughput testing of multiple-simultaneous drug response on GBM spheroids in PEGDA hydrogel (Fan et al., 2016). They validated generation of drug concentration in chip by testing single and combined effects of pitavastatin and irinotecan on U87 spheroid cell viability. Recently, the same group of authors engineered an improved version of the brain cancer chip that that prevents the diffusion of any drug molecule across channels (Akay et al., 2018). As a proof-of-concept experiment, they tested viability effects of concentration gradient generated by simultaneous application of temozolomide and bevacizumab on three GBM patient-derived cell spheroids. To consider effect of cellular heterogeneity on drug treatment response, Pang and collaborators designed a microfluidic platform for single-cell separation based on cell biomechanical properties. In this system, the authors were able to determine the level of vincristine sensitivity according to the cell size and degree of deformability (Pang et al., 2016).

5. Effects of 3D culture on GBM gene expression and phenotype

Cell culture dimensionality significantly affects glioblastoma cell phenotype, such as morphology, migration, proliferation, differentiation and stemness (Table 2). Numerous studies have shown that glioma cells have an altered morphology in 3D systems compared to 2D cell cultures. In 3D matrices, cells are present in a spindle or round form compared to flattened epithelioid shape when grown in monolayers (Gomez-Roman et al., 2016; Huang et al., 2018; Jia et al., 2018; Jiguet Jiglaire et al., 2014; Kievit et al., 2010, 2014; Wang et al., 2016, 2018a; Wang et al., 2019b). Unlike cells in 2D cultures that lack polarity, spindle cells of 3D cultures contain one or two oriented spindly

Table 1
3D GBM cell cultures used to study anti-glioblastoma drug activity.

3D cell cultures	GBM cell types	Drugs	Reference
Scaffold-free cultures			
	U251, primary cell lines	Acetazolamide with TMZ	(Amiri et al., 2016)
	U87, primary cell lines	RO4929097 with TMZ and radiotherapy	(Yahyanejad et al., 2016)
	U87	Curcumin and DOX in micellar carrier	(Sarisozen et al., 2016)
	U251	Pyrrolidine-2 and curcumin	(Zhang et al., 2016)
Spheroids grown in media	T-98G	Curcumin and TMZ in magnetic nanoparticles	(Dilnawaz and Sahoo, 2013)
	U251	Metformin and Ara-a	(Mouhieddine et al., 2015)
	LN229, U87, T-98 G	Sodium Selenite with TMZ	(Berthier et al., 2017)
	U87	^{225}Ac in polymersomes	(de Kruijff et al., 2018)
	Primary cell lines	Niclosamide with TMZ	(Oh et al., 2020)
Spheroid-based high-throughput platform	U87, primary cell lines	Simultaneous cytotoxicity testing up to 3300 compounds	(Quereda et al., 2018)
Organoids grown in media	Patient tissue	TMZ with radiotherapy, gefitinib, trametinib, everolimus	(Jacob et al., 2020)
Scaffold-based cultures			
Single cells in Type I collagen 3D matrix	U178	IL-1β and TNF-α	(Sarkar and Yong, 2009)
Spheroids in Matrigel	U87, U251	APG101	(Merz et al., 2015)
Spheroids in collagen gel	SNB-19, primary cell lines	Imatinib and Docetaxel	(Kinsella et al., 2011)
Spheroids in collagen type I 3D gel	LN18, F98, C6, F98EGFR-vIII, U87	Vorinostat	(An et al., 2010)
Single cells in Matrigel	Primary cell lines	TNF-α	(Iwasaki et al., 1993)
Organoids in hyaluronic acid-collagen hydrogel	Patient derived cells	Dacomitinib	(Maloney et al., 2020)
Single cells in collagen gel	U251, U87	Simvastatin with TMZ	(Shojaei et al., 2020)
3D platforms			
High-throughput alginate micropillar and microwell chip platform	Patient derived cells and normal human astrocyte cell line	70 compounds simultaneously tested	(Lee et al., 2017)
Spheroids forming microfluidic device with PEGDA hydrogel	U87	Pitavastatin and Irinotecan	(Fan et al., 2016)
Spheroids forming microfluidic device with PEGDA hydrogel	Patient derived cells	TMZ and bevacizumab	(Akay et al., 2018)
Single cell separation microfluidic device	U251	Vincristine	(Pang et al., 2016)

TMZ-temozolomide; DOX-doxorubicin; Acetazolamide-carbonic anhydrase IX inhibitor; RO4929097- NOTCH/γ-secretase inhibitor; Pyrrolidine-2-cytoplasmic phospholipase A2α inhibitor; ^{225}Ac-Actinium; APG101-CD95 ligand neutralizing agent; Vorinostat - suberoylanilide hydroxamic acid.

protrusions (Huang et al., 2018). This is particularly evident for highly invading cells that present a neural progenitor-like phenotype with a round small cell body and a long leading process, as shown in experiments with tumor spheres grafted into a 3D collagen matrix (Fayzullin et al., 2019). Moreover, in 3D scaffolds, glioblastoma cells tend to form multi-cellular clusters and to aggregate into tumor cell spheroids (Florczyk et al., 2013; Lv et al., 2016), while cells cultured on 2D surfaces have an epithelium-like morphology and grow into sheets (Wang et al., 2018a). This colony formation ability in 3D culture depends on the GBM cell type and characteristics of 3D scaffold, such as pore size (Jia et al., 2018). As observed in the study of Wang et al., cells form tighter spheroids in 3D scaffolds with closer connections and have more abundant secretory granules on the cell surface (Wang et al., 2018b). Those cells were also richer in mitochondria and rough endoplasmic reticulum and had a higher number of longer microvilli.

Apart from influencing cell morphology, 3D culture environment also enhances the migration ability of the glioma cells, compared with the 2D plated cultures (Jia et al., 2018). The movement pattern in 3D cultures significantly depends on the presence of the tumor core; without a tumor core, cells migrate randomly, while existence of tumor sphere induces oriented cell migration (Fayzullin et al., 2019). It was shown that migration of U87 glioblastoma cells in 3D environment is driven by mesenchymal-amoeboid transition (MAT) upon Rac1 GTPase inhibitor treatment (Huang et al., 2018).

Glioblastoma cell proliferation is generally slower in 3D compared to 2D cultures (Dai et al., 2016; Florczyk et al., 2013; Gomez-Roman et al., 2016; He et al., 2014; Jia et al., 2018; Jiguet Jiglaire et al., 2014; Kievit et al., 2010, 2014; Lv et al., 2016; Wang et al., 2016), and it is accompanied by decreased expression of the proliferative marker Ki67 (Jia et al., 2018). According to the Gomez-Roman study, this behavior can be changed in response to VEGF supplementation which increases 3D cell proliferation but has no effect on 2D growth conditions (Gomez-Roman et al., 2016). It was observed that in a 3D environment, GBM cells accumulate in G0/G1 cell cycle phase but without significant difference in apoptosis rate compared to 2D culture, suggesting their decreased proliferation and increased quiescence in 3D scaffolds (Lv et al., 2016). Although 2D culture initially had higher proliferative capacity, it was shown that upon prolonged 2D cultures (longer than 10 days), cellular proliferation decreases after some point of time and then proliferation rate of 3D bioprinted cultures becomes higher (Dai et al., 2016; Wang et al., 2018a, 2019b).

Fernandez-Fuente and colleagues first showed that there is no difference in GBM cell differentiation level (GFAP level) between 2D and 3D cultures (Fernandez-Fuente et al., 2014). However, a later study of Lv et al., reported decreased GFAP level as a marker of increased dedifferentiation of glioblastoma cells in 3D compared to 2D culture (Lv et al., 2016). Several observations are consistent with the later finding and show that cultures in different 3D scaffolds have a greater proportion of stem cell-like cells, with CD133 positive phenotype, compared to monolayer cultures (Kievit et al., 2014; Wang et al., 2018a, 2018b; Wang et al., 2019b).

Along with increased stem-like phenotype, angiogenic potential of GBM cells is also altered in 3D compared to 2D cultures. For example, accelerated vasculature formation, with enhanced recruitment of CD31 positive cells, was observed in U87 tumors from cells pre-cultured in 3D chitosan alginate scaffolds (Kievit et al., 2010). Moreover, complexity of 3D culture can affect angiogenic potential as well. Wang et al., reported that 3D bioprinted GSC culture had increased VEGFA secretion and formed more tube-like structures than conventional GSC suspension culture (Wang et al., 2018b).

Corresponding to increased stemness, three-dimensionally grown glioblastoma cells also have increased *in vivo* tumorigenic potential. They form larger tumors that are developing much faster, compared to tumors derived from monolayer cell cultures (Kievit et al., 2014; Wang et al., 2018a, 2019b). However, Kievit et al., reported that this initial rapid tumor growth is not sustainable and after some time tumors

Table 2
Effects of 3D cell culture and microenvironment on GBM cell phenotype.

