UNIVERSITY OF GENEVA

bachelor monograph

BRAIN ORGANOIDS: POTENTIAL, ADVANTAGES AND LIMITATIONS OF A MODEL SYSTEM FOR GLIOBLASTOMA

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Abbreviations

GBM	Glioblastoma Multiforme
GSC	Glioblastoma Stem Cell
CSC	Cancer Stem Cell
iPSC	Induced Pluripotent Stem Cell
ES or ESC	Embryonic Stem Cell
ASC	Adult Stem Cell
ECM	Extracellular Matrix
TME	Tumor Microenvironment
GBM organoid	Glioblastoma organoid
GBO	Glioblastoma Organoid (a very similar model of the GBM organoid)
NeoCOR	Neoplastic cerebral organoid
GLICO	GLIoma cerebral organoids
CAR-T	Chimeric Antigen Receptor-T cell
TIL	Tumor Infiltrating Lymphocyte
EB	Embryoid Body

Abstract

Among the different types of brain cancers, glioblastoma (GBM) has the most severe prognosis with a median survival of 14 months and a survival expectancy beyond 2 years very poor. GBM is associated with the highest stage of brain cancer, stage 4 astrocytic tumors. This grade is assigned when there is histological evidence of vascular proliferation and necrosis. This differs from grade 3 which is characterized by anaplasia and increased mitotic activity or cytological atypia for grade 2 (1,2).

The lack of a research model is partly responsible for the dramatic prognosis of this disease. The recent emergence of brain organoids has opened up a spectrum of research in GBM modelling. First, cerebroids technology offers opportunities to study biological aspects of GBM such as driving mutations and signaling pathways. Some models partially recapitulate the tumor microenvironment (TME), which is a source of failure of numerous therapies. In addition, these model systems are used to develop personalized medicine, via the creation of biobanks. Cerebroids can be derived directly from patient tumor cells. The genetic and epigenetic profiling of these organoids is then used to test and/or predict the response of the tumor to certain therapies or drugs. Finally, some GBM organoid models are used to develop and refine cancer therapies such as immunotherapy, chemotherapy, or radiotherapy. For example, the tolerance of cerebroids to radiation can be tested by ionizing and highlighting cleaved caspase 3 in them. Alkylating agents such as temozolomide (TMZ) and bis-chloroethyl nitrosourea (BCNU) can be administered to the organoid model to test cytotoxicity in chemotherapy. Some of these organoid models of GBM recapitulate the tumor immune microenvironment, making it possible to study the effects of immunotherapy. For example, by including CAR-T cells in the organoid culture (co-culture) it has been possible to measure the killing of CAR-T cells. Organoids derived from patient GBM cells have the particularity of maintaining the phenotype and genetic expression of the original tumor which makes them important for personalized treatment, especially for testing drug response.

In this work I have tried to review the applications, advantages and limitations of some of the main organoid models of GBM. I have focused on the GBM models GLICO and NeoCOR, which are made from a cerebroid, and the organoid models GBM and GBO, which are constructed from a tumor biopsy only.

Introduction

The mouse model has been and remains an important model organism for understanding brain development and function. However, there are important differences between humans and mice, particularly regarding the neocortex formation, neural stem cell divisions and the absence of gyrification in mice. Post-mortem human brain tissue also has significant drawbacks with difficulties in processing and preservation of the tissue. 2D cultures are interesting tools offering uniform accessibility to factors (growth or differentiation) and allowing high-throughput screens. However, spatial gradients of different factors or nutrients, as well as cell-cell and cell-extracellular matrix interactions are limited or absent. These interactions are important for the regulation of the developmental stages of neurons (3,4). In 1999, Clive N. Svendson and Augustin G. Smith reported that if, in the future, an in vitro technique would allow to make human brain tissue, many problems in the medical field and in basic research could be solved (5). This technology currently exists and consists in the production of biological entities called cerebral organoids, cerebroids or mini brains.

Cerebroids are a type of organoids. Organoids are in vitro models existing for different organs. For example, gastruloids mimic embryonic development at the gastrula stage, embryoid bodies model embryonic early development and embryoids assimilated to more organised embryoid bodies mimic the development of the embryo at early stages (6).

With regard to organoids, for example, the intestinal organoids developed in 2009 by Sato et al. were obtained by culturing Lgr5+ cells (ASC). They formed cryptic and villous-like structures that recapitulated functions of the small intestine. The retinal organoid appeared in 2011. Aggregates of cultured EBs formed these optic cup organoids within which rods and cones reside. In 2013, at the same time as the brain organoid emerged, the organoid of the liver, pancreas and kidney appeared. The liver organoid formed by lgr5+ cells (ASC) in culture with Matrigel allows to obtain mature and functional hepatocytes which are transplantable in murine models with liver pathology. The pancreas organoid also allows transplantation which makes them valuable candidates for regenerative medicine (7,8).

There are other types of 3D structures that are used as brain model systems (*box I*). Neurospheres for example are interesting for toxicology studies or personal medicine. Dopaminergic neurons from neurospheres can be transplanted to people with Parkinson's disease. Neurospheres *(see box I "neurospheres")* are smaller, do not include cytoarchitecture, and are initiated from redifferentiated iPSCs unlike cerebroids *(see interview in Annex I)*. These examples are all multicellular structures in three dimensions like

organoids, however their derivations, compositions, limitations, and applications are different from organoids.

As organoids recapitulate in vitro some physiological aspects of different biological systems, they are now widely used as models in basic and applied research. Organoids are derived from embryonic stem cells (ES cells), induced pluripotent stem cells (iPSCs) or adult stem cells (ASCs), depending on the type of organoid that should be obtained or the scientific questions that is addressed (9). The word "organoid" emerged in the 1970s, first referring to the 3D cell culture leading to the formation of organ-like structures (10). Currently, organoids can be defined as organs in character and morphology. They are composed of cells that can self-organize into a 3D structure similar to an organ. These cells have a function and a morphological similarity with the cells composing the organ in vivo (9).

Organoids are valuable tools for developmental biology research to study organogenesis, the roles of different morphogens, processes that lead cells to a specific cell fate, and to explore signaling pathways (6,11). Organoids offer new possibilities and new challenges for the scientific and medical community. The potential applications are multiple. They enable the study of biological tissue, the modelling of diseases and offer potential in the field of regenerative medicine. In the case of disease modelling, organoids offer the possibility of studying the mechanism of the disease, of screening drugs, and of personalized medicine, particularly with the help of biobanks (linking organoid cryopreserved and tested for different therapies to transcriptional profiles of patients) *(see box I " Tumor biology")* (12).

Mini brains emerged in the Institute of Molecular Biotechnology in Vienna. Madeline Lancaster, a cellular biologist, observed an unexpected

box I – non-organoid 3D model systems

Neurospheres:

Neurospheres are 3D multicellular structures like organoids. They are the product of neuronal progenitors derived from IPSCs guided to form either glial cell spheres only, neuronal cell spheres only, or a mixture of both cell types. The final structure obtained is smaller and less complex in cytoarchitecture than a brain organoid. (see annex "interview") (4).

Embryoid Body (EB):

The embryoid bodies correspond to the differentiating aggregated ESCs or iPSCs (6,11).

Neural rosettes:

Neural rosettes are the stage at which cultured ESCs have become neuroprojenitors. The structure of the neural rosette has many similarities to the neural tube that forms during neurogenesis in vivo (12).

result as she was manufacturing 2D neural rosettes *(see box I "neural rosettes")*. The cells that did not stick to the surface of the dish behaved differently, forming self-organized 3D floating balls. This observation led the laboratory to develop the first brain organoid derivation protocol (13). Since their emergence, cerebroids have proven their usefulness in the study of human brain development and neurological diseases. Autism, schizophrenia, microcephaly and more recently the impact of SARS-CoV-2 on the brain have been studied using this model (14–16). The first experiment by Lancaster et al. was to derive their cerebroids from IPSCs, both from healthy patients and from patients with microcephaly caused by Zika. The resulting brain organoids contained regions such as the cortex, an enlarged VSZ (external subventricular zone) which was not found in mice. The microcephaly, which in rats showed almost no phenotype, could be reconstructed more faithfully by these organoids. In addition, it was possible to find the causal mutations of the disease and its mechanism(17).

box II - TME

Tumor microenvironment (TME):

A tumor can generate a microenvironment at the tumor site that challenges the immune system. Thus, even if the immune system triggers an adequate anti-tumor response, it will not function at the tumor site. TME is tumor specific (18). In vivo, the TME includes the physical and chemical environment, the cancer cells, the stromal cells, the cells of the immune system and the vascular system and their interactions (4). Immune cells include brain resident and infiltrating myeloid, NK, DC and T regulatory cells. In GBM, TME is characterized by a depletion of T cells and many myeloid-derived suppressor cells (18).

More recent studies have looked at the potential of organoids in modelling GBM cancers (4,18–20). The harmfulness of GBM is associated with several factors such as its high mitotic activity, invasiveness and inter- and intra-tumor heterogeneity. In addition, the presence of a complex tumor microenvironment (TME) *(see box II "Tumor microenvironment (TME)")* makes modelling complicated. Genetically modified mice, mouse embryonic brains and 2D culture of GBM stem cells have led to a better understanding of tumor biology. However, they are characterized by many shortcomings. Genetically modified mice and GBM spheroids do not easily reproduce tumor heterogeneity, mouse embryonic brains are laborious to produce and human cancer stem cells in 2D culture do not present essential components of the TME. The 2D cultures or spheroids accumulate additional mutations during their manufacture, making them an unreliable model (21,22).

Brain organoids have emerged as an ideal tool to model GBM tumorigenesis. For example, they recapitulate few elements of the TME and hypoxia observed in many tumors organoids. In addition, cerebroids allow to capture the heterogeneity of the tumors of individual patients and offer a good reproducibility of generating these organoids. In applied research, this technology has been used to develop drug screens and improve therapies such as immunotherapy *(see box III "immunotherapy")*, chemotherapy and radiotherapy. The possibility of creating a biobank opens the field of predictive and personalized medicine (18). However, these models have limitations such as deficient immune responses, lack of vascular system, controversial reliability (4,22). They are also valuable models for basic research as they allow to study general brain biology, such as the invasion mechanisms of GBM cells (23).

In this monograph, the potential applications of the different GBM organoid models will be discussed in light of their limitations and advantages. To provide a clear context, this text will begin with a description of brain organoids including their manufacture. This will help to apprehend, later on, the GBM organoid models.

box III- Tumor and therapies

Tumor biology:

GBM is characterized by proliferation, diffuse infiltration, necrosis and significant angiogenesis. A better understanding of tumor biology is a key issue to improve treatments such as targeted therapy and personalized medicine. The identification of biomarkers is essential to improve the predictive potential and prognosis of patients. Knowledge of GBM signaling pathways is also important for the development of targeted therapies. Challenges associated with GBM are stem cell resistance, interaction with TME and tumor heterogeneity (24).

Immunotherapy:

Immunotherapy is designed to use the natural abilities of the immune system to fight cancer by tipping the balance in the right direction (24). Immunotherapy aims to increase immune surveillance and/or modulate the immune microenvironment locally (22). It therefore focuses either on increasing the elimination of cancer cells by the immune system (with the tools of cell therapy or vaccines), or on decreasing the tumor's escape mechanisms with checkpoint inhibitors, for example (24).

Manufacturing

Important parameters for deriving organoids

There are many protocols to derive many different organoids. With the same initial cells, completely different organoids can be obtained depending on the cells environment *(see Figure 1 and the box IV "ECMs (extracellular matrix)")* (12,24). To obtain a specific type of organoid, the protocol must consider three parameters: the initial population of cells, the degree of exogenous signals delivered by the matrix as well as the physical characteristics of the culture environment (12). The following paragraphs will discuss these three points in relation to the formation of cerebroids.

The maturation of the final organoids depends on the starting cells. Among the different starting cells used for organoid culture are ASCs (adult stem cells), CSCs (cancer stem cells), iPSCs (induced pluripotent stem cells) and ESCs (embryonic stem cells). CSCs produce organoids that are at a more developed stage, closer to the adult organs. On the other hand, organoids obtained by aggregation of iPSCs or ESCs are more reliable to study organogenesis, but organoids that reach the adult stages are rare. iPSCs are particularly interesting for disease modelling because they can be derived from patients. These cells are less ethically problematic than ES cells from humans (12,25).

box IV - ECMs

ECM (extracellular matrix) :

ECMs (extracellular matrices) support growth and cell adhesion in vivo and constitute the environment from which the cells will organize themselves. For the fabrication of organoids, Matrigel or hydrogel is often used to mimic the ECM found in vivo. There are two types of material mimicking ECM: natural ECMs or chemically defined ECMs. First, Matrigel is a natural ECM produced from mouse sarcoma. It contains mainly components of the ECM in vivo. Cells can adhere to the Matrigel, but it is not a solid scaffold like those generated by bioengineering. A limitation of Matrigel is reproducibility. Indeed, Matrigel is a complex, naturally derived product and therefore has a high variability of composition, making the control of the environment difficult. Another limitation of Matrigel is the risk of transmission of antigens or pathogens, which make organoid transplants complicated. Hydrogels circumvent some of the Matrigel limitations (e.g., better mechanical and environmental controls). The composition of Hydrogel is synthetically defined. However, Hydrogel is less bioactive and thus requires additional manipulations to increase its biocompatibility between this material and the organoid **(25)**.

In the case of cerebroid formation, the starting cells are largely homogeneous iPSCs and sometimes ESCs (18,20,26). As far as tumoroids modelling GBM are based on homogeneous or heterogeneous CSCs (12,21,24).