Cell characteristics	Properties of 3D cell culture									
	Third dimension	Inclusion of ECM components in 3D scaffold	Stiffer scaffold	Interstitial flow	Hypoxia	Nutrient supply	Endothelial cells	Astrocytes	Mesenchymal cells	Immune cells
Morphology	Spindle/ round[1]	Spindle/ round[2]	More round	More homogeneous	ND	ND	Round/ elongate[2]	Round	ND	ND
Proliferation	–	ND	–	ND	+/–[3]	+	–	+/–[2.4]	+	+
Migration	+	+/-[2]	–	ND	+	ND	ND	+	ND	ND
Invasion	ND	+	–	+	+	ND	+	+	+/–[5]	+
Stemness	+	+	ND	ND	ND	+	+	+	ND	+
Angiogenic potential	+	ND	+	ND	ND	ND	+	ND	ND	+
Tumorigenic potential	+	+	ND	ND	ND	ND	ND	ND	ND	ND
Therapy resistance markers expression	+	+	ND	ND	ND	ND	ND	ND	ND	ND
Apoptotic factors expression	Pro – Anti +	ND	ND	ND	Pro +	ND	ND	ND	ND	ND

+ increased; - decreased; ND – not determined; [1] depending on 3D cell culture model; [2] depending on scaffold composition; [3] depending on O_2 concentration; [4] depending on seeding ratio; [5] depending on cell type.

developed from 3D pre-cultured cells began to grow at a similar rate as the 2D pre-cultured tumors (Kievit et al., 2010).

As cell appearance and behavior is determined by gene expression, it was reasonable to assume that the dimensionality of cell culture would also significantly affect regulation of gene expression. Indeed, when grown in 3D cultures, glioblastoma cells showed different levels of expression of various genes compared to culturing in monolayers (He et al., 2014; Levin et al., 2012). Numerous studies have observed altered expression of several classes of markers (mRNA and/or protein) in 3D cultures, including:

- Stemness related markers - most frequently general stemness markers such as CD133 (Jia et al., 2018; Kievit et al., 2014; Lv et al., 2016; Martínez-Ramos and Lebourg, 2015; Wang et al., 2018a, 2018b; Yang et al., 2015), CD44 (Kievit et al., 2014; Martínez-Ramos and Lebourg, 2015; Wang et al., 2018a), Nestin (Dai et al., 2016; Florczyk et al., 2013; Jia et al., 2018; Kievit et al., 2014; Martínez-Ramos and Lebourg, 2015; Wang et al., 2018a; Yang et al., 2015), Nanog (Jia et al., 2018; Lv et al., 2016), SOX2 (Jia et al., 2018; Lv et al., 2016); Oct4 (Jia et al., 2018; Yang et al., 2015); Snail (Kievit et al., 2014; Wang et al., 2018a), as well as less frequently observed Frizzled 4, GLI, HES (Kievit et al., 2014), LIN28A, LIN28B, CXCR4 and CSPG4 (Ma et al., 2016b), RHAMM (Martínez-Ramos and Lebourg, 2015), Musashi-1 (Florczyk et al., 2013); interestingly, the degree of overexpression of some stem-related genes, such as MSI1, MSI2 and BMI-1 and c-Myc, was shown to be cell type dependent in 3D environment (Jia et al., 2018);
- Markers of glial differentiation and neural development - β-tubulin III (Dai et al., 2016) and GFAP (Dai et al., 2016; Florczyk et al., 2013); however, there is inconsistency in the literature regarding GFAP because some authors reported no difference in its level between 2D and 3D cultures (Kievit et al., 2014);
- DNA damage repair genes – MGMT (Lv et al., 2016);
- ABC transporters - ABCG2 (Florczyk et al., 2013) and ABCB1 (Ma et al., 2016b), although Lv et al., reported no significant alterations in ABC transporter gene expression in 3D compared to 2D environment (Lv et al., 2016)
- Markers related to invasion and EMT - MMP1 (Jia et al., 2018), MMP-2 (Florczyk et al., 2013; Jia et al., 2018; Kievit et al., 2010; Pedron and Harley, 2013), MMP3 (Jia et al., 2018), MMP7 (Jia et al., 2018), MMP-9 (Florczyk et al., 2013; Pedron and Harley, 2013), N-cadherin (Jia et al., 2018; Kievit et al., 2014), TWIST1 (Florczyk et al., 2013), Twist2, Snai1 and Snai2 (Kievit et al., 2014), vimentin (Jia et al., 2018); it is worth mentioning that E-cadherin levels is are suppressed upon 3D cultures (Kievit et al., 2014)
- Markers of angiogenesis and response to hypoxia - HIF-1α (Florczyk et al., 2013; Pedron and Harley, 2013; Wang et al., 2018a, 2018b; Yang et al., 2015), VEGF (Dai et al., 2016; Kievit et al., 2010; Pedron and Harley, 2013), laminin (Kievit et al., 2010), fibronectin (Kievit et al., 2010; Pedron and Harley, 2013), VEGFR2, and CD31 (Wang et al., 2018b)
- Cell-cycle related genes - p21, p27, CCNA1, CCNB1, CCND1 and CCNE1 (Jia et al., 2018)

Excluding specific genes/proteins, whole signaling pathways are also differentially regulated in 3D compared to 2D GBM cellular systems. Specifically, it was reported that components of pro-apoptotic signaling cascade (caspases, poly (ADP-ribose) polymerase (PARP) and p53) were downregulated, while anti-apoptotic signaling factors (PDL-1 and Livin) were upregulated in 3D collagen scaffold (Jia et al., 2018). In addition, key proteins of Wnt, SHH and Notch signaling pathways (Notch1, 2 and 3, Wnt3a, Wnt5a and SHH) were also highly expressed in this 3D system (Jia et al., 2018).

6. Effects of the TME on GBM cell phenotype in 3D models

Besides dimensionality, other characteristics of 3D culture, also significantly shape glioblastoma phenotype (Table 2).

6.1. Composition of 3D scaffolds

In the first place, the type of 3D scaffold material, its composition and formulation, considerably determines glioblastoma behavior and gene expression. For example, patient-derived primary GSC exhibited opposite migratory profile in Matrigel and collagen scaffolds, moving in spherical multicellular aggregates in Matrigel *versus* single elongated cells within collagen matrix (Herrera-Perez et al., 2015). The type of collagen used for scaffold fabrication also affects GBM cell morphology. Cells gain a round morphology in collagen-IV, while in collagen-I/III, with the strong fibrillary structure, they acquire spindle shape and prominent migratory phenotype (Rao et al., 2013a).

Addition of ECM components to 3D scaffolds considerably influences GBM cell characteristics. Incorporation of HA in 3D collagen-hydrogels causes cells to get a more rounded morphology and to decrease migration (Herrera-Perez et al., 2015; Rao et al., 2013a). Pedron at al., showed that addition of methacrylated hyaluronic acid (HAMA) to adhesive

(GelMA) and non-adhesive (PEG) 3D hydrogels induced cell clustering and increased expression of invasion-related genes (VEGF, HIF-1, MMP-9, and Fn) (Pedron et al., 2013). Several groups also reported that the presence of HA in 3D scaffolds increased expression of stem cell markers, particularly CD133, and EMT-related genes in GBM cell lines (Kievit et al., 2016; Martínez-Ramos and Lebourg, 2015; Wang et al., 2018a), also leading to increased tumorigenicity in nude mice (Wang et al., 2018a). Accordingly, Li et al., developed 3D HA scaffold-based bioreactor (AlgTubes) for scalable culturing of high quality and high quantity glioblastoma TICs (Li et al., 2018). They made coaxial alginate tubes filled with HA and dispersed primary glioblastoma cells. Such a bioreactor system with a high HA content ensured efficient mass transport, protection from the hydrodynamic pressure, efficient expansion of glioblastoma TICs and maintenance of stem phenotype (Li et al., 2018).

Similar to HA, addition of agarose to collagen matrices also promoted a round morphology and amoeboid motility and slowed down migration of GBM cells due to increased elasticity, reduced porosity and presence of steric barriers within such composite 3D scaffold (Ulrich et al., 2010). Excluding HA, the presence of other ECM components, such as laminin, contribute to altered GBM behavior in 3D cultures. Coating of electrospun polystyrene (ESPS) scaffolds with different laminin isoforms was shown to increase expression of integrin alpha 6 and beta 4, as well as several stem markers and ABC transporters in U251 cells (Ma et al., 2016b). Additionally, including of laminin in polyacrylonitrile scaffold leads to higher migration and lower proliferation (Saleh et al., 2019).

6.2. Biomechanical properties of 3D scaffolds

The addition of ECM components and ligands to 3D scaffolds, as well as crosslinking 3D scaffold material, alters biomechanical properties, stiffness and porosity, of the scaffold causing changes in GBM cell behavior. For example, Kaphle et al., showed that 8S-StarPEG, used to crosslink collagen, could increase hydrogel viscosity and decrease collagen degradation and cell migration (Kaphle et al., 2019). Generally, increasing hydrogel stiffness causes cells to be more rounded and proliferate less and it also inhibits their motility and invasion (Heffernan et al., 2015; Rao et al., 2013a; Unal et al., 2020; Wang et al., 2014, 2020b; Zhu et al., 2020). It is interesting to note that, in spite of the general behavior pattern, different glioblastoma cells may act differentially in the same 3D matrix (Ananthanarayanan et al., 2011), which is particularly evident for primary glioblastoma cells (Grundy et al., 2016).

Altering scaffold stiffness and crosslinking also affects gene expression. Increased expression of HIF-1, VEGF, and MMP-9, as well as reduced CD44 expression, was observed in response to greater stiffness and/or crosslinking density (Pedron and Harley, 2013; Wang et al., 2014). Apart from stiffness, varying scaffold porosity may influence GBM cell gene expression. In the Jia et al., study, pore size of the 3D collagen scaffold significantly and differentially affected expression of malignancy, stemness, cell cycle and EMT-related markers in three glioblastoma cell lines. This effect was mainly observed at the protein level rather than at the mRNA level, but without clear influence on their biological functions (proliferation, colony formation, migration and invasion) (Jia et al., 2018).

In more complex 3D systems, media perfusion is introduced to mimic interstitial flow (IF) in tumors. Presence of IF generates fluid shear stress (SS) which can alter glioblastoma cell behavior and gene expression, even though it is of lesser magnitude in tumor tissue than in vasculature system. Panchalingam et al., developed stirred-suspension bioreactor protocols for growing human-GSCs in suspension culture under low- and high-shear stress forces (Panchalingam et al., 2010). High SS conditions resulted in a higher cell expansion and lower mean diameter of neurospheres. Also, the size of the neurospheres formed under high-shear stress conditions was more uniform, suggesting that high-shear GSC tissue may lead to homogeneous cell culture morphology. It was previously shown using a 3D Modified Boyden chamber with media perfusion that SS either induced cell death or decreased cell motility which was accompanied by downregulation of MMP-1 and MMP-2 activity (Qazi et al., 2011). However, later studies have shown that IF stimulates glioblastoma cell invasion, mediated by CXCR4-CXCL12 chemotactic signaling and/or HA receptor (CD44)-dependent mechanosensing (Kingsmore et al., 2016; Munson et al., 2013).

6.3. Induction of hypoxia

Hypoxia, as one of the major hallmarks of glioblastoma as well as solid tumors, is an important microenvironment factor that should be mimicked in 3D cultures in order to study GBM behavior and response to therapy in the most comprehensive way (Li Petri et al., 2020; Raz et al., 2014). Therefore, several studies have investigated its effects on 3D GBM cell culture phenotype. Xu and colleagues studied the effects of 1% and 0.2 % oxygen levels on GBM cells in PDMS microfluidic chip with collagen hydrogel (Xu et al., 2015). They observed increased induction of EMT and migration under hypoxic conditions, as well as upregulated expression of HIF-responsive and EMT-associated genes. However, the effects on cell proliferation depended on the degree of oxygen levels. At an environment of 1% O_2, cell proliferation was increased whereas under highly hypoxic conditions (0.2 % O_2) it was decreased. Similar results, namely increased invasion, mesenchymal transition and spheroid growth and expansion, were observed by mimicking *in vivo* hypoxia conditions using a genetic approach, by transfecting HIF1α or HIF2α into GBM cells (Ma et al., 2016a). However, long incubation (in 7 days) of hypoxic 3D culture of patient-derived glioblastoma cells, the size of spheroids did not change over time albeit they were dramatically smaller than under normoxia at the end of cultivation period (Rosenberg et al., 2018). In these 7-day spheroid cultures, cellular proliferation marker was mainly decreased, and hypoxia-induced markers (HIF-1α, carbonic anhydrase IX, VEGF) were upregulated under hypoxic conditions compared to normoxic conditions, while expression of stem cell markers varied across spheroid cultures in response to hypoxia. Furthermore, Ayuso et al., developed a microfludic device that enabled generation of both oxygen and nutrient gradients within 3D cultures with the possibility to monitor cell death, viability, proliferation and ROS production (Ayuso et al., 2016). Using this platform they were able to mimic blood-vessel obstruction and consequently oxygen and nutrient deprivation, therefore inducing cell migration and formation of the characteristic pseudopalisade structure within 3D cell culture (Ayuso et al., 2017). Detailed proteomic analysis of 3D GBM cell cultures revealed that proteins and phosphoproteins are differentially expressed in response to hypoxic conditions (Levin et al., 2012). Namely, both pro-survival and pro-apoptotic proteins were activated, as well as migration-associated proteins, while the amount of proteins promoting cell cycle was reduced.

6.4. Nutrient deprivation

Effects of nutrient supply on GBM cell phenotype were also examined in the study of Panchalingam et al. (2010). In their stirred-suspension bioreactor-based culture of human GSCs, the authors introduced different feeding strategies and examined the effect that the 2-day and 6-day fed-batch (40 % medium replacement every 2 or 6 days) had on the expansion and phenotype of human GSC expanded cells. The results showed that the 2-day fed-batch mode resulted in the highest expansion after 32 days of culture (90 -fold cell expansion, larger neurosphere diameter, enrichment of CD133+ cells and maintenance of their genomic and phenotypic characteristics (Panchalingam et al., 2010).

6.5. Introduction of stromal cells

The complexity of the GBM TME is particularly reflected in the presence of various stromal cells and their intense interaction with

tumor cells. Therefore, numerous models were developed to study GBM co-cultures with various stromal constituents in a 3D setting. So far, most extensively were investigated the 3D GBM co-cultures with endothelial cells. Co-culturing glioblastoma and HUVEC cells in 3D hydrogels was shown to stimulate overall co-culture growth throughout the time, especially when the proportion of GBM cells in co-culture was increased (Avci et al., 2015). The presence of glioblastoma cells in 3D hydrogels enhanced proliferation, sprouting and migration of HUVEC cells (Chen et al., 2009; Nguyen et al., 2016a, 2016b). Such stimulation of angiogenesis is mediated through VEGF secretion by glioblastoma cells or exogenously added VEGF at low concentrations (Chen et al., 2009). This effect was even more pronounced under hypoxic conditions (Chen et al., 2009). On the other hand, HUVEC cells slowed down the growth rate of GBM cells, caused them to form spheres around or on the top of endothelial cells (Kievit et al., 2016) and promoted their invasive phenotype (Chonan et al., 2017). In the novel 3D model of Wang et al., adult patient-derived GBM tumor xenograft cells were shown to have significantly increased cell proliferation in the presence of mouse brain microvascular endothelial cells encapsulated in alginate microfibers mimicking microvessels in hydrogel (Wang et al., 2019a). Co-culturing GBM and endothelial cells in 3D models induced the acquisition of GBM stem-like phenotype with increasing expression of corresponding markers CD133, CD44 and Id1 (Kievit et al., 2016). Recently, McCoy and colleagues showed that enrichment in GBM stem cell population and their increased invasiveness were mediated by interleukin-8 signaling in 3D models of patient-derived GBM spheroids co-cultured with brain endothelial cells (McCoy et al., 2019), while Truong et al., proved in 3D organotypic microfluidic platform that these effects of microvasculature environment on patient-derived glioblastoma cells involved activity of CXCL12-CXCR4 signaling (Truong et al., 2019). Similarly, Wang et al., reported that endothelial cells increased the expression of CXCR4 in GBM cells in their 3D co-culture system (Wang et al., 2019a). Co-culturing also affected the expression of several other groups of genes and proteins: induced expression of angiogenesis related genes, such as PECAM1/CD31, KDR/VEGFR2, and PIK3R1 (Avci et al., 2015), induced the expression of differentiated cell marker tubulin β3 (TUBB3), upregulated expression of cell–ECM adhesion-associated proteins (integrin α2, integrin β3, type II collagen α1 and vitronectin) and downregulated the expression of genes associated with cell–cell adhesion such as cadherin 1 and catenin α2 (Chonan et al., 2017). Ngo and Harley established a tri-culture comprising of U87 cells, HUVECs and normal human lung fibroblasts (NHLFs) and explored their interaction in 3D hydrogel depending on the ECM composition (Ngo and Harley, 2017). This study revealed that U87 cells arranged in close proximity to endothelial cells and their morphology changed from rounded in the absence of HA to elongated in its presence. On the other hand, U87 cells induced the regression of the microvasculature network in a cell density- and time-dependent manner, irrespectively of HA presence.