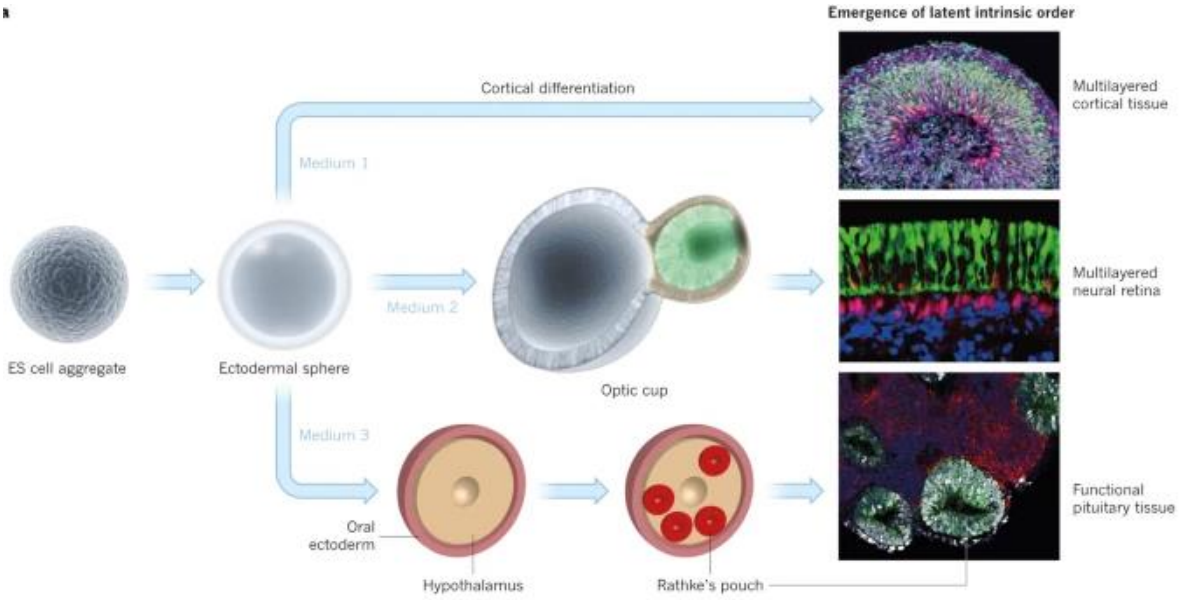

Figure 1: With ES culture, it is possible to make a stratified cortical tissue (top), an optic cup (middle), or a multilayered neural retina (bottom), depending on the medium. figure from (24).

An organoid is formed by a temporal distribution of different signals influencing the proliferation, differentiation, migration and selection of cells in culture (27,28). These signals are given to the organoid depending on the medium in which the iPSC or ESC cells are placed. In the first protocol developed by Lancaster et al. the cells used as starting material are ESCs.

The method of making brain organoids is called "unguided". It is characterized by a neuroectodermal induction without addition of extrinsic factors such as growth factors or small molecules in the culture medium. The cells are self-organizing and differentiate mainly spontaneously. However, it should be noted that the Matrigel *(see box IV "ECM")* used to coat the starting cells may contain small doses of extrinsic signaling factors (12,15). Neuroectodermal induction allows to obtain neural epithelia in a controlled way, by limiting the presence of layers other than the ectoderm *(see box V " The different germ layers")* (29,30).

With concerns to the environment of cerebroids formation, Matrigel is used after the initial cells have been induced towards a neural fate. Subsequently, the organoids are transferred to a bioreactors to optimize oxygen and nutrient acquisition in the brain organoid *(see box V" bioreactors"and Figure 2)* (12,17).

Box V- concept for the derivation of brain organoids

The different germ layers:

In the early stages, an embryo consists of three germ layers: the endoderm, the mesoderm and the ectoderm. The endoderm layer is determined to form the digestive system as well as the respiratory system. The mesoderm leads to the formation of mesenchyme, connective tissue and blood cells. Finally, the ectoderm gives rise to the nervous system and the neural crest, so it is this layer that is sought after for the formation of cerebroids (6).

Bioreactors:

Figure 2 illustration of a bioreactor adapted from (21).

Bioreactors can be defined as dynamic platforms for cell culture. They generate small shear forces that provide a circulation of the medium around the cells. This circulation allows a better exchange between the medium and the cells. Bioreactors create gradients (for example of nutrient or waste), which leads to a better orientation and stratification of organoids. There is a multitude of different bioreactors depending on the needs of the different cells (31).

Brief overview of organoid derivation

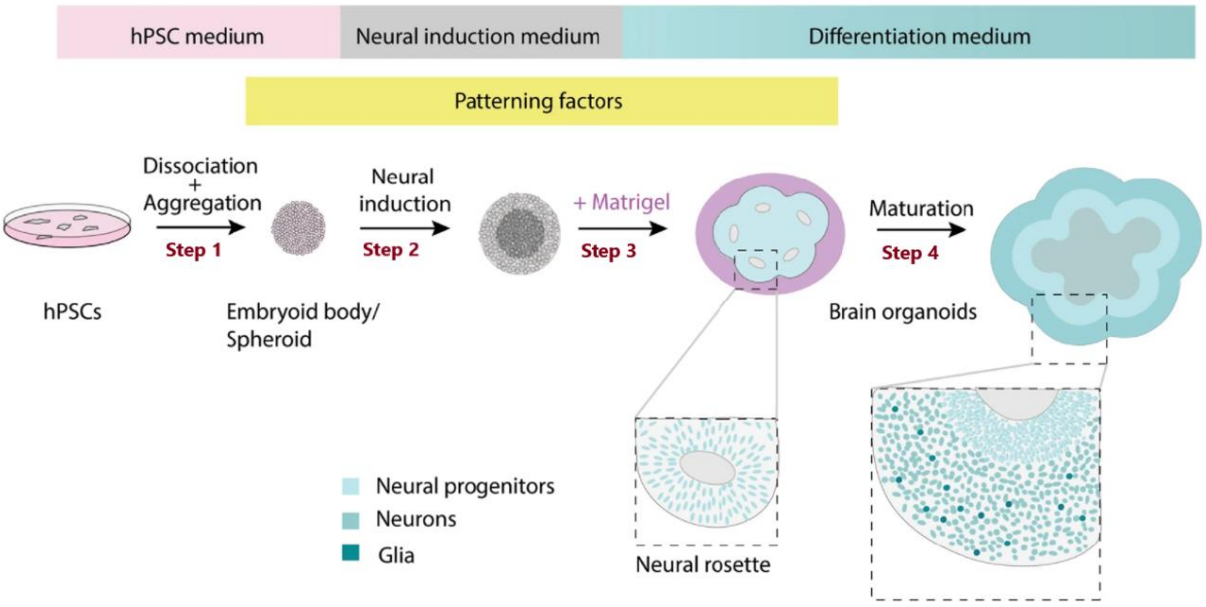

Figure 3: an overview of the main stages in the formation of a cerebroid. The steps (1 to 4) correspond to those mentioned in the text. Figure adapted from (31)

In order to provide an overview of the main steps in the fabrication of *cerebroids (see Figure 3)*, the protocol of Madeline A. Lancaster established in the laboratory of Juergen A. Knoblich's laboratory is presented in details. This will provide a clear understanding of further organoid models of GBM, in particular the GLICO and neoCOR organoid models which involve cerebroid fabrication.

The method presented below allows the formation of a neural identity after 8-20 days and after 20-30 days, specific brain regions appear, which is quite similar to the time it takes for fetal neural development (31).

Step 1

The first step is the formation of embryoid bodies (EBs) *(see box I "Embryoid Body (EB)")* from the ESCs/iPSCs, this takes 5 days (26). The starting cells (iPSC or ES) are grown in suspension in petri dishes containing a nutrient medium including, for instance, inhibitory Rho kinase to limit cell death (26,31). The cells are dissociated and then reaggregated, which promotes their spontaneous differentiation, resulting in EBs. In the EB, the different embryonic layers are present (ectoderm, mesoderm, endoderm) but they are not yet organized in well-defined cell layers (17).

Step 2

The second step consists in changing the solution that induces neural tissue (6-10 days) (17,31). To be more specific, the EBs formed in step one, are placed in a media inducing neuro-ectodermal fate, inhibiting the formation of the other germinal layers (32,33). The culture is also done in suspension (26).

Step 3

The third step consists of placing the neuroectoderm tissue in Matrigel droplets (11-15 days). This is the stage where the organoid begins its differentiation (31). The Matrigel forms a scaffold that stimulates the self-organization of neuroectodermal tissue, leading to multiple 3D epithelial structures called "neural rosettes" also known as "buds". The neural rosettes have a similar architecture than the neural tube in vivo, including a liquid-filled cavity which corresponds to the apical lumen, and an epithelial morphology (33). At this stage, stem cells and neural progenitor cells proliferate and differentiate to produce neurons and glial cells (31). The neural identity is established ***after 8-20 days***. The neuroepithelium surrounds filled cavities reminiscent of the ventricle. If fibroblasts are also detected at the stage or neurons may be, this suggests that neural induction has been incomplete. This incomplete induction may be caused by a lack of morphogen. (17,26).

Step 4

The last step is the maturation of the organoid (15-30 days). The "neural rosettes" are placed into a rotating bioreactor, in order to increase the absorption capacity of nutrients and oxygen. The organoid differentiates and grows further, eventually forming a cerebroid (31).

The formation of discrete brain regions is established between ***20-30 days***. At this stage, regions similar to the hindbrain, the hippocampus, the dorsal cortex, and the limit between the midbrain and the hindbrain start to form (34). Other structures do not systematically emerge, such as the retina which is observed in 11% of organoids, the ventral forebrain in 34% or the choroid plexus (but without detectable gyrus, appearing in 71% of cases (17).

After 2 months, the maximum size is obtained *(Figure 4, Figure 5)*. The organoid is about 4 mm in diameter (17). Its size does not increase more, certainly due to the lack of circulation system, the limited exchange of oxygen and nutrients through the organoid. A necrotic nucleus forms inside the organoid while the different brain regions develop outside.

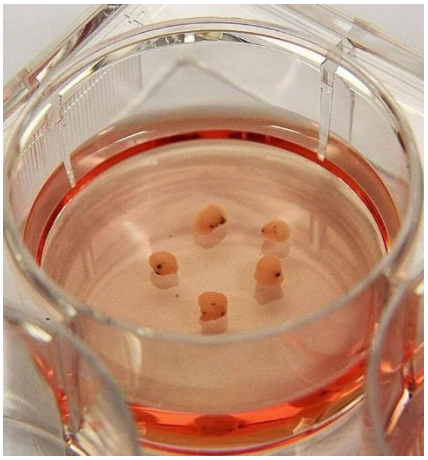

Figure 4: mini brain developed in the biotechnology laboratory in Standford. It is one of the few organoid photos showing what can be seen of an organoid formed without the use of a microscope, adapted from (35).

Reminiscent layers of the cortical and superficial layers appear around ***75 days*** (26). It seems that a cortical pre-plate is formed by neurons that have migrated out of these layers.

Neurons acquire different identities from the cortical plate ***after 5-6 months.*** However, layers 2 and 6 are not found compared to what happens in vivo. The organoids reduce, probably due to the loss of neural cells and the lack of projectors.

If the cerebroids have been placed into rotating bioreactors, they can live for up to ***15 months*** (17).

Several tests have confirmed that the cortical organization, the formation of functional cerebral cortical neurons and their organization in discrete brain regions can be recapitulated by this type of organoids.

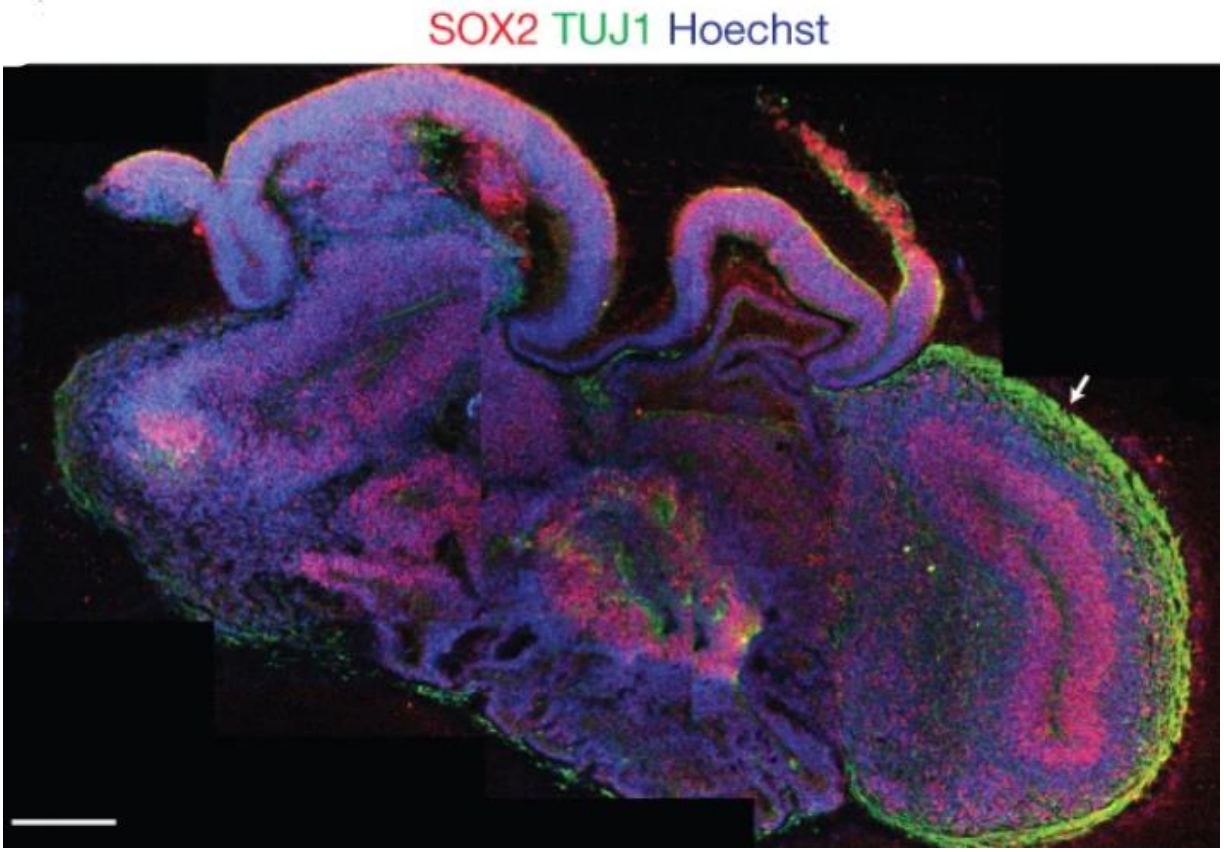

Figure 5: section of a cerebroid with immunohistochemistry, showing in red (SOX2) the neuronal projectors, in green (TUJ1) the neurons, adapted from (17).