Other stromal cells also significantly influenced GBM cells behavior in 3D co-culture models and *vice versa*. According to Kievit and collaborators, astrocytes affect GBM cells similarly to HUVEC cells. They slowed down GBM cell growth rate when cultured in a higher cell ratio (astrocytes:GBM 5:1), stimulated them to form spheres and promoted their stem-like phenotype (Kievit et al., 2016). On the other hand, when grown in a ratio 1:1 in HA-gelatin hydrogel, astrocytes promoted tumor cell proliferation (Civita et al., 2019). 3D GBM co-cultures with normal astrocytes showed that ECM molecules secreted by GBM cells increased glial fibrillary acidic protein (GFAP) expression in astrocytes, while astrocytes secreted soluble factors that increased GBM cell migration (Grodecki et al., 2015). Moreover, it was demonstrated in the study of Gritsenko et al., that 3D astrocyte scaffold stimulated GBM cell invasion, both along astrocyte layers and through the scaffold (Gritsenko et al., 2017). In more complex 3D systems developed by Herrera-Perez et al., human endothelial colony forming cells were introduced to co-culture of various patient-derived cell lines and astrocytes to investigate their mutual effect on GBM cell invasion (Marisol Herrera-Perez et al., 2018). Astrocytes significantly induced invasion of all three examined GBM cell lines. However, addition of endothelial precursor cells had diverse effects on invasion depended on the cell type and their genetic background.

Mesenchymal stem cells (MSC) also modulated the GBM phenotype in corresponding 3D co-culture models. MSC promoted GBM cell proliferation in transwell chamber system either through a TGFB1-mediated paracrine signaling or by direct interaction, independently of TGFB1 cytokine secretion (Oliveira Rodini et al., 2018). Breznik et al., observed that glioblastoma cell lines increased MSC motility when co-cultured in collagen I or matrigel. On the other hand, MSC had a differential effect on GBM cell invasion depending on the cell type, suppressed the invasion of U87 cells but increased the invasion of U373 cells (Breznik et al., 2017).

Immune cells as important constituents of the tumor stroma also influence GBM cell invasion. For example, Coniglio et al., showed that tumor associated macrophages (THP-1 cells) significantly induced invasion of human glioblastoma cell line U87 and similarly rat microglia stimulated invasion of murine glioblastoma cell line GL261 in 3D matrices (Coniglio et al., 2016). Besides, human microglial cells were shown to promote GBM cell proliferation (Leite et al., 2020). Cui and colleagues went a step further and developed 3D tri-culture microfluidic angiogenesis platform to investigate the interaction between GBM, endothelial cells and immune cells (Cui et al., 2018b). They observed that GBM cells switched uncommitted macrophages into macrophages with immunosuppressive phenotype. These tumor-associated macrophages further stimulated sprouting and angiogenesis of co-cultured endothelial cells (Cui et al., 2018b). Tang et al., developed a tetra-culture model with GSC, astrocytes, neural precursor cells and macrophages. This system supports the upregulation of different glioblastoma signatures, such as invasion, angiogenesis, hypoxia and stemness. On the other hand, macrophages in a co-culture system are polarized to M2 phenotype, showing that way mutual effect of cellular components of TME (Tang et al., 2020).

7. Effects of drug treatment on GBM cells in 3D models

Regardless of the type of 3D GBM culture is used in drug screening, they are generally more resistant to drug treatments than monolayer cultures and therefore more closely resemble chemotherapy response in GBM patients. A number of studies has investigated the difference in response to drugs between 2D and 3D cultures and some examples are listed in Table 3. Regarding drug resistance development, Han et al., constructed a specific microfluidic device for the detection and study of drug resistance acquisition in GBM (Han et al., 2016). This so called, Cancer Drug Resistance Accelerator (CDRA) chip consists of 488 hexagonal microchambers with two microchannels for antiparallel supply with drug and media. It was designed to generate a drug concentration gradient and observe the emergence of drug resistant cell population throughout the time. Different mechanisms were reported to underlie the development of drug resistance in 3D cell cultures. Ayuso et al., observed that TMZ had a mild effect on U-251 cell viability in their microfludic system. They explained this resistance as a consequence of reduced GBM cell proliferation in 3D hydrogel that affects TMZ activity, which is dependent on DNA replication (Ayuso et al., 2016). Pang and collaborators observed that biomechanical properties of GBM cells significantly affected sensitivity to vincristine in their microfluidic platform for single-cell separation based on cell (Pang et al., 2016). They revealed that smaller and/or more deformable tumor cells were more resistant to the drug.

According to data of Wang et al., several groups of genes demonstrated increased expression in 3D cell cultures, contributing to a higher degree of drug resistance (Wang et al., 2016). These included genes associated with drug detoxification, drug efflux (ABCC5, ABCC3, and MVP), resistance to apoptosis (ESR1, RARG, ERBB4, MET), anti-apoptotic genes (BCL2, B2M), resistance against oxidative stress (NFKB

Table 3
Studies showing drug resistance in 3D compared to 2D cell cultures.

3D cell cultures	GBM cell types	Drugs	Relative resistance (3D *vs* 2D)	Reference
3D culture with chitosan-hyaluronic acid scaffolds	U-118	DOX, TMZ	no difference for DOX, 2-fold for TMZ	(Florczyk et al., 2013)
3D bioprinted cell culture with gelatin/alginate/fibrinogen hydrogel	U87, SU3 patient-derived cell line	TMZ	1.5-fold for SU3 cells, 2.2-fold for U87	(Dai et al., 2016)
3D culture with collagen scaffolds	U87, primary GBM cells	TMZ, CCNU, Cisplatin	U87 cells: 3-fold for DDP, 42-fold for CCNU, 6-fold for TMZ primary cells: 3-fold for DDP, 18-fold for CCNU, 6-fold for TMZ	(Lv et al., 2016)
3D culture with chitosan-hyaluronic acid scaffolds	Primary GBM cells	TMZ, BCNU, CCNU, Everolimus	3-fold for TMZ, 9-fold for BCNU, 16-fold for CCNU, no difference for Everolimus	(Wang et al., 2017a)
3D bioprinted culture with gelatin/alginate/fibrinogen hydrogel	U118	TMZ	2.5-fold	(Wang et al., 2019a)
3D aggregates on agarose hydrogels	BMG-1	Cisplatin, Bleomycin	1.3-fold for cisplatin, no difference for bleomycin	(Ravi et al., 2017)
Spheroids and 3D culture with gelatin foam	Patient-derived cells	Irinotecan, 5-Fluorouracil	about 10-fold for both drugs	(Yang et al., 2015)
3D bioprinted culture with alginate/HA/collagen I	U87, primary cell line	TMZ, cisplatin	2-fold for TMZ, 8-fold for cisplatin for U87, 24-fold for cisplatin for primary cells	(Hermida et al., 2020)

TMZ-temozolomide; DOX-doxorubicin; CCNU-comustine; BCNU-carmustine.

family members, PPAR, SOD1, HIF1A), DNA repair (MGMT, XPC, TOP2B and BRCA2) and DNA replication arrest (CDKN13 and CCND1). Interestingly, the authors did not observe a significant difference between 3D scaffold and 2D cultures in the expression of genes mainly involved in multidrug resistance (*e.g.*, ABCB1) and detoxification (*e.g.*, CYP3A4). In line with these findings are results published by Lv and colleagues that observed upregulated expression of MGMT, as a possible mechanism of resistance to alkylating agents in 3D environment, but no changes in the expression of major ABC transporters (ABCB1, ABCC1, ABCC2, ABCC4, and ABCG2) (Lv et al., 2016). However, some authors have reported increased expression of ABCG2 in GBM cell lines resistant to drug treatment in 3D cultures (Florczyk et al., 2013; Kinsella et al., 2011). Florczyk et al., also observed that cell line with the highest degree of resistance to alkylating agents in 3D systems, besides upregulating ABCG2 expression, also increased the expression of ABCB1 (Florczyk et al., 2013). Moreover, results from previously described 3D system, Cancer Drug Resistance Accelerator chip, showed that increased drug efflux activity was the main cause of emerging DOX resistance in U87 cells (Han et al., 2016). The authors of this study isolated resistant U87 cells from the chip and performed exome and transcriptome sequencing and identified several mutated genes (*CHD1* and *FLNA*) related to DOX resistance, as well as significant number of differentially expressed genes associated with immune response, DOX metabolism and NFκB signaling. Increased resistance to apoptosis is another mechanism that contributes to resistance to alkylating agents (Samiei et al., 2020; Wang et al., 2016), as well as DOX and resveratrol (Kim et al., 2011), in 3D cell cultures. Regarding the latter study, authors observed increased production of anti-apoptotic factors, survivin and Bcl2, in 3D cultures in response to DOX and resveratrol as known apoptosis inducers.