glioblastoma multiforme (GBM)

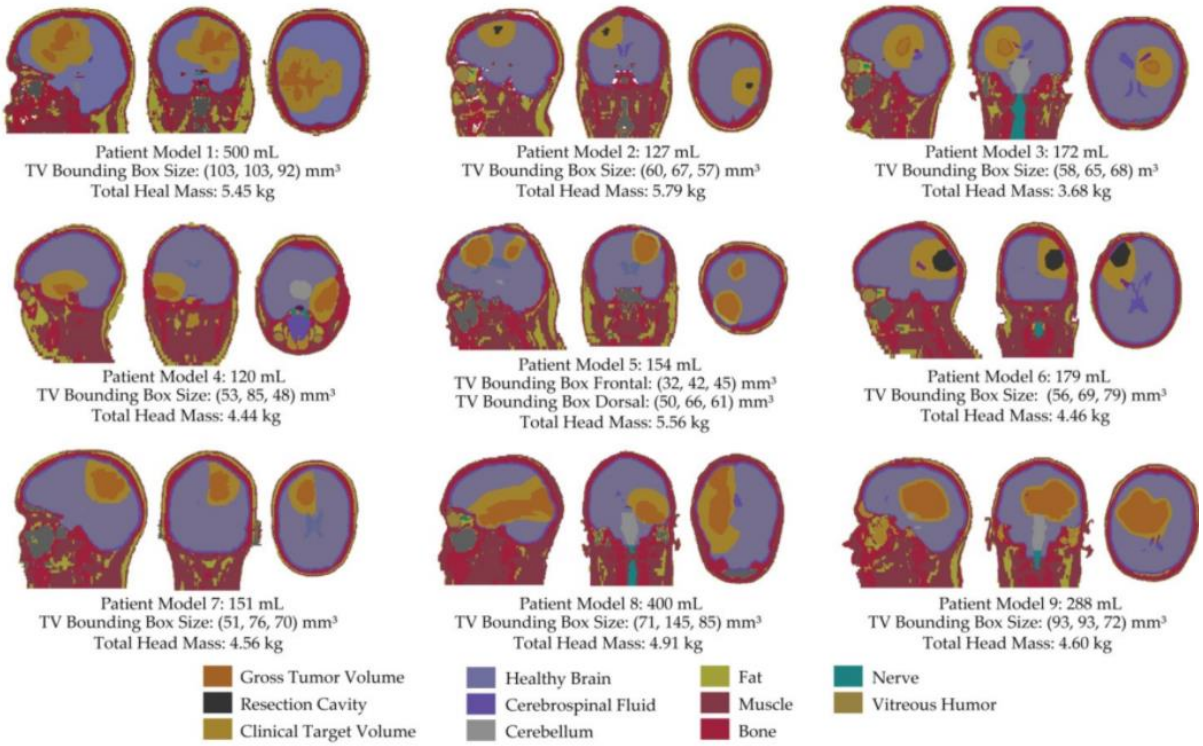

Figure 6 : showing the heterogeneity between patients of GBM multiforme adapted from (35). Here we see that depending on the patient the affected area, the size and the stage of the tumor varies. Patient-Specific Planning for Thermal Magnetic Resonance of GBM Multiforme

GBM is the most malignant glial cell cancer of the brain. It is very common in adults and has a bad prognosis (4,20,36). There are three subtypes of GBM defined by expression patterns based on transcriptional features: proneural, classical and mesenchymal (37). A major reason for its fatality is related to its ability to invade healthy brain tissue and its intra- and inter-tumor heterogeneity *(see Figure 6)* (22). The invasiveness of GBM makes complete surgical resection difficult. On the other hand, tumor heterogeneity is an important cause of its frequent recurrence and resistance to treatment including chemotherapy and radiotherapy. Genetic and phenotypic plasticity is partly responsible for this resistance. These residual cells escaping treatment are the cause of GBM recurrence (20).

The inter-tumor heterogeneity characteristic of GBM is related to the variation between different patients but also between tumor regions within the same patient. Intra-

tumor heterogeneity results from a combination of factors such as genetic abnormalities, epigenetic alterations, transcriptome, microenvironment, and developmental status. There are several levels of variation such as heterogeneity caused by different transcriptional subtypes in different cellular states. At the cellular level, there are different types of tumor cell behavior (37). The presence of different developmental states of GBM cells as well as the existence of different stem cells is another cause of cellular heterogeneity. The proliferation of GBM cells is hierarchical (18).

As far as tumor invasion is concerned, the factors determining it are currently unknown. GBM very frequently invades the brain parenchyma and the perivascular space. The invasion of healthy tissues is often accompanied by degradation of the extracellular matrix and a decrease in cell volume. This change in cell morphology is caused by a change in the ionic gradient allowing it to acquire greater motility (20).

The current treatments for patients are: immunotherapy, targeted therapies, post-operative chemotherapy and surgical resection followed by radiotherapy(4). These treatments are cumbersome without significantly improving the diagnosis, since the survival rate is generally no more than 2 years (20).

The lack of progress in improving the prognosis of the disease is due to a lack of a model to represent the genotype-phenotype association, to screen for drugs (18) and a lack of data on the mechanisms leading to the heterogeneity typical of GBM (20). For example, relapse can be blocked if the "top of the hierarchy" tumor stem cells are inhibited (18). The current challenge is to find a model of GBM that can more accurately represent its invasion and heterogeneity (4).

Different models have been available for some time: the animal model, xenotransplantation, 2D culture and spheroids. However, they do not represent the cell composition, the TME such as hypoxia, as well as the physiological behavior of the cells (4).

For example, the transgenic xenograft model (genetically modified mice) in an immunocompetent mouse brain allows to interrogate the oncogenic mutations leading to tumor initiation. However, this model does not represent the heterogeneity found in the original tumor. An alternative model to overcome this limitation is the patient-derived xenotransplantation (PDX). It is constructed using patient biopsies where cells are extracted and then xenografted into an immunodepressed mouse brain. This model has the merit of offering a microenvironment to the tumor cells including the presence of blood vessels which is similar to what happens in vivo (although not human). However, the manufacturing cost and the non-human brain microenvironment are problematic (20).

Although 2D surface adherent culture has proven to be a useful tool in understanding the role of cancer stem cells (CSCs), this tool also has limitations such as the lack of complex cell-cell and cell-environments interactions (18). Organoid models are now available, offering

better genomic stability than the 2D model (4), as well as a more reliable representation of the complexity of GBM (20). Organoid models of GBM represent a suitable tool to study the invasion of GBM and the consequences on the TME of the affected brain (22). The interactions between TME and the cells are more accurately recapitulated, which is particularly important for studies of tumor invasion and resistance (20). Moreover, patient-specific modeling is made possible by these models and opens the field of specialized medicine. For example, the efficiency of different treatments such as immunotherapy or drug response can be evaluated for each individual tumor. Patient-derived organoids also participate in improving the understanding of tumor biology, including relapse and drug library screening. Predicting tumor progression and identifying new therapeutic targets are among the most promising applications of GBM organoids (18).

The different organoid models of GBM

Fabrication of different types of GBM organoids and their own characteristics

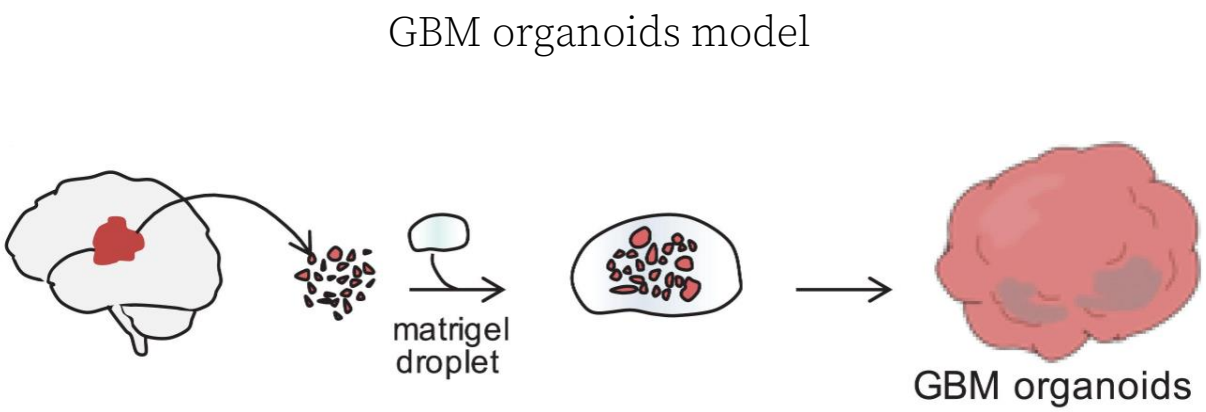

Figure 7: GBM organoid manufacturing procedure. The two key steps are the collection of GBM cells from biopsies and the embedding of these cells in Matrigel. Figure from (18).

In 2016, the first GBM organoid was developed by Hubert et al. (18). They are sometimes called "tumoroids" because of they are mainly made of tumoral cells (20). The principle of construction of this type of organoid is to take GBM stem cells (GSCs) isolated from tumors in order to obtain a self-organized 3D structure mimicking the tumor development (18).

The manufacturing of GBM organoids *(Figure 7)* is an adaptation of the protocol established to form brain organoids detailed previously (38). GSCs are sampled from patient biopsies (20) and individualized by enzymatic digestion (19). Then, these cells are coated in Matrigel and put in an adherent culture in serum. The neurobasal medium in which the cells are placed is often supplemented by addition of B27 medium as well as growth factors such as EGF (epidermal growth factor) and FGF2 (fibroblast growth factor) (38). They reach 3 to 4 mm in two months and are viable for more than a year.

GBM organoids recapitulate major aspects of tumor architecture, hypoxia, stem cell density gradients and heterogeneity (38).

Altogether, these observations show that GBM organoids is the best model system to address questions about the role of tumor stem cells (SOX2+) according to their location. It is an organoid model that represents certain tumor characteristics such as the hypoxia gradient.

GBO (Glioblastoma organoids) model

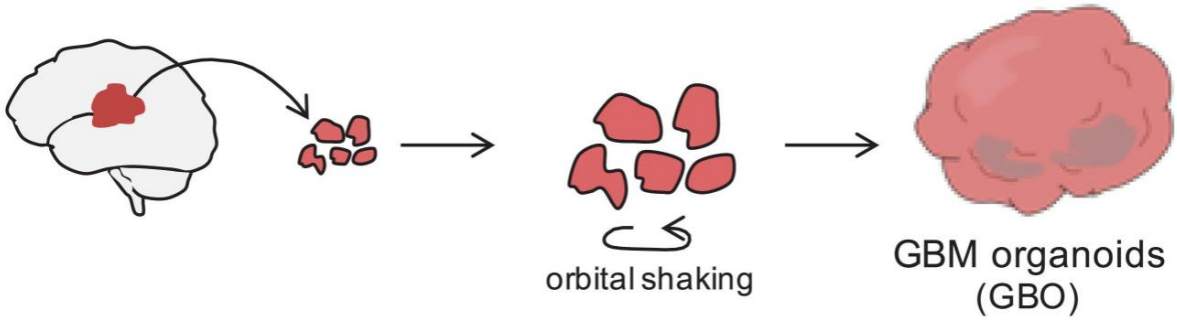

Figure 8: Summary of the manufacture of the GBO organoid. What differs from the GBM model is the larger cutting of the tumor biopsy and culturing in an orbital shaker. Figure from (18).

Similar protocols have emerged after the GBM organoid protocol *(see Figure 8)* (18). A model of major importance for personalized medicine was introduced in 2020 Jacob et al., the GBO, also for "GBM organoid" but refers to a specific protocol. Unlike the GBM organoid, the GBO model does not extract the cells from patient biopsies (22). Instead, the tumor sample is fragmented more coarsely, about 1mm. Once cut, the tissue is placed in a medium containing a neurobasal mixture (19). Contrary to GBM organoids, the culture medium of GBO is defined and does not contain serum, EGF/bFGF or Matrigel (22), which makes it less restrictive in the fate of the cells in culture which gives the possibility to obtain cells that are part of the TME in the final organoid (18). The whole is placed in an orbital shaker which optimizes the distribution of nutrients and oxygen (22). Afterwards, the organoids formed within 1 to 2 weeks are re-cut to form pieces of 0.5 mm in diameter. This step prevents the internal necrosis of the organoid (18,22).

With this protocol it is possible to find characteristics of the TME such as hypoxia gradients, micro vascularization and immune cells (19,22).

In application, GBOs are used preferentially in biobank formation, which will allow genotype-drug studies opening the way to personalized medicine.

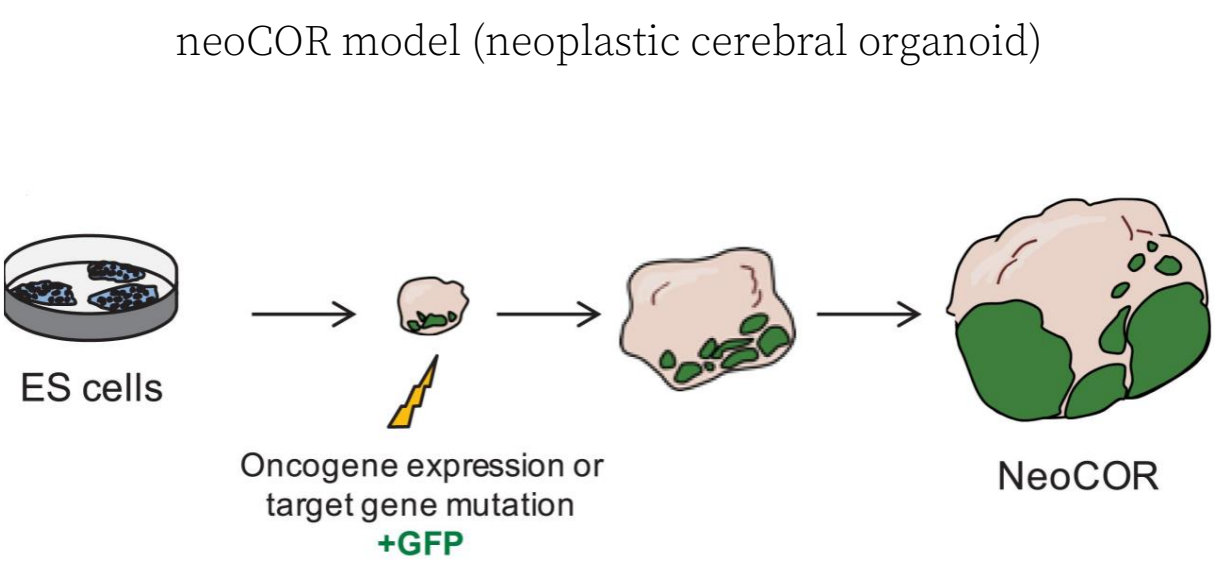

Figure 9: Summary of the fabrication of the NeoCOR model. Here the fabrication of the brain organoid is an integral part of the protocol. The organoid can have as initial material ESCs or iPSCs (1) even if it is not represented on the picture. In parallel, cells of the organoid in formation are genetically modified to make them GBM. They are tagged with GFP which allows to follow them. Figure from (18).