Drug resistance in 3D models may be at least partially reversed by combined drug application. Fernandez-Fuente and collaborators showed that sunitinib resistance in three-dimensional GSC culture may be reversed through the inhibition of the Akt and ERK signaling pathways, using PD98059 and LY294002, respectively (Fernandez-Fuente et al., 2014). Shojaei et al., described that simvastatin, a mevalonate biosynthesis inhibitor, can increase TMZ-induced apoptosis by inhibiting autophagy flux (Shojaei et al., 2020). Similarly, in our recent study we were able to sensitize TMZ- resistant RC6 cells by combining TMZ with coenzyme Q10 in 3D microfludic device within collagen hydrogel (Stojković et al., 2016).

8. Effects of 3D culture microenvironment on GBM cell response to drug treatment

The effects of drug treatments on 3D glioblastoma culture are determined not only by culture dimensionality but also by the presence of various microenvironment factors, as summarized in Table 4.

Two publications reported that the efficacy of EGFR-targeted therapies in 3D GBM culture depends not only on EGFR status but also on the chemical composition and physicomechanical properties of the ECM. Pedron et al., showed that EGFR mutated, patient-derived GBM cells, that are sensitive to erlotinib, had decreased response to this TKI inhibitor in the HA-containing GelMA hydrogel, due to crosstalk between CD44 and EGFR signaling pathways (Pedron et al., 2017). Xiao and colleagues made a similar observation in their 3D brain-biomimetic platform. They confirmed that patient-derived GBM cells are less responsive to erlotinib and lapatinib in the presence of HA-bound hydrogels and this effect was also dependent on the stiffness of the hydrogel (Xiao et al., 2018b). Recently, the same group showed that HA and RGD-functionalized proteins in 3D hydrogel concomitantly contributed to GBM cell resistance to the alkylating agents TMZ and carmustin (Xiao et al., 2019). This effect was mediated by Src signaling upon joint activation of CD44 and integrin. Another ECM component, fibronectin, was also shown to influence GBM cell response to targeted therapy. Efficiency of MEK inhibitor, PD0325901, on GBM cell growth, motility and dispersal from spheroid is dependent on high fibronectin concentration in the ECM (Shannon et al., 2017). Recently, two additional research groups demonstrated that growing cells in stiffer scaffolds leads to higher TMZ resistance (Wang et al., 2020b; Zhu et al., 2020).

As previously mentioned, hypoxic conditions promote an invasive phenotype of GBM upon 3D cultures, however the lack of oxygen also influences their response to therapy. Namely, Musah-Eroje and Watson showed that growing GBM cells in 3D culture increased resistance to TMZ which became even more pronounced under hypoxic conditions (Musah-Eroje and Watson, 2019).

The presence of stromal cells in composite 3D GBM cultures further contributes to tumor cells' drug resistance. Namely, astrocytes and endothelial precursor cells are able to diminish the cytotoxic effect of the STAT3 inhibitor (SH-4-54) in 3D culture of stem-like GBM cell line (Marisol Herrera-Perez et al., 2018). Earlier work of Yang and colleagues also showed that addition of the astrocytic cell line to different GBM cell spheroid cultures protected them from cytotoxic insult of TMZ and DOX (Yang et al., 2014). Additionally, Civita et al., reported that lower response to TMZ, vincristine and clomipramine can be a result of mitochondrial exchange between reactive astrocytes and GBM cells through tunneling nanotubes formed between them (Civita et al., 2019). Xiao et al., established U87 cells co-culture with rat neurons and glial

Table 4
Effects of various microenvironment conditions on drug response in 3D glioblastoma cell cultures.

3D cell cultures	GBM cell types	Drugs	Microenvironment conditions	Major findings	Reference
3D culture with HA decorated GelMA hydrogels	Patient-derived xenograft cells	Erlotinib	0, 0.3, 0.5, 0.7, 1 and 2 wt% hyaluronic acid methacrylate	EGFR mutated cells were 2 fold resistant in the presence of HA in hydrogel	(Pedron et al., 2017)
Gliomaspheres and 3D culture with HA and RGD containing hydrogels	Primary cell lines	Erlotinib, Lapatinib	0.5 % or 0.1 % (w/v) HA, with or without RGD peptide, 1 or 2 kPa compressive modulus	Cells cultured in 3D hydrogels with a high HA content (0.5 % w/v), RGD tripeptide and low compressive modulus (1 kPa) were the most resistant to erlotinib	(Xiao et al., 2018b)
Gliomaspheres and 3D culture with HA and RGD containing hydrogels	Primary cell lines	TMZ, carmustine	0.5 % and 0.1 % (w/v) HA, with or without RGD peptide	High content HA scaffolds, particularly those with RGD peptide, were more resistant to TMZ and carmustin than low-HA hydrogel culture and gliomaspheres	(Xiao et al., 2019)
Spheroids	Primary cell lines	PD0325901	30 and 300 μg/mL serum fibronectin	PD0325901 increased spheroid stiffness and viscosity under high fibronectin serum concentration	(Shannon et al., 2017)
Neurospheres	U251, U87 SNB19	TMZ	Normoxia (20 % oxygen), hypoxia (1 % oxygen)	U87 and U251 neurospheres were more resistant to TMZ than monolayer culture, particularly under hypoxic conditions	(Musah-Eroje and Watson, 2019)
3D culture in collagen type I-hyaluronan matrix	Primary cell lines	SH-4-54	Tri-culture with normal primary astrocytes and ECFCs	Stem marker positive cells were 1.25 fold more resistant to STST3 inhibitor in 3D matrix with stromal cells than in 3D matrix only	(Marisol Herrera-Perez et al., 2018)
Spheroids	A172, U251, LN18, C6, primary cell lines	TMZ, DOX	Co-culture with TNC-1 astrocytes	Most cell lines were resistant to TMZ and DOX in the presence of astrocytes	(Yang et al., 2014)
3D culture in GCNT matrix	U87	blebbistatin	Co-culture with rat neurons and glial cells	Cortical cells reduced U87 migration velocity by 34 % in GCNT matrix	(Xiao et al., 2018a)
3D culture in HA-gelatin hydrogel	Primary cell line	TMZ, CLM, VCR	Co-culture with microglia	Cells were more resistant to all drugs in co-culture with microglia	(Leite et al., 2020)
3D culture in HA-gelatin hydrogel	Primary cell lines	TMZ, CLM, VCR	Co-culture with astrocytes	Cells were more resistant to all drugs in presence of astrocytes	(Civita et al., 2019)
3D bioprinted culture with GelMA and GMHA	Xenografted tumor cells	TMZ, Erotinib, Gefitinib	Tetra-culture with astrocytes, neural precursor cells and macrophages	The resistance was enhanced in tri-culture and it was potentiated with addition of macrophages	(Tang et al., 2020)

HA-hyaluronic acid; GelMA-methacrylamide-functionalized gelatin; RGD-arginine-glycine-aspartate; TMZ-temozolomide; ECFCs-endothelial colony forming cells; PD0325901-MEK inhibitor; SH-4-54-STAT3 inhibitor; blebbistatin-myosin II inhibitor; GCNT-graphene–carbon nanotube, GMHA-glycidyl methacrylate-HA.

cells within 3D graphene–carbon nanotube matrix and evaluated the effect of myosin II inhibitor, blebbistatin, on tumor cell migration. They observed that the presence of cortical cells reduced blebbistatin activity reflected in decreased U87 cell motility (Xiao et al., 2018a).

9. Preclinical models of the blood-brain barrier

9.1. *The blood-brain barrier limits the efficient treatment of brain tumors*

The BBB consists of brain endothelial cells which form the complex network of brain capillaries. It enables the supply of brain with the essential nutrients and oxygen but at the same time, its important function is to protect the brain from any environmental insult. Besides, neurons must be protected against physiological fluctuations such as temperature oscillations, variations in O_2 or CO_2 levels, and variations in the concentrations of different factors produced by the organism. Therefore, the BBB has the least permeable capillaries also due to tight junctions between endothelial cells. The tight junctions between these endothelial cells consist of transmembrane proteins (occludin, claudin and junctional adhesion molecule), cytoplasmic attachment proteins (cingulin, zona ocludens-1, -2, -3) as well as cytoskeletal proteins (He et al., 2018). Besides tight junctions, there are other physical components of the BBB such as astrocytic end-feet, pericytes and a basement membrane (Dyrna et al., 2013). The entire neurovascular unit of the BBB consists of five cell types: endothelial cells, astrocytes, pericytes, neurons, and microglia (Abbott, 2013). Among brain cells which assist the BBB, astrocytes secrete factors necessary for the BBB function (Colgan et al., 2008), pericytes decrease vascular permeability (Daneman et al., 2010), induce polarity to the astrocyte end feet (Allt and Lawrenson, 2001) and synthesize factors necessary for the differentiation of the BBB (Dore-Duffy et al., 2006), while microglia clears the cellular debris (Sumi et al., 2010).

Brain tumors and brain metastasis compromise the integrity of the BBB due to the formation of specific blood-tumor barriers. The blood-tumor barrier (BTB) possesses its individual characteristics including different and non-uniform permeability with higher activity of the multidrug efflux transporters of the ABC superfamily (Arvanitis et al., 2020). The efflux mediated by P-glycoprotein (ABCB1) can be present on membranes of both endothelial and tumor cells. P-glycoprotein extrudes toxic compounds on the expense of ATP which is essential for the P-glycoprotein function (Borgnia et al., 1996; Henderson and Piquette-Miller, 2015; Li et al., 2016b; Zhang et al., 2021). Thus, the drug cannot reach the effective concentration necessary for its anticancer activity. The high expression and the ATP-driven efflux mediated by P-glycoprotein is the mechanism of MDR responsible for the low intracellular penetration of chemotherapeutics.

Thus, although the presence of GBM alters the normal vascular function of BBB rendering it more permeable, disruption of barrier due to the BTB cannot provide efficient drug penetration (Dhermain et al., 2010). Rather, cell migration is increased across a more permeable BTB, thereby facilitating the spread of GBM cells in distant places within the brain parenchyma (Jia et al., 2014).

9.2. *In vitro models of the blood-brain barrier*

Although different approaches were used to open the BBB for the efficient treatment of brain diseases, the lack of *in vivo* validation and controlled clinical trials delayed the progress. Even if *in vivo* experiments provide the natural environment, the versatility of results obtained from animal models cannot be accurately translated into humans (Perel et al., 2007). This could be surpassed by using the results from the *in vitro* models of BBB. The quality of the *in vitro* models is usually determined by several parameters including the transendothelial electrical resistance (TEER), permeability of specific marker substances (mannitol and

sucrose) and the expression of BBB markers (Zona Ocludens-1, Claudin-5, Occludin, and endothelial von Willebrands factor) (Wolff et al., 2015). The *in vivo* TEER across the functional BBB is 1500–8000 Ωcm^2 (Crone and Olesen, 1982). Therefore, achieving the approximate values for the *in vitro* models is imperative.

The most used type of *in vitro* BBB models has polystyrene or polycarbonate membranes with 400 nm pores (Colgan et al., 2008) separating the endothelial cells grown in the upper (luminal) compartment of the Transwell (Corning Inc., Corning, NY) from the astrocytes and pericytes usually cultured on the lower (abluminal) side of the membrane. To obtain the most accurate model, it is important to choose the right cell types such as brain derived endothelial cells which are almost impossible to obtain from healthy donors. As an alternative human pluripotent stem cells induced to differentiate into brain microvasculature are used (Lippmann et al., 2012). However, the majority of experimental work was performed with immortalized cell lines such as human cardiac microvascular endothelial cells (hCMEC)/D3 and human brain microvascular endothelial cells (HBMECs) (Eigenmann et al., 2013). To increase the tightness of endothelial cells grown in a monoculture, the astrocyte-conditioned medium (Siddharthan et al., 2007) as well as glia-conditioned ECM are used (Hartmann et al., 2007). Besides, it was shown that by introducing the shear stress through microfluidics, together with astrocyte conditioned medium, the TEER of primary HBMECs increased up to 1500 Ωcm^2 (Siddharthan et al., 2007).

In respect to co-cultures, two models can be studied: contact and non-contact. In the contact model, endothelial cells are grown on the luminal side of a Transwell membrane, whereas astrocytes, pericytes, or neurons are grown directly on the abluminal side of the membrane allowing a close-range impact on endothelial cells. In non-contact model, non-endothelial cells are grown on the bottom of the well making the effect on the endothelial cells solely through excreted molecules. These two models complement and provide more comprehensive understanding of the BBB functioning (Wolff et al., 2015). It was discovered that astrocytes and pericytes were more efficient in preserving BBB properties and increasing TEER in contact than in non-contact models (Al Ahmad et al., 2009; Hayashi et al., 2004). However, the permeability of endothelial cells did not change in co-culture models implying that a monolayer is sufficient for studying drug permeability (Nakagawa et al., 2007).

9.3. *Microfluidic devices with neurovascular units*

Although Transwell cell culture systems are affordable and relevant for the BBB studies, they lack the dynamic mechanical microenvironment and complex architecture of the BBB. Therefore, in recent years, the interest in microchip BBB models constantly increases (Booth and Kim, 2012; Griep et al., 2013; Kaisar et al., 2017; Maoz et al., 2018; Prabhakarpandian et al., 2013; Wang et al., 2017b). Systems developed to resemble the neurovascular unit offer a great potential for BBB research (Achyuta et al., 2013; Bhatia and Ingber, 2014; Prabhakarpandian et al., 2013). These microfluidic devices with multiple physiologic parameters of the BBB and the cytoarchitecture present *in vivo* should be able to serve as high-throughput drug screening platforms. Besides, microfluidics technology combines dynamic fluid flow and 3D setting enabling mimicking of *in vivo* situation of each patient's pathology thus having the potential to serve as a platform for the development of personalized therapy (Bhatia and Ingber, 2014).

Advantages of microfluidic devices over Transwell BBB models are better mimicking of the microenvironment with a potential to imitate organ-level functioning, ability to analyze BBB properties such as permeability, TEER and shear stress, real-time readouts, and testing of pharmacodynamics and pharmacokinetics (Chin and Goh, 2018).

However, these multi-physiological systems have some limitations that need to be overcome in the future. Dimensions of mimicking blood vessels within majority of devices are around 100 μm in diameter, while human cerebral capillaries are around 10 μm (Wiedeman, 1963). In addition, the blood vessel compartment usually does not recapitulate the circular cross section found *in vivo*. Commonly, the geometry in the microdevices involves square or rectangular cross sections. These factors significantly impact the intensity and uniform distribution of shear stress on the endothelial cells (Phan et al., 2017). Moreover, many of the BBB models have a single channel and represent a single blood vessel (Brown et al., 2015; Deosarkar et al., 2015; Wang et al., 2017b). More appropriate and more complex devices should build a network of hierarchical branching vasculature. Another limitation is the non-natural contact among endothelial cells, basal lamina, pericytes and astrocytes that need to be integrated and incorporated in 3D milieu (Phan et al., 2017).

Xu et al., presented a newly fabricated microfluidic device which integrated the physical endothelial-astrocyte interaction, 3D ECM, and dynamic vascular flow (Xu et al., 2016). Although the authors used rat-derived brain microvascular endothelial cells (BMECs) and astrocytes to construct the BBB model, they were able to confirm key features of the BBB (the expression of the BBB markers, TEER in range of the *in vivo* values, and permeability of endothelial cells). Moreover, this multiple functional unit design mimics different BBB regions, which makes this BBB assay suitable for use in a high throughput manner. The results showed that the exposure to dynamic flow as well as presence of astrocytes significantly increased expression of endothelial tight junction proteins (ZO-1, claudin-5) and adhesive protein known as vascular endothelial cadherin (VE-cadherin) in BMECs. On the other hand, the presence of astrocytes increased the expression of P-glycoprotein and GLUT-1, while the flow alone did not alter their expression.

The presence of either dynamic flow or astrocytes increased TEER of BMECs. However, the addition of astrocytes, in the presence of dynamic flow, enhanced the TEER values up to those relevant *in vivo*. The TEER reached a steady-state within 60 h.

Interestingly, the presence of astrocytes increased the impermeability of BMECs as well as dynamic flow alone. However, when the dynamic flow was applied along with the presence of astrocytes, it was not able to enhance the already achieved impermeability.

The authors studied the potential of different human cancer cell types to cross the BBB established in their microfluidic device (Xu et al., 2016). Their results confirmed the clinical empirical findings that lung cancer, breast cancer and melanoma are able to cross the BBB, while liver cancer cells cannot migrate through BBB (Paolillo and Schinelli, 2016). Despite the inherent aggressiveness of GBM, U87 cells could not transverse the BBB. This result confirmed the already known fact that GBM almost never metastasizes out of the central nervous system (Simonetti et al., 2017).

The co-culturing of U87 cells and astrocytes resulted in a homogenous cell layer within 72 h. However, when lung cancer, breast cancer and melanoma cells were co-cultured with astrocytes, they didn't intermix but rather formed cell spheres comprised solely of these cancer cells (Xu et al., 2016).