In cancer, specific genetic alterations such as overexpression of oncogenes and deletion of tumor suppressor genes can cause the switch from healthy to tumor cells (18). These key alterations were screened and reproduced by genetic engineering on differentiating organoid brain cells. It is therefore a manufacturing protocol based on the brain organoid derivation (19). This method named NeoCOR (neoplastic cerebral organoid) was developed simultaneously by Bian et al. and Ogawa et al. in 2018 (18). Unlike GBM organoids and GBOs, the tumor in NeoCOR or GLICO (discussed next) represents only a fraction of the organoid *(see Figure 9).* In addition, the initial material is a healthy brain organoid in formation, not a biopsy as in the GBMs or GBOs models (20).

In this NeoCOR model, no GBM cells are introduced. Instead, genetic modifications induced during NeoCOR fabrication generate tumorigenesis in the healthy organoids. These modifications take place before the complete maturation of the brain organoid (19). Tumorigenesis is achieved using transposons and/or CRISPR-Cas9. This will allow to overexpressing oncogenes (i.e., MYC) and/or destabilizing tumor suppressor genes (i.e., NF1 (37)) in neural stem cells or progenitor cells, which are then re-introduced in growing organoids by nucleofection (18). By expressing a fluorescent marker in cells that have been genetically modified, it is possible to analyze the tumorigenic genes induced within the

organoid. It has been reported that tumor growth was very similar to the patient's tumor especially in terms of invasion (20).

Overall, the NeoCOR organoid model is formed by mutations induced in a developing cerebroid and it is useful to analyze allows the biology of the tumour, for example by identifying mutations that induce tumorigenesis, and to study the early stages of GBM initiation.

GLICO model (GLIoma cerebral organoids)

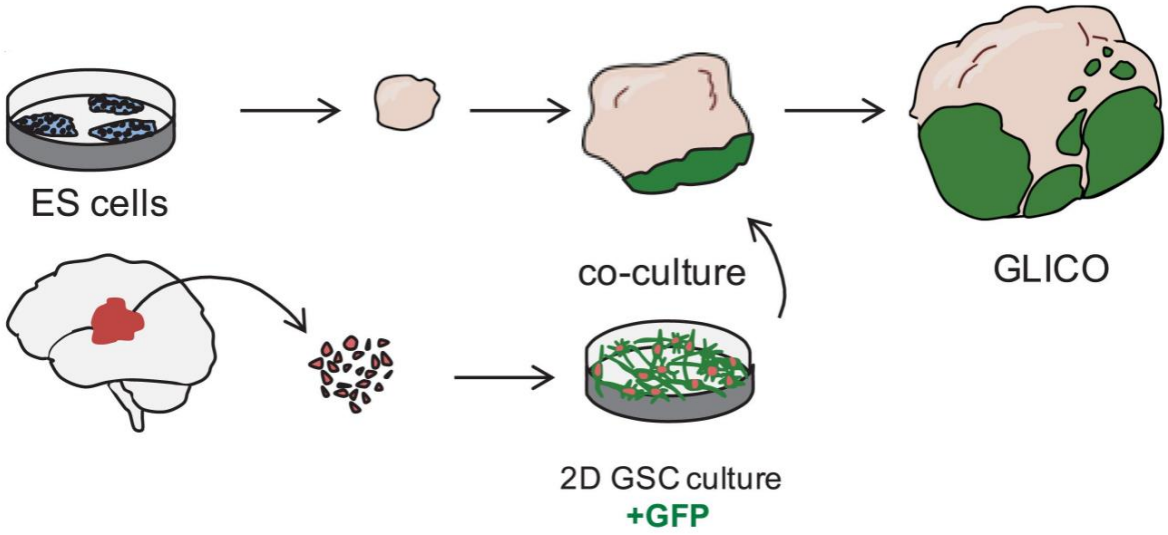

Figure 10 : Summary of the manufacturing of the GLICO model (18). Similar to the NeoCOR model, the fabrication of the brain organoid is an integral part of the protocol. What differs from NeoCOR is that in parallel to the formation of the organoid, GBM cells are harvested from the patient, cultured and then fused to the organoid. This method is called co-culture. The GBM cells are also tagged with GFP which allows them to be followed. Figure from (18).

The GLICO (GLIoma cerebral organoids) model system was made by da Silva et al. and Linkous et al. in 2019 (18). The specificity of the GLICO model is that it is based on the co-culture between different GSC lines tagged with GFP and a mature cerebral organoid *(Figure 10)* (18). It has been shown that after the formation of spheroid (or GBM organoid) from patient-derived GBM cells, these structures could be fused to a cerebral organoid derived from ESCs (2) or iPSCs (1). The tumor cells attach to the cerebral organoid and then invade it. The cancer cells infiltrate the brain organoid showing different invasion patterns (18). The injection step of patient derived GSCs is critical (19) because inclusion often requires removal of the ECM to avoid cancer cells growing on the surface of the matrix rather than inside the mini brain. It was observed that the cell behavior accurately mimics the behavior of the tumor in vivo (18). The genetic signatures of the tumor cells appear to be maintained over time, giving this organoid hybrid model an interesting potential for therapeutic and preclinical applications.

By sharing characteristics with the NeoCOR model, GLICO model overcomes some NeoCORs limitations. Both organoid model are based on a healthy brain organoid (20). It therefore recapitulates the tumor-brain interactions as the cancer cells reside in a healthy environmental medium, the brain organoid itself (19). In distinction to the GBO and GBM

organoids, the two organoid models NeoCOR and GLICO make invasion studies of GSCs possible. As it is GBM cells that are introduced and not just a few mutations as in the case of the NeoCOR, genomic complexity is found in the GLICO organoid (18), which makes of it the best candidate to investigate GBM TME interactions or GBM invasiveness (20).

In summary, GLICOs are best suited to study invasive behavior of GBM cells and for analyzing TME with the interactions between healthy brain cells and tumor cells. More generally, this model is an interesting tool to study tumor biology and to screen for drugs. It is a hybrid model composed of a co-culture of cerebroid and spheroid GSCs.

Bioprinted GBM model

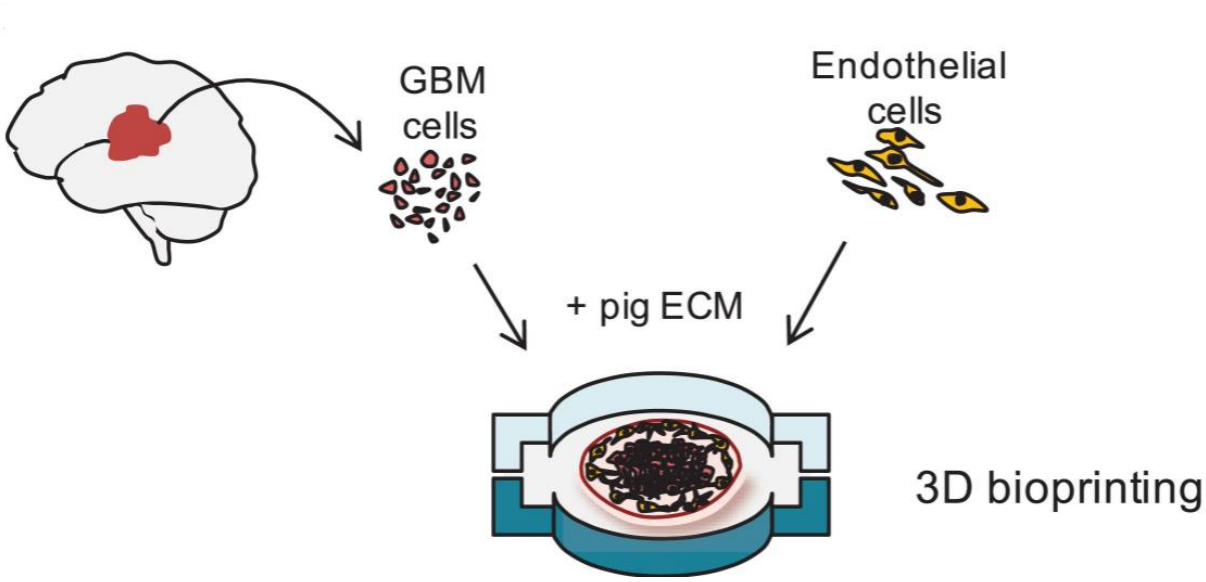

Figure 11 : Summary of the fabrication of GBM models by 3D bioprinting. (18) In this model, the required material are GBM cells that can be harvested from patients, as well as a cell matrix coming here from pigs. The bioprinting system will superimpose layers of GBM cells coated with the matrix on a support which can be a chip. Figure from (18).

Box VI- Bioprinting:

3D bioprinting is an additive manufacturing technique in the field of bioengineering (39,40). The superposition of cell layers is computer assisted resulting in the formation of material mimicking a living tissue or organ part such as blood vessels, bones and heart (41). An emerging technology of bioprinting is the ink-jet bioprinting. The cells are coated with biocompatible material (bio-ink) that can contain signaling molecules to mimic the extracellular matrix. These coated cells are ejected in a spatial distribution that most closely matches the structure and composition of the organ in question (39,40). This method avoids damaging the cells by preventing the generation of heat that is higher than physiologically sustainable. Advances in bioprinting to mimic the physiological environment have led to the development of organ-on-chip technology. Bioprinting is used to form the microfluidic channels of the organs-on-chips (42).

This last model of GBM on a bioprinted chip was made by Yi et al. in 2019 (18) *(see box VI "Bioprinting"and Figure 11)*. The bioink is composed of a decellularized pore brain extracellular matrix containing ECM proteins. On the chip, different types of GBM cells dissociated from patient tumors are coated with bioink as they are printed for example on endothelial cell layers. This bioink serves to reveal the invasion of GBM cells into endothelial cells. Bioprinted GBM organoids recapitulate the presence of hypoxic gradients and the appearance of different tumor compartments (18,20).

Other components can be additionally incorporated such as the macrophage printing performed by Tang et al. This offers the possibility to model the interactions between the immune system and the tumor. Bioprinted GBM organoid contains several elements of the TME allowing cell-cell and cell-matrix interactions. This model requires less time to be manufactured (one to two weeks), which provide fast results to tests such as drug screening. However, the spatial organization is not comparable to that found in brain organoids. Normal brain tissues are absent, the substrates are too homogeneous and the inaccuracy in the printing of the different layers are challenges of this method (18,20).

In summary, bioprinted GBM organoids are not the best disease model for drug testing. Its short manufacturing time is a major advantage.

Investigating tumor biology with different GBM organoid models.

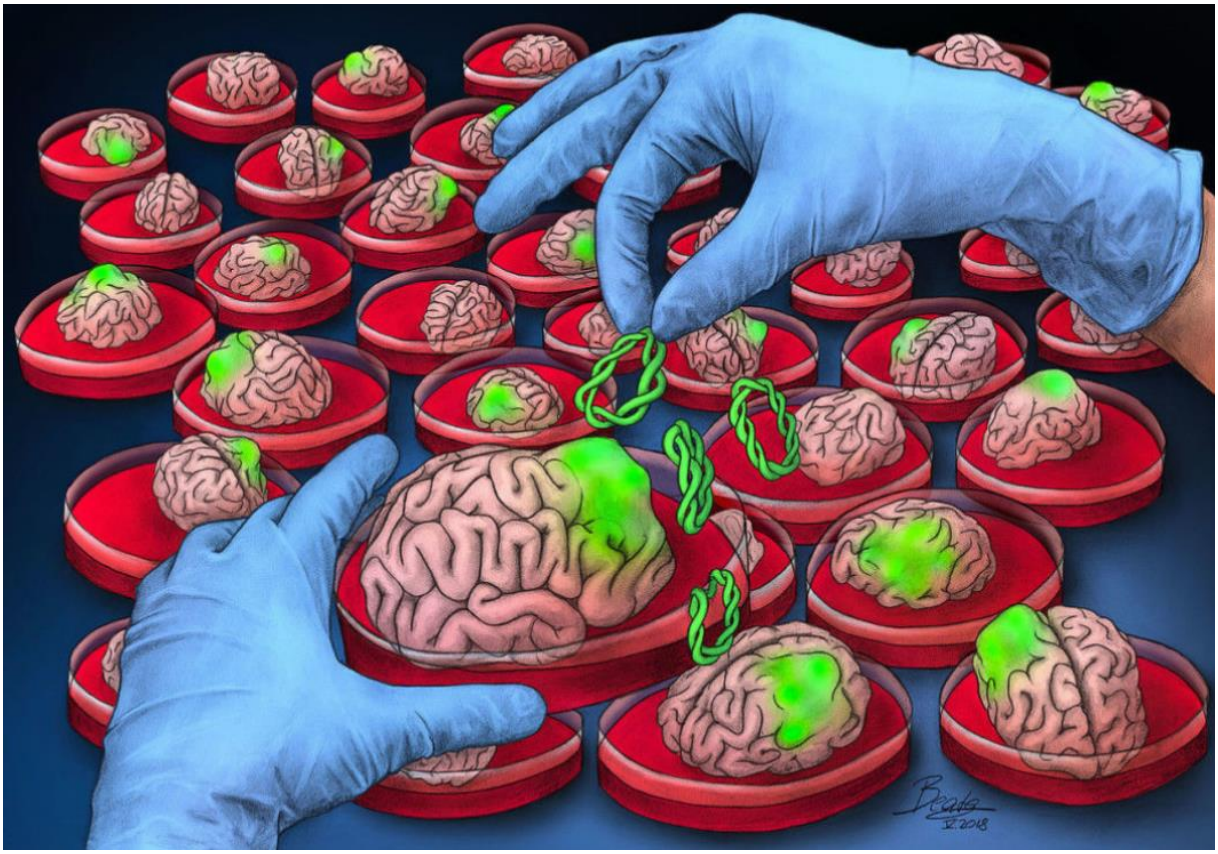

Figure 12: This artistic image illustrates the manufacture of one type of GBM organoid: the neoCOR. Organoid models are tools in cancer research. However, this image is intended to be disturbing, highlighting the ethical dilemmas associated with brain organoids, particularly the concern about the consciousness of these models. Figure from (39).{Citation}

GBM and GBO organoids models enable to study the localization of stem cells within the tumor *(Figure 13)*. Indeed, it has been reported that GSCs (SOX2+) are localized mainly at the periphery of the organoid. This region is characterized by an abundant concentration of nutrients, oxygen and other factors. GSCs in the organoid periphery have a high division rate and a short life span. This region of the organoid is therefore similar to the perivascular niche in which the GSCs are located. Similarly, the distribution of these stem cells would be governed by a threshold effect explaining their weak presence in the center of the organoid analogous to the hypoxic niche in vivo. These GSCs of the hypoxic niche, unlike those located at the periphery, are quiescent (18,22,38).

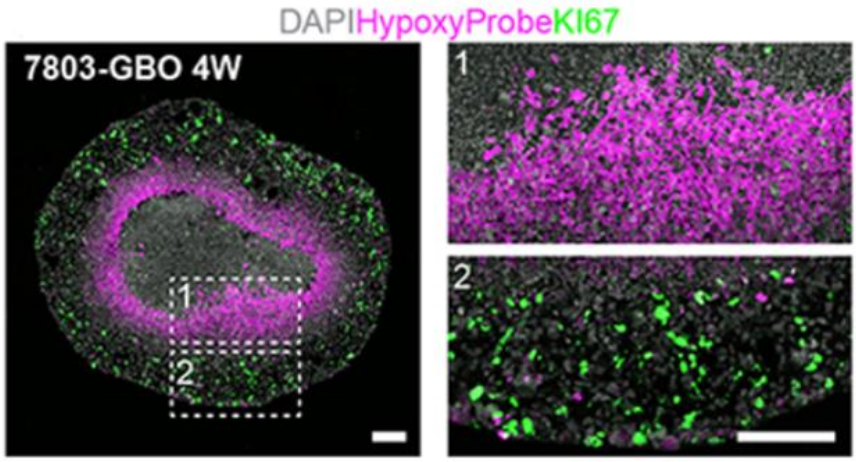

Figure 13: Highlighting the hypoxia gradient (tagged in violet) in a GBO organoid with immunostaining. Proliferating cells (tagged in green) are found in the periphery (box "2") but are absent from the hypoxic core (box "1"). Figure from (22).

In addition, for the organoids GBO and GBM it is possible to study some aspects of cell infiltration by performing a xenograft in mouse brains *(see Figure 14)* (22). Xenografting of these organoids into a mouse brain induced fatal tumor development (20,38).

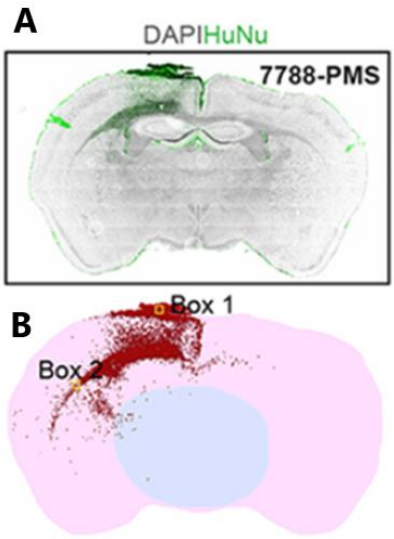

Figure 14: Xenograft of a GBO organoid sample into an immunodeficient mouse brain at two months post-transplant. A) slice shows the invasion of GBO-derived cells by immunohistochemical labelling (targeting human Hunu antigens). B) shows the quantification of the invasion and migration of cancer cells away from the site of origin of the graft. Figure from (22).

The NeoCOR organoid model allows to analyze and better understand the tumor biology also. This organoid model enables testing for genotypes conferring invasive phenotypes (4). It can also be used to test which combinations of mutations introduced in the cells allow the invasion of healthy cells of the organoid and more generally tumor development *(Figure 15).* For example, it was discovered that tumors linked to the overexpression of the MYC oncogene were at the origin of three distinct genotypes: CDKN2A -/- / CDKN2B -/- / EGFR OE / EGFRvIII OE, NF1 -/- / PTEN -/- / TP53-/- , or EGFRvIII OE / CDKN2A -/- / PTEN -/-. Moreover, this model is mainly composed of healthy brain tissue (brain organoid) which makes it possible to analyze the interactions between the tumor and the tissue. It is therefore a unique tool to study the early stages of tumorigenesis (19,20).

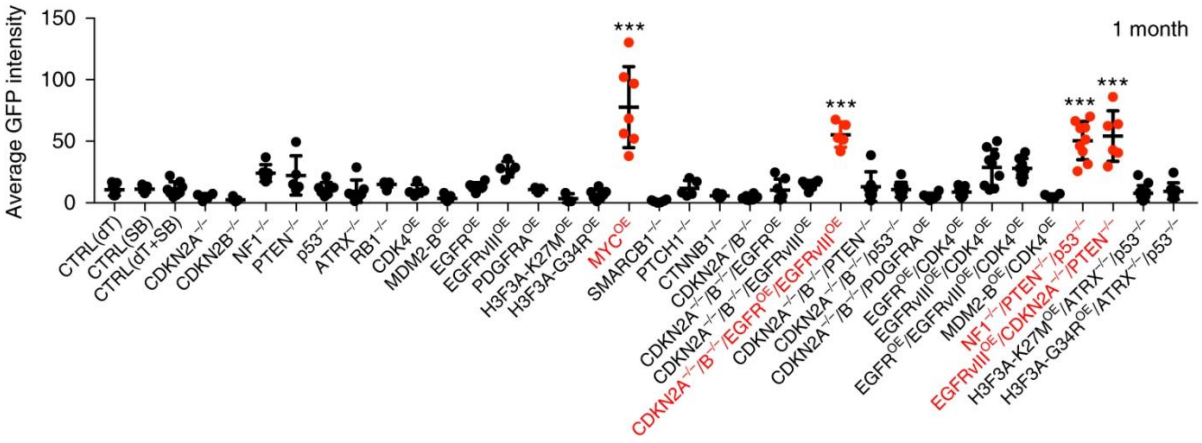

Figure 15: Quantification of GFP fluorescence intensity according to the mutation combination performed to form NeoCOR organoid tumorigenesis. In graph ,4 mutation combinations showed significant growth after one month (MYCOE , CDKN2A-/-/CDKN2B-/-/EGFROE/EGFRvIIIOE, NF1-/-/PTEN-/-/p53-/- , and EGFRvIII/PTEN-/-/CDKN2A-/-). Figure from (40)

NeoCORs have revealed that depending on the modified genes, the tumors have different cellular identities and therefore, there are reproducible subtypes of GBMs in the organoid (18).

A group of researchers Pine et al. compared the different models according to their transcriptomic profiles. The organoid model that most closely resembled the original tumor was the GLICO model. They also determined that GBM cells in this model expressed key genes attributed to GBM as markers of invasiveness. In addition, a new tumor cell subtype, the external radial glia, was identified using GLICO (20).

TME can be captured by the GLICO model specifically as it includes the interaction between healthy and cancerous cells (4). If the GBM cells used in the GLICO were harvested from a patient, the GLICO model specifically represents the patient's TME. Single-cell sequencing of these cells revealed the expression of a stem cell marker SOX4 and a notch development marker as well as an invasiveness marker BCAN within the TME. The addition of exogenous immune components into the organoid is enabled by co-culturing. Peripheral blood lymphocytes, or TILs can be added for example (41).

Some examples of therapies based on GBM organoids

Immunotherapy

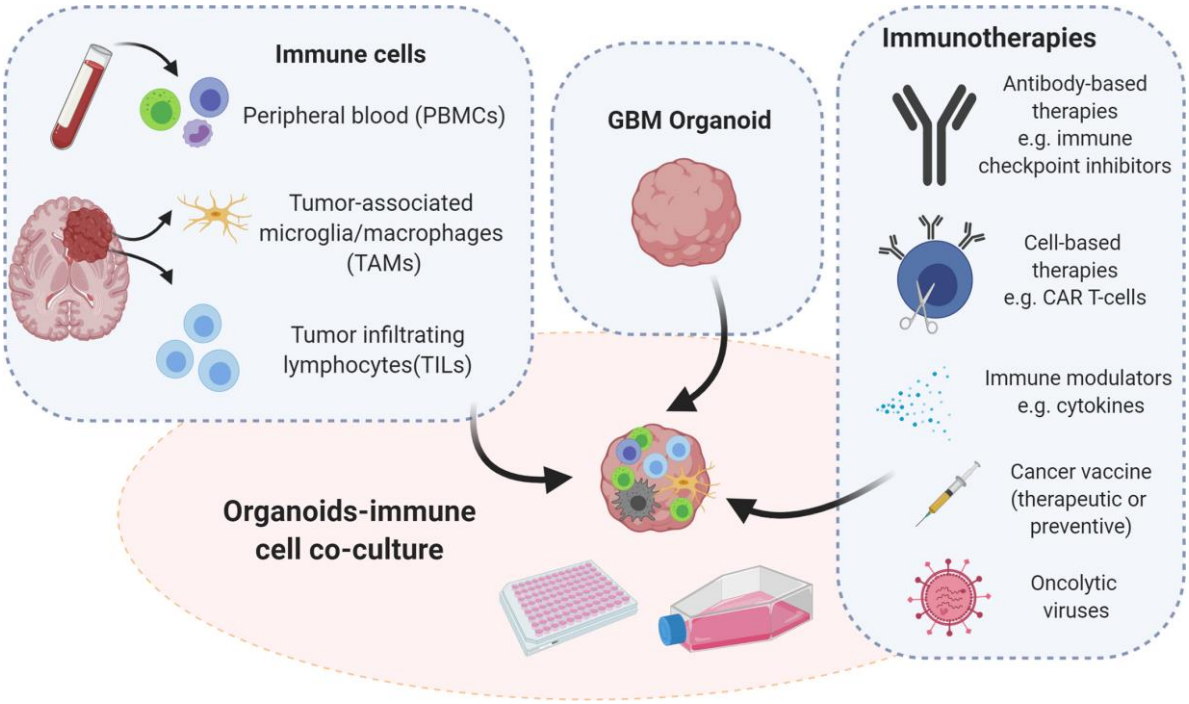

Figure 16: Overview of the different applications of GBM organoids focusing on immunotherapy. Adapted from (42).

The TME of GBM is characterized by multiple tumor escape mechanisms (19). GBM has a highly immunosuppressive microenvironment composed of myeloid-derived suppressor cells as well as regulatory T cells. Different protocols exist to obtain an immunocompetent organoid, involving the addition of immune cells *(see Figure 16)*. A regularly used method is the co-culture of the GBM organoid with isolated immune cells like lymphocytes. These immune cells are collected from peripheral blood, either from the same GSCs donor (autologous[1]), from a healthy donor (allogeneic[2]), or from the tumor tissue itself (autologous). A co-culture with immune cells autologous to the tumor tissue enables the development of a personalized organoid for the patient. In some cases, these immunocompetent organoids can lead to the activation of T cells, allowing the elimination of

[1] when the (cells) donor is the same person as the GBM patient to be treated
[2] when the (cells) donor is not the same person as the GBM patient to be treated

the tumoroid, a discovery that offers an interesting therapeutic potential. First, it provides an opportunity to analyze the interactions between T cells and cancer cells in a patient (43). These analyses include the study of the efficiency of tumor destruction. In addition, it may be possible to use these activated effector T cells in the organoid by re-injecting them into the patient to help eliminate the tumor. This technique would be an alternative to cell therapy using TILs.

Cellular therapies focused on CAR-T

In GBM, CAR-T *(see box VII "CAR-T therapy")* often target EGFRVIII, IL-13 α2 and HER antigens. Organoids are particularly suitable for testing the killing efficiency of different CAR-T cells. For example, the GLICO organoid model makes it able to tag CAR-T cells and visualize the interactions between CAR-T cells and the target (4).

The GBO organoid model can be cocultured with CAR-T cells. This application makes it possible to study treatment responses according to the antigen targeted by CAR-T cells *(see Figure 18)*. Statistics on the efficiency of a CAR-T treatment can be done with GBO according to the patient's genotype. For example, patients with mutation in EFGRvIII showed an effective response to CAR-T targeting EGFRvIII (4). In parallel, GBO organoids with mutant EGFRvIII expression cocultured with CAR-Ts targeting EGFRvIII showed the destruction of tumor cells but also of healthy cells expressing this receptor. The destruction of the cells can be seen by the presence of cleaved caspase 3 and T lymphocytes close to this cellular debris (19,22,44).

It is also possible to use this immunology technology with NK cells (CAR-NK) but it has not yet been tested for GBM organoids (19).

Box VII - CAR-T (Chimeric antigen receptor-T cell therapy) :

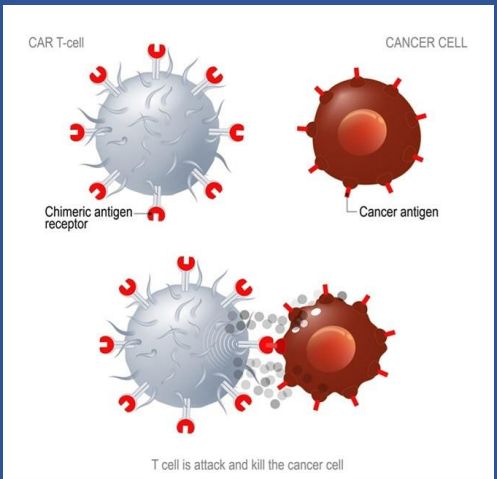

Figure 17: Illustration CAR-T cell that destroys a cancer cell adapted from (44).