Another more recently developed hypoxia-enhanced BBB chip used human induced pluripotent stem brain microvascular endothelial cells (iPS-BMVECs) which enabled the maintenance of high TEER levels up to seven days (Park et al., 2019). Hypoxic conditions in the presence of human astrocytes and pericytes were used to differentiate iPS-BMVECs and thus better mimic barrier characteristics. This approach ameliorated previously identified problems with BBB models using human iPS-BMVECs which could only maintain high TEER levels for approximately two days, while the expression levels of MDR efflux pumps in iPS-BMVECs could not reach the levels observed *in vivo* (Vatine et al., 2017). Hypoxic conditions with 5% O_2 induced the expression of HIF1-α, Wnt signaling, P-glycoprotein, GLUT-1, VE-cadherin and VEGF (vascular endothelial growth factor) in iPS-BMVECs, while the expression of ZO-1, Claudin-5 as well as platelet endothelial cell adhesion molecule (PECAM-1) was detected in these cells when co-cultured with human astrocytes and pericytes in a BBB chip. Besides, hypoxia-induced iPS-BMVECs from BBB chip were able to generate their own basement

membrane ECM containing high levels of collagen IV, laminin, perlecan (a heparin sulfate proteoglycan), and fibronectin. Increased expression of some ABC transporters (BCRP, MRP1 and MRP4) as well as many solute carriers (SLC) was observed after three-day cultivation in the BBB chips. All these characteristics were not observed when iPS-BMVECs were differentiated under normoxic conditions.

9.4. Drug testing in 3D models of the blood-brain barrier

The most pragmatic way to confirm the advantages of using *in vitro* models of BBB, particularly technologically advanced microfluidic devices, is to obtain the analogous results with drugs approved for clinical use. Thus, different drugs were applied in the microfluidic device reported by Xu et al. (2016). Eight clinically approved chemotherapeutic drugs were tested on their ability to cross the BBB and their cytotoxic effects on GBM. TMZ as a lipophilic and BBB permeable pro-drug (Ramalho et al., 2019) was compared to a broad-spectrum of anticancer agents with low BBB permeability. The results showed that only TMZ was able to induce apoptosis in GBM cells when the BBB was present. The rest of the tested compounds were hydrophilic and efficient only in the absence of BBB implying that their inability to cross the BBB reduces their anticancer potential against GBM.

Importantly, 400 μM of TMZ was able to induce apoptosis around 80 % of U87 cells in the microfluidic device with established BBB. Therefore, Xu et al., demonstrated the reliability of their microfluidic BBB model in evaluating anticancer drugs' potential (Xu et al., 2016). Even more, this platform has the ability to perform parallel experiments suggesting that it could be used for high throughput screening for anti-glioma agents.

In another study (Park et al., 2019), the functionality of the BBB in the microfluidic chip was tested using verapamil as P-glycoprotein inhibitor, Rhodamine 123 as P-glycoprotein substrate and citalopram as a serotonin-specific reuptake inhibitor - drug commonly used to treat depression. Surprisingly, the authors found that citalopram permeability was increased in the presence of verapamil indicating that P-glycoprotein suppresses the transcytosis of this drug. For the first time, results obtained in the BBB chip recapitulated the *in vivo* findings regarding citalopram (Doran et al., 2005), whereas no other *in vitro* BBB models was able to identify citalopram as a P-glycoprotein substrate (Feng et al., 2008).

Doxorubicin permeability was also tested in this BBB chip under a physiological flow of 100 μL/h (Park et al., 2019). The authors reported that after verapamil application, doxorubicin influx significantly increased through the vascular channel of the BBB chip. This was also comparable with the P-glycoprotein activity *in vivo* (Kalvass et al., 2013). All these results point to the importance of using differentiated iPS-BMVECs, their plating under physiological flow and at the interface with human brain astrocytes and pericytes if one wishes to create the closest mimicking conditions for the investigations of the BBB functionality *in vitro*. However, BBB-on-a-chip models need considerable optimizations and therefore, they still cannot be ideally used for the high-throughput screening of drugs. Another challenge is to explore whether iPS-BMVEC based BBB chips can explain differences in patient-specific response to drugs.

10. Mathematical models as a way to complement research on biomimetic models

Mathematical models describe real systems by means of abstraction and mathematical formalism. They may enable extrapolation beyond the situations originally analyzed, allowing for quantitative predictions, inference of mechanisms, falsification of underlying biological hypotheses and quantitative descriptions of putative relationships between different components of a system. They cannot replace experimental results obtained by biomedical models, but may complement experimentation in providing a broader picture. This may help novel findings or solutions for some cancer-related problems. Thus, a combination approach using experimental and mathematical models has the potential to provide robust findings. Interestingly, GBM is the tumor type that has attracted the most interest of applied mathematicians. Here we will describe only results integrating theory and experiments, *i.e.* validated models with the potential of having biomedical value and not those of interest for pure mathematicians because of technical reasons. The field is still in its initial stages and much progress has been made from the initial mathematical models dealing with simple biological scenarios in either 2D cultures or spheroids to the feature-rich ones in use in recent works.

One of the first studies combining mathematical models and experimental work was done by Stein et al., who proposed a continuum mathematical model describing GBM invasion observed in experiments on the patterns of growth and dispersion of U87MG tumor spheroids in a 3D collagen-I gel. The authors identified and characterized discrete cellular mechanisms underlying invasive cell motility from the experimental data (Stein et al., 2007). Another work (Kim et al., 2009) developed a mathematical method to study 2D *in vitro* experiments reported by Khain and Sander (Khain and Sander, 2006), who showed two different migration patterns: one pattern of wild type U87 cells exhibited radially symmetric migration of individual cells and another pattern of mutant U87-ΔEGFR cells exhibited formation of branches moving outward, with a slower speed. The mathematical model explained both growth patterns to be a result of the effect of cell-cell adhesion on migration.

A complementary analysis of Stein et al., experiments was performed by Kubo and Miyata (Kubo and Miyata, 2017). Another study (Jiang et al., 2014) explored different motility models to describe the U87MG GBM cell line on different substrates and found super-diffusion to provide the best fit. They explained their findings as a result of that type of invasive behavior allowing a better access to external nutrients. Further studies on the same datasets using cellular automata mathematical models were developed (Tektonidis et al., 2011). Yet, an additional study investigated mathematically with the help of a lattice-gas cellular automaton model, the impact of the migration/proliferation dichotomy on avascular glioma invasion in terms of invasion velocity and width of the infiltration zone (Böttger et al., 2012). Khain et al., studied the differential dynamics of tumor cells in spheroids and in wound-healing assays both under hypoxia and normoxia and developed a stochastic mathematical model to understand the results (Khain et al., 2011). The same group investigated, by combining theory and experiments, the effect of cell–cell adhesion on glioma front propagation and the properties of the invasive interface (Khain et al., 2012). Moreover, Aubert et al., studied migration of glioma cells on substrates of collagen and astrocytes using GL15 spheroids and constructed a mathematical model based on a cellular automaton where the various dynamic effects were introduced through adequate evolution rules (Aubert et al., 2008). Using the model, they investigated the role of homotype and heterotype gap junction communication and showed that it was possible to reproduce the experimentally observed migration patterns (Aubert et al., 2008). Related studies have explored recently the invasive patterns of primary cultures and U87 cell line with discrete, individual cell-based mathematical models to understand the relationship between cell-adhesion properties and the different invasive morphologies displayed in the experiments (Oraiopoulou et al., 2018). A summary of the early mathematical modeling activity of glioma invasion in simpler *in vitro* scenarios can be found in a previous review (Alfonso et al., 2017).

Following these initial research studies focused on developing simple mathematical models involving mostly cell-motility and adhesion properties, several works have used the combination of mathematical and biomimetic models to obtain a deeper insight into other biological processes. Kingsmore et al., studied the role of interstitial fluid flow (IFF) in patient-derived GBM stem cells (Kingsmore et al., 2016). Using 3D *in vitro* assays and correlative *in vivo* studies, they demonstrated an increased invasion with flow in GSC lines. Flow-stimulated invasion was

reduced by blockade of CXCR4, CXCL12, and/or CD44, revealing that GSC invasion may be mediated simultaneously by different mechanisms. Characterization of CXCR4+, CXCL12+, and CD44+ populations in different GSC lines revealed different percentages of the subpopulations for each line. The authors developed and validated a mathematical agent-based model to identify the contributions of each subpopulation to flow-stimulated invasion. The mathematical model predicted that IFF-stimulated invasion was driven primarily by CXCR4+ and CXCL12+. Kim et al., combined a special transwell assay with a mathematical model to demonstrate that microglia can stimulate tumor cell invasion by secreting the growth factor TGF-β (Kim et al., 2017). The mathematical model was also used to make new predictions to guide future experiments aimed at the development of new therapeutic approaches. Ayuso et al., constructed an experimental model of GBM by embedding U-251 MG cells within a collagen hydrogel in a custom-designed microfluidic device (Ayuso et al., 2017). By controlling the medium flow through lateral microchannels, they could control blood-vessel obstruction events associated with this disease. They showed that nutrient and oxygen starvation triggered a strong migratory process leading to pseudopalisade generation *in vitro*. The results validated the hypothesis of pseudo-palisade formation in GBM and revealed an excellent agreement with a partial-differential equation mathematical model based on a hypoxia-driven phenomenon (Ayensa-Jiménez et al., 2020; Ayuso et al., 2017).

Mathematical models in combination with *in vitro* experiments have been also used for drug-response studies in GBM. A very interesting work parametrized a partial-differential equation-based computational model of GBM growth and treatment response using *in vitro* data from SF268 during lapatinib exposure (Stein et al., 2018). Then, the authors explored *in silico* what would be the most effective treatment strategy within the clinical toxicity limits of lapatinib. Despite the inability of lapatinib to induce tumor regression with a continuous daily schedule, the modeling approach predicted that continuous dosing would be the best strategy to slow down tumor growth and to decrease overall tumor burden, compared to pulsatile schedules currently known to be tolerated. This was found to hold even when considering drug resistance, reduced lapatinib tumor concentrations due to the BBB, and the phenotypic switch from proliferative to migratory cell phenotypes that occur under the hypoxic TME. Rabé et al., studied the development of drug resistance in glioblastoma using an integrated approach involving experimentation and mathematical models (Rabé et al., 2020). They did not use advanced biomimetic devices but a longitudinal study including a combination of mathematical models, RNA sequencing, single cell analyses, functional and drug assays in a human glioma cell line (U251). However, the methodology shows the potential of the combination of mathematical modelling with *in vitro* studies for unveiling mechanisms in complex situations. The model suggested the presence of a transient state. The experimental analysis found it to be characterized by slow growth, a distinct morphology and a shift of metabolism. The specific gene expression pattern associated with this population revealed chromatin remodeling. Indeed, the histone deacetylase inhibitor trichostatin (TSA), specifically eliminated this population and thus prevented the appearance of rapidly growing TMZ-resistant cells. Thus, the study allowed the identification of a population with tolerant features, which could constitute a therapeutic target.

A different line of research is the use of mathematical models for the improvement of the design of biomimetic devices. For instance, a recent study (Marino et al., 2018), reported the use of different fluid-mechanical models to optimize the construction of a 1:1 scale, biomimetic, and biohybrid model of the BBB fabricated through two-photon lithography and used as scaffolds for the co-culturing of endothelial-like bEnd.3 and U87 glioblastoma cells. Another research group (Koens et al., 2020) used a computational model to adjust parameters and understand oxygen heterogeneities in a new double-layer microfluidic device.

Finally, a recent paper combining mathematical models and patient's imaging data has underlined the substantial role played by evolutionary dynamics in tumor growth dynamics (Pérez-García et al., 2020). The mathematical models predicted that three spatial dimensions would be necessary to capture the rich evolutionary dynamics observed in genuine cancers with experimental systems. The idea behind the calculations is that evolutionary dynamics typically take places in peripheral spatial locations where phenotypic and/or genetic alterations can consolidate in macroscopic populations and 3D is the only configuration allowing for a sufficient space for those changes to occur and consolidate.

In summary, mathematical models, when used in combination with GBM *in vitro* biomimetic models, have a substantial potential to aid in hypothesis generation and falsification, interpretation of experimental results and the design of better devices. As they become more mature they can help in better understanding the complex interplay arising in rich 3D biomimetic systems and to extrapolate the experimental observations on those *in vitro* systems and translate them to *in vivo* scenarios.

11. Future perspectives and directions

In the past decade researchers have made a great effort to develop a variety of 3D glioblastoma cell models. These models are valuable tools for *in vitro* studies of glioblastoma pathogenesis and also represent good platforms for drug screenings. 3D glioblastoma models are physiologically more relevant and reliable than conventional 2D cell cultures and they are increasingly replacing monolayer cultures in laboratory practice. They should ultimately substitute animal models in preclinical drug characterization, reduce the costs of drug discovery and development, avoiding at the same time ethical concerns regarding *in vivo* experiments. However, there are still important issues to be considered and properties to be improved in order to fully adopt 3D glioblastoma cell culture models as standard platforms for preclinical drug screening and development. The greatest challenge in developing 3D glioblastoma models is to mimic the full temporal and spatial complexity of the tumor: organization of its structures, heterogeneity of cell types, specificity of ECM composition, dynamics of TME conditions, and simulation of different cancer-related processes, such as metastasis.

As reviewed above, spheroids represent the first step in making more complex cell cultures. They have cell-cell and cell-ECM interactions and established biochemical gradient, but are still lacking the 3D architecture of the tumor. On the other hand, organoids, as miniature organs, appear to most closely resemble native tumor structure *in vitro* but lack reproducibility and have limitations in continuously providing tumor-related physicochemical conditions and preserving long-term culture. Introducing 3D bioprinting technology also gives an opportunity to achieve structural complexity of tumor tissue in a controlled manner. However, bioprinted cultures, as with organoids, lack an important physicomechanical component namely fluid flow. Therefore, in the future, organoids and bioprinted cultures should be combined with other approaches and platforms that provide a dynamic microenvironment, for example perfusion bioreactors and microfluidic devices, for establishing a complete mimic of 3D glioblastoma cell culture. In this regard, microfluidic devices are so far the most comprehensive of all 3D cell culture models. Yet, there are several challenges to be addressed in the future development and use of microfluidic-based 3D glioblastoma cell cultures. In the first place, the choice of biomaterials remains a great challenge and requires further development by biomaterial and tissue engineering. Focus should be put on improving biomaterial characteristics for 3D GBM cell culture scaffolds, on increasing their long-term stability, mimicking the specific composition of GBM ECM (*e.g.* enriched in HA), maintaining consistency of their composition and physicomechanical properties, particularly in response to the presence of GBM cells and their dynamic interaction with scaffold. Particular attention should be paid to the choice of biomaterials- regarding the type of drugs applied in studies to avoid false positive or false negative

results on chemosensitivity due to variable drug permeability and absorption. Other challenges, which also affect drug efficiency in GBM, remain to be addressed in the future design of microfluidic-based 3D GBM platforms. Those include establishing heterogeneity of GBM cells, mimicking interactions between GBM cells and other brain cell types, as well as modeling GBM motility.

Development of composite 3D glioblastoma cell culture platforms can be an expensive and time consuming process and their operation often requires the use of additional specialized equipment (*e.g.* peristaltic pumps). Therefore, improvements in design and engineering of 3D glioblastoma cell cultures should be made in the future, particularly to ensure easy handling and widespread use. Moreover, in the near future, 3D glioblastoma cell cultures should be designed to be applicable for high-throughput and high-content screening, to enable their wider and more frequent commercial usage.

An important aspect in the application of 3D glioblastoma cell platforms is monitoring processes within them, not only on phenotypic but also on molecular level. So far, end-point analyses or time-lapse fluorescent and confocal microscopy were mainly used to analyze cancer cell behavior in 3D platforms. However, in order to obtain as much relevant data, particularly as a function of time, it is necessary to couple 3D cultures with more advanced monitoring techniques such as MRI and MALDI imaging that will enable long-term real time monitoring of 3D cell cultures and identify the spatial distributions of biomolecules within cells.

3D cancer cell culture analyses generate large amounts of data that will further increase with the development of high-throughput and high-content 3D screening platforms. Fast development of machine-learning software and their implementation for 3D cancer cell culture analyses will provide quick and automated evaluation of the obtained results which will further increase applicability of 3D cell cultures for widespread commercial use.

Regardless of how complex 3D GBM cell cultures will be made in the future, they will still represent simplified models of the real tumors. Application of mathematical modeling can help to overcome this problem of reductionism in *in vitro* conditions. Mathematical simulations based on the obtained *in vitro* data could predict GBM behavior *in vivo,* particularly response to treatment and potential development of chemoresistance, thereby increasing usefulness of 3D glioblastoma cell cultures for drug screening and precision medicine.

Overall, upgraded 3D GBM models, that will be developed in the future with integrated advanced imaging techniques, cutting-edge artificial intelligence-based analyses and mathematical simulations will be invaluable and irreplaceable tool for better understanding of GBM, its response to therapy and overcoming chemoresistance, as well as for commercial application in preclinical drug screening, drug development and personalized medicine.

Although *in vitro* BBB models cannot fully recapitulate the activity of the BBB *in vivo*, they help us studying BBB development and BBB function under pathological conditions. Particularly useful BBB models are iPSC-based models offering the possibility to understand the individual changes that can be crucial for defining drug concentrations in GBM patients. Other advances in the BBB-on-a-chips include introduction of appropriate ECM, all cellular components of the neurovascular unit and shear stress due to the dynamic flow. These advanced BBB models were developed in order to evaluate drug permeability and toxicity aiming to help in the determination of new GBM therapeutic approaches.

Declaration of Competing Interest

Ignacio Ochoa and Luis Fernández are promoters and consultants for BeOnChip S.L. and EBERS Medical Technology S.L. (Zaragoza, Spain). Both cited companies have had no role in the decision to publish nor were involved in the writing of this manuscript.

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