Cellular therapies consist of injecting specific T lymphocytes into the tumor site to activate the immune response *(see Figure 17)* (44). These lymphocytes are in a first step generated outside the tumor in order to make them specific to the tumor either by the expression of a preselected TCR or by the expression of a CAR. CAR-T cells are lymphocyte T cells that have been engineered to express an antibody (CAR) targeting a tumor cell-specific surface protein. The binding between CAR and tumor antigens leads to the destruction of the tumor cells (19).

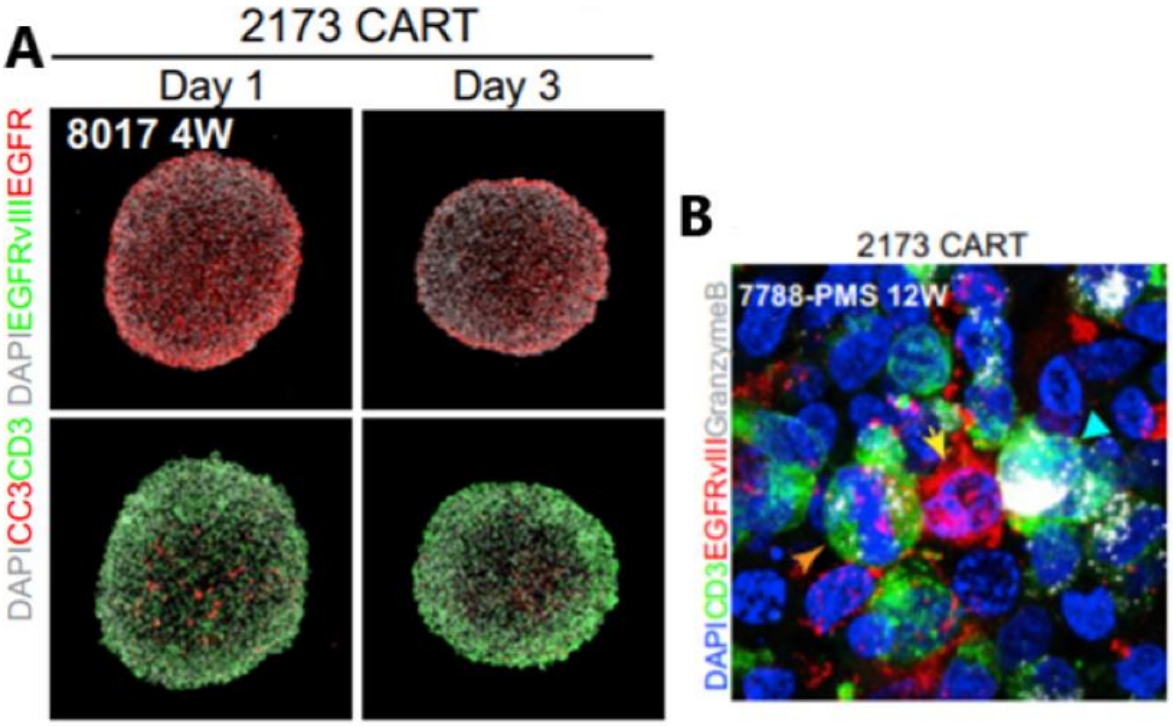

Figure 18:

(A) Confocal image of a GBO co-cultured with CAR-T 2173 cells showing EGFR, EGFRvIII, cleaved caspase (CC3) and CD3 immunostaining.

(B) Demonstration of activated and proliferating T cells (red arrow for granulating T cells and blue arrows for mitotic T cells) close to apoptotic cells (yellow arrows) in GBOs cocultured with CAR-T2173. Figure from (22)

Checkpoint molecule inhibitors

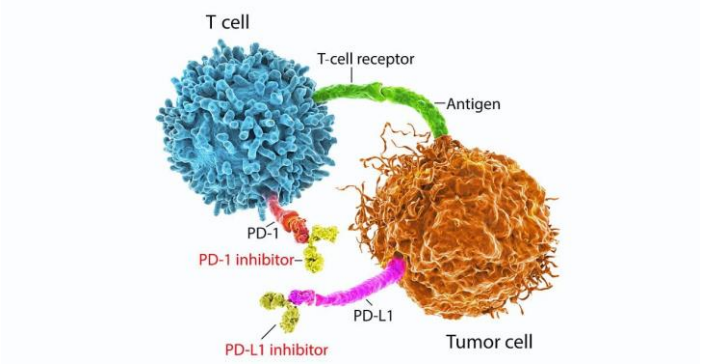

Figure 19 : Illustration of a molecular checkpoint inhibitor (anti PD-L1 and anti PD-1) preventing T cell inactivation normally mediated by binding to the cancer cell (45).

Tumor-infiltrating lymphocytes (TILs) are used to test the efficacy of checkpoint inhibitor molecules *(see box IIX " Checkpoint molecule inhibitors" and Figure 19)*. Unlike peripheral blood lymphocyte co-culture, in this case the TILs are part of the TME (19). Therefore, they can be collected simultaneously during the acquisition of GBM tissue, which facilitates the fabrication of the organoid.

box IIX - Checkpoint molecule inhibitors:

Checkpoint molecule inhibitors target the TME to make it accessible to T cell destruction. The purpose of this approach is to maintain an active anti-tumor T cell response (45).

In the near future, the air-liquid interface (ALI) culture system could be used for GBM organoids (4). This system has been shown to work well on several cancer organoids. It allows to study the interactions between TME including immune cells and epithelia. The ALI system applied to intestinal organoids has for example demonstrated the appearance of oxygen gradient to increase cell viability and differentiation (46). The ALI PDO (generated Patient-Derived tumor Organoids) system is the product of a co-culture (47). This co-culture is composed of patient- or mouse-derived tumor epithelium organoids, to which is added lymphocyte T cells which will fuse with the tumor. When the anti PD-1/PD-L+ checkpoint inhibitor is added, the tumor-infiltrating lymphocytes (TILs) had the expected effect: the T cells remained active longer. In another similar experiment, they obtained the same results, however they prolonged the survival of TILs by adding IL2 or antiCD3/CD28 (19). ALI PDO can be used as a predictive tool to analyze molecular checkpoint inhibitors (47). The use of the TILs for modeling is however challenging, because

few TILs are obtained from tumor tissue samples. In patient GBM, the predominant lymphocytes are in fact T regulators which participate in the immunosuppression of the TME. Moreover, within an organoid the TME is lost with time. In addition, the isolation of TIL cells by enzymatic digestion can interfere with the formation of the organoid (19).

Radiotherapy and chemotherapy

Organoids are also a suitable model to analyze the sensitivity of tumors to chemotherapy and radiotherapy *(see Figure 20)*. Tumor cells from NeoCOR and GLICO organoid models can be tagged, for example with GFP or luciferase. This allows tracking and quantifying the cells. This quantification can therefore be used as a viability test. For example, in one experiment, patient GBM cells were labeled with luciferase. They were cocultured with a brain organoid to form GLICO models. These were tested for 2 chemotherapeutic agents: TMZ (temozolomide) and BCNU (bis-chloroethylnitrosourea). The results showed that depending on the genotype of the cocultured cells, only the TMZ treatment was effective or only BCNU, which was not recapitulated in 2D culture since with the same samples the culture showed an equal and high sensitivity to both chemotherapeutic agents (2,20).

NeoCOR and GLICO organoid model recapitulates both the invasion of cancer cells as well as the formation of microtubules *(see Figure 21)*. Tumor microtubes constitute a support for proliferation, invasion, communication of GBM cells and make them resistant to radiation (4). Calculating the level of cleaved caspase-3 found in the organoid is one method to quantify cell death due to e.g. ionizing radiation (2). Using the GLICO hybrid organoid or GBM organoid, they observed that sensitivity to chemotherapy or radiotherapy mainly affects non-stem tumors cells while GSCs are resistant (18,38). Indeed, isogenic GSCs fused in the GLICO organoid show a phenotype of resistance to chemical agents and genotoxic stress. In contrast to CSCs, non-stem tumor cells are radiosensitive. A recent discovery made using organoids showed that the introduction of antisense oligonucleotides targeting RNAs from GBM cells decreased tumor growth and made these cells radiosensitive (4).

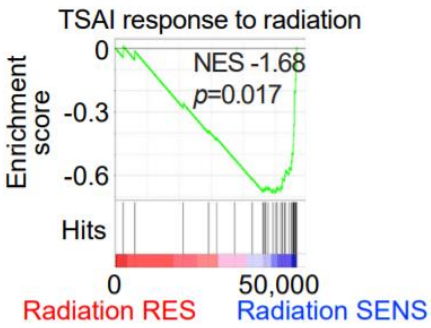

Figure 20: Graph showing a reduction of proliferative cells (KI67+) when GBOs are treated with radiation and temozolomide treatment. Figure from (22).

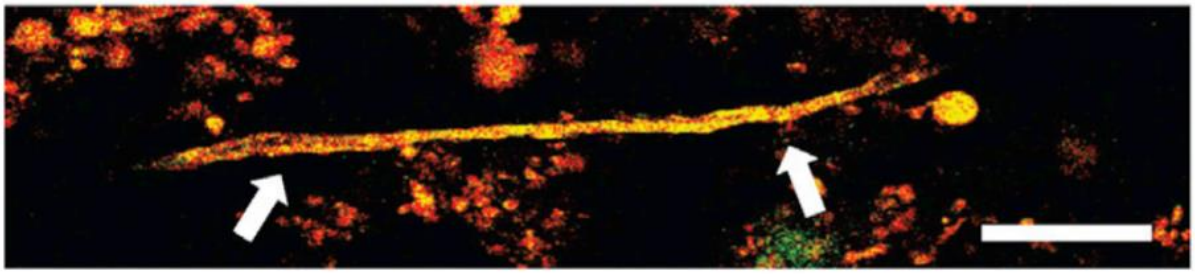

Figure 21: Two-photon microscopy of a fluorescently labelled tumor microtube forming in a GLICO organoid. (scale bar, 80 mm). Figure from (2).

Biobanks

Biobanks are formed by systematic collections of human body substance samples (48). Biobanks for GBM organoids focus on the storage of a collection of GBOs derived from patient tissues *(see Figure 22)*. One use of this biobank is to develop a range of different GBOs[3] to cover the spectrum of genomic alterations associated with GBM (18). The goal is to obtain standardized organoid hosts and make them a therapeutic prediction tool in personalized medicine. This application requires to precisely characterize each GBO organoid and test them for drug responses or other therapies. Thus, depending on the genomic profile of a patient, it could be assigned to a specific organoid already established in the biobank to find out which therapy or drug is more likely to be effective for the patient. (4,20). In other words, from this model, RNA and exome profiling can be performed to establish genotype-drug associations. For example, GBO organoids can be used for CAR-T cell immunotherapy analysis as we have seen before (18,22). Another example of drug test, sequencing of donor GSCs detects the deletion of a copy of a PTEN gene (involved in GBM), GBOs are used to test drugs that inhibit the signaling pathway that is activated by this deletion (18).

The possibility to cryopreserve GBOs, combined with the maintenance of key genetic aberrations of GBM, makes this model system ideal for the creation of organoid biobanks recapitulating the heterogeneity of patient tumors. Indeed, as GBO manufacture does not involve dissociation of GBM cells or the addition of serum or ECM, it enables to obtain organoids that recapitulate well the molecular properties, transcriptomic signatures, cell architecture and cellular heterogeneity found in the donor tumor. Moreover, GBOs can be cryopreserved in liquid nitrogen tanks on a long-term basis while maintaining their properties after thawing *(Figure 23)* (22,49).

Biobanks have the potential to help predicting specific drug efficacy and response to immunotherapy. These pre-established analyses will increase the speed of therapeutic processes. However, these analyses were performed in the early stages of the organoid, the maintenance is variable over the long term, which is a notable limitation such as the cost (18).

[3] A biobank of 70 GBM GBO organoids made from tumor material of 53 patients has already been established (18).

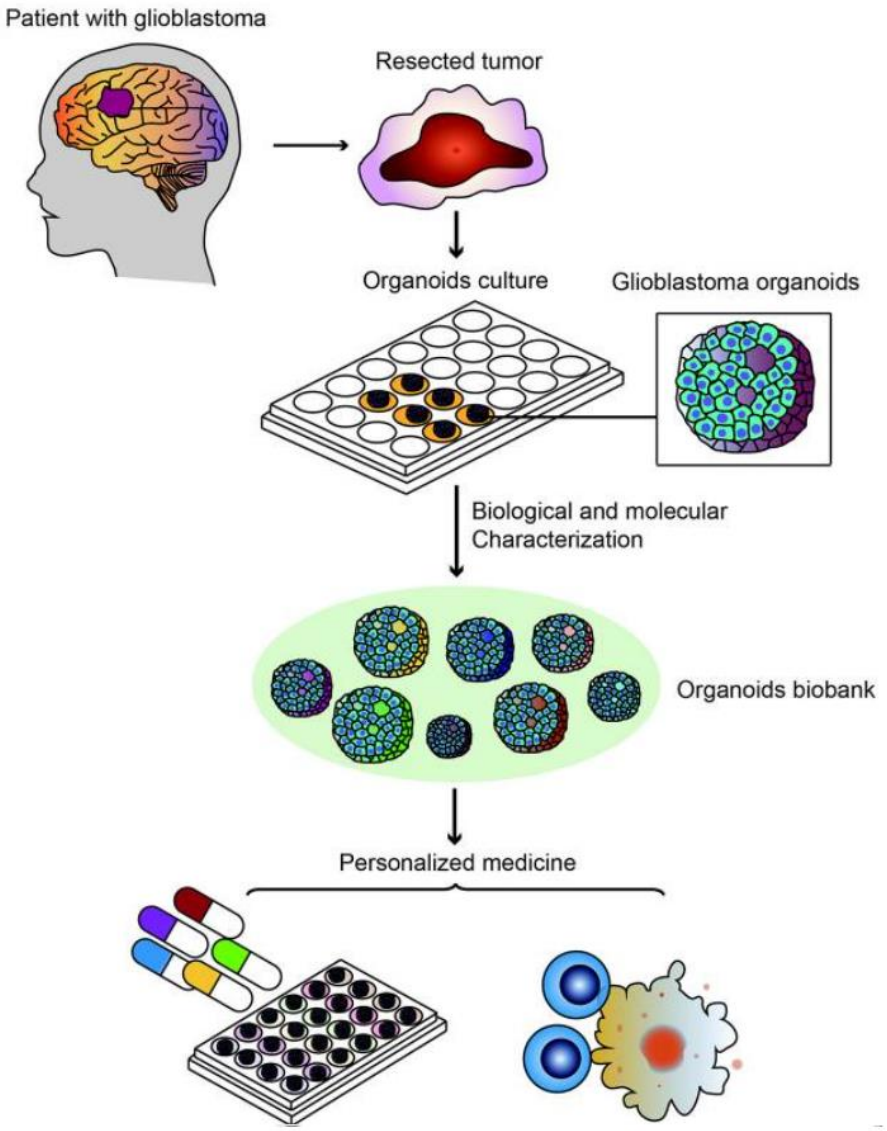

Figure 22: This illustration gives an overview of the manufacturing steps and tests that can be performed on organoids placed in biobanks. First a piece of tumor tissue is removed from a patient to form a GBO organoid. Then the different GBO organoids formed from different donors are placed in a biobank. This biobank captures some of the variability of GBM tumors that exists. From this biobank it is possible to correlate mutational profiles of GBOs with responses to drugs or immunotherapy such as CAR-T. Figure adapted from (49).

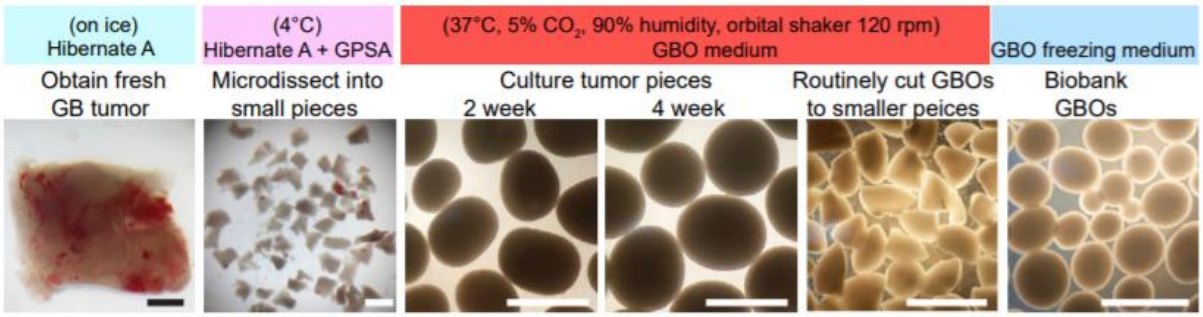

Figure 23: Overview of the procedure for producing GBO organoids in biobanks. Figure from (22)

Limitations of GBM organoids

Specific limitations to the different models

All these organoids model have their own limitations. For example, it is not yet known whether it is possible to reproduce all the subtypes of GBM with NeoCOR organoid model, considering that for other types of cancer, certain genetic aberrations appear at a different time during tumorigenesis. Moreover, as they are formed by a few driving mutations/oncogenes, it is not certain that it recapitulates the heterogeneity, genomic complexity of tumors and tumorigenesis with enough precision to draw robust conclusions (20).

Moreover, due to the heterogeneity of the tumor regions characteristic of the GBM organoids model, the final organoids vary depending on the sample. The fabrication of GBM organoids requires tumor material, which may be limiting. This tumorsphere model represents few TME components. For this reason, GBM organoid is not suitable for questioning immunotherapy (19). Another subjacent limitation of this model is caused by the use of Matrigel *(see box IV "ECM")* (4). One of the future solutions that has already been proven on intestinal cancer organoids is to use a polyethylene glycol (PEG) matrix. PEG is a synthetic hydrogel which gives the possibility to modify it specifically. The GBO model overcomes some of the limitations of the GBM model, notably its method of manufacture which does not require serum or ECM and does not dissociate the cells, thus making it possible to obtain an organoid with more TME components. However, this model has limitations such as the absence of normal brain environment. GBO, GBM, GLICO, NeoCOR and bioprinted organoids does not escape the limitation of lack of vascular system as well as a low level of residual tumor immune cells (20,22).

Reproducibility and model standardization

Cell quantification and viability tests on organoids have limitations such as the lack of shape, size and proliferation status standardized (19). Distinguishing tumor cells from healthy cells is crucial in drug screening to ensure that only tumor cells are targeted. Smaller organoids are better models because nutrients have difficulty being delivered to the organoid center when it is large. Bioprinted organoids associated with a short derivation time represent an interesting potential for establishing automated drug screening (20).

Recent advances and development of GBM organoid models have raised issues in model reproducibility and standardization (20). Standardization of protocols will be a necessary step to decrease the discrepancy between the different properties of GBM organoids. One method for size standardization is to mechanically cut the organoids (19). The other method is to reform the organoids from dissociated individual cells.

The standardization of the analysis methods is also an important point for the progress of the research to allow the comparison (20). For example, it is necessary to be able to quantify survival, proliferation and self-renewal on organoid models (19,20). The evaluation of the proliferation and survival of cancer cells is complicated by the difficulty of counting the cells within the organoid. Furthermore, it has been observed that the invasion rate of GBM cells is higher in more mature organoids.

Self-renewal is analyzed by two methods: microscopy or analysis of living cells using immunohistochemistry. For example, staining with ki67 insertion in cells allows to identify proliferating tumor cells.

Fluorescent labeling of viability markers allows estimation of cell death and the ratio of living to dead cells. For genetically modified organoids, it is possible to use viral barcodes to trace clinical lineages and proliferation capabilities.

What makes GBM aggressive stems in part from its recurrence mediated by residual GBM cells. The plasticity of these cells is one of the characteristics that makes it resistant to treatments. However, data are still lacking as to whether organoid models can model recurrence after treatment.

Deficiency in the immune response

A common disadvantage of all organoid models of GBM (GBM, GLICO, GBO, NeoCOR) is the lack of immune components.

As we have discussed, immunotherapy is based on the microenvironment involving the interaction of immune system cells and tumor cells. However, the organoid TME is marked by the absence of vascular system and immune cells. Immune cells introduced by co-culture or fusion are present only for a short time. The GBO model offers the advantage of preserving the expression of antigens, which facilitates testing the efficacy of CAR-T targets. The ALI AOP culture allows to demonstrate the efficacy of checkpoint inhibitors such as PD-1/PD-L1. Advances in microfluidics, organ-on-chip, 4D imaging, sequencing and transcriptomics may in the future improve the capabilities of the models. One solution to this limitation would be to improve the method of brain tumor organoid culture (4,19).

The absence of a vascular system

Another limitation shared by all current organoid models is that none of them contains the equivalent of a vascular system (19). The lack of endothelial cells as well as tumor vasculature is a limitation, as these are important constituents of the TME (4). In the case of brain organoids, we mentioned that the cause of necrotic core formation was the consequence of a lack of vascularization (50). For GBM model, vascularization is also important, since GBM cells have a cerebral perivascular microenvironment forming a natural niche. GBM is therefore characterized by its invasion of the perivascular spaces (20). Prevention of perivascular invasion requires a model that integrates vascular parameters. Recent approaches allowing the formation of vascular structures in organoids are being developed. Among these approaches, the introduction of VEGF when the organoid is at the embryoid body stage has shown interesting results. The introduction of endothelial cells into developing organoids has also been shown recently (50).

Conclusion/discussion

Ethical dilemma related to research with brain organoids

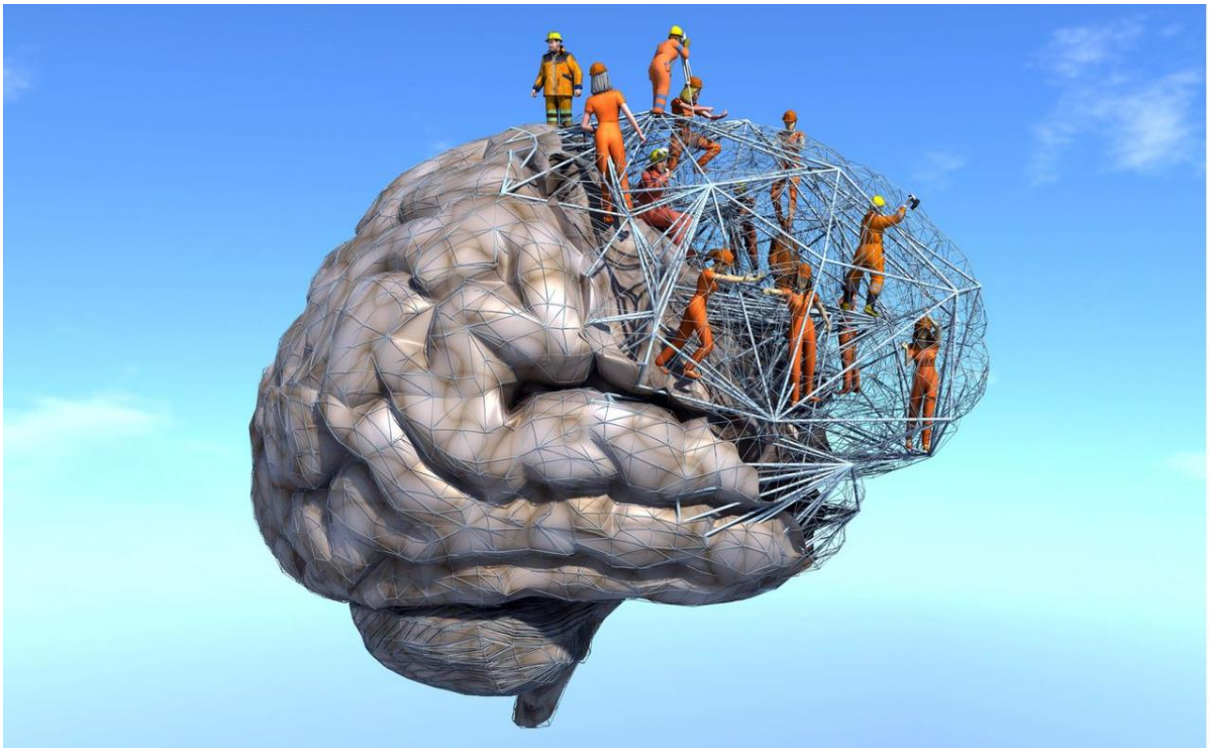

Figure 24: artistic image illustrating the creation of cerebroids in vitro by humans, figure adapted from (9).

On the face of it, brain organoids are more ethically acceptable than some methods performed on animals or human fetuses. On the one hand, human brain organoids hold promise for basic research, as well as for the study of neurodegenerative diseases and mental disorders. So far, organoids have met the 3Rs of laboratory research. They help to reduce animal research, for example in studies on new drugs (9).

On the other hand, the rapid emergence of organoids and their resemblance to human fetuses have raised new ethical questions *(see Figure 24)* (51). A crucial point that triggered the major ethical questions about organoids was the discovery that a cortical organoid had an EEG whose electrical activity resembled that of a premature baby of 25 to 39 weeks (52). Reflections on the potential consciousness of the organoid, or on its status as a complex inanimate system to be reconsidered, have emerged.

The following discussion will focus on biobanks and then on the problem of the potential consciousness attributed to the cerebroid (53).

Biobanks

While biobanks offer a new perspective, especially in the field of specialized medicine such as cancer, several ethical issues are emerging, namely about commercialization, intellectual property and donor rights (54).

The value of human tissues that can be stored indefinitely represents a first challenge, as it is difficult to know exactly how it will be used, and who will benefit from it in terms of care and profit, or even patentability. The possibility of intellectual property being assigned in the event of a novel discovery means that donors no longer have any rights to their own biological material, which is problematic (9).

In the future, donors will have to give their initial consent, after being informed of all possible information (purpose of the study, privacy, etc.) (54). As this is a recent application, it is impossible to know whether donors consent to their cells being used to make organoids that can be stored potentially indefinitely (32). There is also the question of the potential consciousness of organoids (55). If the organoids are considered conscious, the donor will not be able to withdraw consent. This is problematic because, in the future, the organoid could reveal memories of the donor as well as important genetic data about diseases the donor may have.

A final issue that needs to be explored in the future is the relationship between the donor and the organoid. A better understanding of this relationship with this being that is

close to them but lives separately will be important for establishing governance and ethical guidelines (9,54).

The consciousness of cerebroids.

Is the cerebroid conscious? If so, what does this imply about its moral status?

The potential consciousness of organoids faces two problems: scientific uncertainty, and ethical uncertainty.

The first one refers to the difficulty of *scientifically measuring consciousness*. The lack of consensus concerning the brain basis defining consciousness is problematic (56). Currently the measures to detect consciousness scientifically are only applicable to humans. It consists of an introspective and therefore linguistically dependent report (55). In neuroscience consciousness has been defined by the study of patients in coma. Consciousness includes wakefulness, the mechanism that keeps the patient awake, awareness, the mechanism of perception of self and environment, and psychological functions such as emotions and thoughts (9). Thus, despite the electrical activities analogous to a 25-39 week fetus (the stage at which the acquisition of consciousness is possible), this would not mean that the organoid is being directed to consciousness (55). If consciousness would be defined by elementary neural activity there should be no ethical problem (7).

However, according to the ethical committee of INSERM[4], if the electrical activity is not enough to attribute a consciousness to the organoid, this does not exclude that it can have mental states as there is a causal link between the mind and the brain (9). Thus, the second problem is related to the ethical uncertainty concerning the *definition of consciousness* (57). In psychology the notion of consciousness is divided into 4 concepts: **1)** the phenominal consciousness which corresponds to the feelings like pain or pleasure, **2)** the access consciousness which is the whole of the representations (thoughts, desires, etc), **3)** the self-consciousness corresponding to the representation of ourselves and finally **4)** the monitoring consciousness including the reflexive consciousness, the interrogation on our mind (51). These subdivisions possessing an independent ethical value, create by themselves debates about the relevance of moral consciousness. Is the attribution of a phenomenal consciousness enough to prohibit research? For others, only the acquisition of self-consciousness would require a limit to research. This type of consciousness is one step above phenomenal consciousness, implying a higher cognition that can allow the possible sensation of distress (55).

[4] Institut national de la santé et de la recherche médicale

Assuming that the cerebroids have a consciousness, their moral status as a "thing" is to be re-evaluated. If they are attributed a moral patient status, the actors connected to the cerebroids should adopt a behavior of moral agents having moral obligations towards the cerebroids (51). In the case that the moral status of the organoid acquires rights, especially those of respect to life and autonomy, the destruction of organoids when they are no longer useful and the storage of organoids in biobanks would be questioned (54).

In the future, it would be important to carry out work on several different organisms (rodents, etc.) to conceptualize consciousness more simply (56).

What would be the future solutions?

There are many directions to consider in order to reduce the ethical impact of organoids. New guidelines are needed. If possible, they should be adaptable rather than dogmatic to better guide research (9). They should address issues such as donor consent, governance, property (55).

To achieve these next guidelines, it would be ideal to integrate ethicists with researchers so that they can have a proactive approach (32). With the emergence of biotechnologies, the work of ethicists is taking a turn, giving rise to parallel ethics. This new profession is formed by combining the fields of bioethics and research philosophy. They constitute a discipline that complements the other analyses. Their goal is to work in collaboration with researchers to guide the development of technology towards better practices. They must be able to anticipate the plausible and the possible applications and abuses (56). Ethicists will have to think in terms of social justice, fairness of distribution, benefits and accessibility of applications. In a more global way, it would be good if new ethical examinations were made. At present they are limited to checking the origin of the stem cell lines. In the future, regional or national institutions, and in most cases worldwide, should carry out additional examinations (32).

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I also had the opportunity to visit a laboratory at the Campus Biotech dedicated to neurospheres. This visit and the following interview (attached) were made possible by a family friend, Luc Stoppini, who specializes in applied biology and tissue biology. I also thank his colleague Aurélien Roux, who also took the time to explain many concepts to me. I am indebted to them, because even if the subject they were dealing with was not exactly GBM organoids as in this monograph, they helped me to understand in which context organoids have evolved and what are the characteristics that make them different from other 3D or 2D cultures.

On the other hand, I was not very familiar with English, so I thank William Favre, who, in addition to his support, helped me.

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Bibliography

1. Glioblastoma Organoids [Internet]. [cité 1 août 2021]. Disponible sur: https://encyclopedia.pub/3960

2. Linkous A, Balamatsias D, Snuderl M, Edwards L, Miyaguchi K, Milner T, et al. Modeling Patient-Derived Glioblastoma with Cerebral Organoids. Cell Rep. 19 mars 2019;26(12):3203-3211.e5.

3. Koo B, Choi B, Park H, Yoon K-J. Past, Present, and Future of Brain Organoid Technology. Mol Cells. sept 2019;42(9):617-27.

4. Zhang C, Jin M, Zhao J, Chen J, Jin W. Organoid models of glioblastoma: advances, applications and challenges. Am J Cancer Res. 1 août 2020;10(8):2242-57.

5. Eiraku M, Watanabe K, Matsuo-Takasaki M, Kawada M, Yonemura S, Matsumura M, et al. Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals. Cell Stem Cell. 6 nov 2008;3(5):519-32.

6. Simunovic M, Brivanlou AH. Embryoids, organoids and gastruloids: new approaches to understanding embryogenesis. Development. 15 mars 2017;144(6):976-85.

7. Corrò C, Novellasdemunt L, Li VSW. A brief history of organoids. Am J Physiol Cell Physiol. 1 juill 2020;319(1):C151-65.

8. Balak JRA, Juksar J, Carlotti F, Lo Nigro A, de Koning EJP. Organoids from the Human Fetal and Adult Pancreas. Curr Diab Rep. 2019;19(12):160.

9. Baertschi B, Atlan H, Botbol-Baum M, Bed'hom B, Combrisson H, Dosquet C, et al. Organoids Research: What are the ethical issues? :20.

10. Simian M, Bissell MJ. Organoids: A historical perspective of thinking in three dimensions. J Cell Biol. 2 janv 2017;216(1):31-40.

11. PhD RP. Key Applications of Organoids [Internet]. [cité 18 sept 2021]. Disponible sur: https://blog.crownbio.com/key-organoid-applications

12. Rossi G, Manfrin A, Lutolf MP. Progress and potential in organoid research. Nat Rev Genet. nov 2018;19(11):671-87.

13. Nowogrodzki A. How cerebral organoids are guiding brain-cancer research and therapies. Nature. 26 sept 2018;561(7724):S48-9.

14. BR27132-Organoids.pdf [Internet]. [cité 3 août 2021]. Disponible sur: https://www.stemcell.com/media/files/brochure/BR27132-Organoids.pdf

15. Marshall M. How COVID-19 can damage the brain. Nature. 17 sept 2020;585(7825):342-3.

16. SARS-CoV-2 targets neurons of 3D human brain organoids. The EMBO Journal. 15 oct 2020;39(20):e106230.

17. Lancaster MA, Renner M, Martin C-A, Wenzel D, Bicknell LS, Hurles ME, et al. Cerebral organoids model human brain development and microcephaly. Nature. sept 2013;501(7467):373-9.

18. Azzarelli R. Organoid Models of Glioblastoma to Study Brain Tumor Stem Cells. Front Cell Dev Biol [Internet]. 2020 [cité 27 juill 2021];0. Disponible sur: https://www.frontiersin.org/articles/10.3389/fcell.2020.00220/full

19. Klein E, Hau A-C, Oudin A, Golebiewska A, Niclou SP. Glioblastoma Organoids: Pre-Clinical Applications and Challenges in the Context of Immunotherapy. Front Oncol [Internet]. 2020 [cité 27 juill 2021];0. Disponible sur: https://www.frontiersin.org/articles/10.3389/fonc.2020.604121/full

20. Rybin MJ, Ivan ME, Ayad NG, Zeier Z. Organoid Models of Glioblastoma and Their Role in Drug Discovery. Front Cell Neurosci [Internet]. 2021 [cité 27 juill 2021];0. Disponible sur: https://www.frontiersin.org/articles/10.3389/fncel.2021.605255/full

21. Andreatta F, Beccaceci G, Fortuna N, Celotti M, De Felice D, Lorenzoni M, et al. The Organoid Era Permits the Development of New Applications to Study Glioblastoma. Cancers (Basel) [Internet]. 9 nov 2020 [cité 20 mars 2021];12(11). Disponible sur: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7695252/

22. Jacob F, Salinas RD, Zhang DY, Nguyen PTT, Schnoll JG, Wong SZH, et al. A Patient-Derived Glioblastoma Organoid Model and Biobank Recapitulates Inter- and Intra-tumoral Heterogeneity. Cell. 9 janv 2020;180(1):188-204.e22.

23. Modeling glioblastoma invasion using human brain organoids and single-cell transcriptomics | Neuro-Oncology | Oxford Academic [Internet]. [cité 20 mars 2021]. Disponible sur: https://academic.oup.com/neuro-oncology/article/22/8/1138/5820825

24. Sasai Y. Cytosystems dynamics in self-organization of tissue architecture. Nature. janv 2013;493(7432):318-26.

25. Narsinh KH, Plews J, Wu JC. Comparison of Human Induced Pluripotent and Embryonic Stem Cells: Fraternal or Identical Twins? Mol Ther. avr 2011;19(4):635-8.

26. Lancaster MA, Knoblich JA. Generation of cerebral organoids from human pluripotent stem cells. Nat Protoc. oct 2014;9(10):2329-40.

27. P Rothenbücher TS, Martínez-Serrano A. Human cerebral organoids and neural 3D tissues in basic research, and their application to study neurological diseases. Future Neurology. 1 févr 2019;14(1):FNL3.

28. Hofer M, Lutolf MP. Engineering organoids. Nat Rev Mater. mai 2021;6(5):402-20.

29. Gopalakrishnan J. The Emergence of Stem Cell-Based Brain Organoids: Trends and Challenges. BioEssays. 2019;41(8):1900011.

30. Kim J, Sullivan GJ, Park I-H. How well do brain organoids capture your brain? iScience. 19 janv 2021;24(2):102063.

31. Sidhaye J, Knoblich JA. Brain organoids: an ensemble of bioassays to investigate human neurodevelopment and disease. Cell Death Differ [Internet]. 1 juin 2020 [cité 27 déc 2020]; Disponible sur: http://www.nature.com/articles/s41418-020-0566-4

32. Hyun I, Scharf-Deering JC, Lunshof JE. Ethical issues related to brain organoid research. Brain Research. 1 avr 2020;1732:146653.

33. Bredenoord AL, Clevers H, Knoblich JA. Human tissues in a dish: The research and ethical implications of organoid technology. Science [Internet]. 20 janv 2017 [cité 4 mai 2021];355(6322). Disponible sur: https://science.sciencemag.org/content/355/6322/eaaf9414

34. Lee C-T, Bendriem RM, Wu WW, Shen R-F. 3D brain Organoids derived from pluripotent stem cells: promising experimental models for brain development and neurodegenerative disorders. J Biomed Sci. déc 2017;24(1):59.

35. Oberacker E, Diesch C, Nadobny J, Kuehne A, Wust P, Ghadjar P, et al. Patient-Specific Planning for Thermal Magnetic Resonance of Glioblastoma Multiforme. Cancers. janv 2021;13(8):1867.

36. Kesari S. Understanding Glioblastoma Tumor Biology: The Potential to Improve Current Diagnosis and Treatments. Seminars in Oncology. 1 déc 2011;38:S2-10.

37. An integrative model of cellular states, plasticity and genetics for glioblastoma [Internet]. [cité 27 juill 2021]. Disponible sur: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6703186/

38. Hubert CG, Rivera M, Spangler LC, Wu Q, Mack SC, Prager BC, et al. A Three-Dimensional Organoid Culture System Derived from Human Glioblastomas Recapitulates the Hypoxic Gradients and Cancer Stem Cell Heterogeneity of Tumors Found In Vivo. Cancer Res. 15 avr 2016;76(8):2465-77.

39. Kjørstad E, Nuse IP. Neuroscientists cultivate mini-versions of human brains in the lab. Could they become conscious? [Internet]. 2019 [cité 4 mai 2021]. Disponible sur: https://sciencenorway.no/a/1599900

40. Fig. 2: Clonal mutagenesis in organoids induces tumor overgrowth. | Nature Methods. [cité 8 sept 2021]; Disponible sur: https://www.nature.com/articles/s41592-018-0070-7/figures/2

41. Yuki K, Cheng N, Nakano M, Kuo CJ. Organoid Models of Tumor Immunology. Trends in Immunology. 1 août 2020;41(8):652-64.

42. fonc-10-604121-g002.jpg (2126×1250) [Internet]. [cité 1 août 2021]. Disponible sur: https://www.frontiersin.org/files/Articles/604121/fonc-10-604121-HTML/image_m/fonc-10-604121-g002.jpg

43. Dijkstra KK, Cattaneo CM, Weeber F, Chalabi M, van de Haar J, Fanchi LF, et al. Generation of tumor-reactive T cells by co-culture of peripheral blood lymphocytes and tumor organoids. Cell. 6 sept 2018;174(6):1586-1598.e12.

44. O'Rourke DM, Nasrallah MP, Desai A, Melenhorst JJ, Mansfield K, Morrissette JJD, et al. A single dose of peripherally infused EGFRvIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. Sci Transl Med. 19 juill 2017;9(399):eaaa0984.

45. Cours Sciences_Dutoit 2021_etu.pdf [Internet]. [cité 27 juill 2021]. Disponible sur: https://moodle.unige.ch/pluginfile.php/1201809/mod_resource/content/1/Cours%20Sciences_Dutoit%202021_etu.pdf

46. Ootani A, Li X, Sangiorgi E, Ho QT, Ueno H, Toda S, et al. Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. Nat Med. juin 2009;15(6):701-6.

47. Neal JT, Li X, Zhu J, Giangarra V, Grzeskowiak CL, Ju J, et al. Organoid Modeling of the Tumor Immune Microenvironment. Cell. 13 déc 2018;175(7):1972-1988.e16.

48. directives_assm_biobanques_material_humain_formation_recherche_2006.pdf.

49. Yang H, Zhang N, Liu Y-C. An organoids biobank for recapitulating tumor heterogeneity and personalized medicine. Chin J Cancer Res. juin 2020;32(3):408-13.

50. Silvia N, Dai G. Cerebral organoids as a model for glioblastoma multiforme. Current Opinion in Biomedical Engineering. 1 mars 2020;13:152-9.

51. Farahany NA, Greely HT, Hyman S, Koch C, Grady C, Pașca SP, et al. The ethics of experimenting with human brain tissue. Nature. avr 2018;556(7702):429-32.

52. Trujillo CA, Gao R, Negraes PD, Gu J, Buchanan J, Preissl S, et al. Complex Oscillatory Waves Emerging from Cortical Organoids Model Early Human Brain Network Development. Cell Stem Cell. 3 oct 2019;25(4):558-569.e7.

53. Derita J, Health JBS of P. Organoid Intelligence [Internet]. Johns Hopkins Bloomberg School of Public Health. [cité 1 mai 2021]. Disponible sur: http://caat.jhsph.edu/programs/oi/

54. Ethics parallel research: an approach for (early) ethical guidance of biomedical innovation | SpringerLink [Internet]. [cité 3 mai 2021]. Disponible sur: https://link.springer.com/article/10.1186/s12910-020-00524-z

55. Sawai T, Hayashi Y, Niikawa T, Shepherd J, Thomas E, Lee T-L, et al. Mapping the Ethical Issues of Brain Organoid Research and Application. AJOB Neuroscience. 26 mars 2021;0(0):1-14.

56. Sharma A, Zuk P, Scott CT. Scientific and Ethical Uncertainties in Brain Organoid Research. The American Journal of Bioethics. 2 janv 2021;21(1):48-51.

57. Buchanan M. Organoids of intelligence. Nature Physics. juill 2018;14(7):634-634.

Annex I

Interview with Luc Stoppini and Aurelien Roux

Luc Stoppini :

"In organoids, there is a certain form of organization. Our neurospheres are all the same, there is not this cerebral stratification of the cortical type or others. So with iPSCs it is possible to make neural lineages. Assembloids are the next step where we complexify."

What are the different types of cells used?

Luc Stoppini :

"IPSCs allow us to make neural lineage, muscular lineage, and for example to form tissues with these specific progenies. The neural and muscle lineage can be assembled to make neuromuscular junctions. There are cells of semi-stem origin either from a human embryo or iPSC. We focus on neural lineages, i.e. iPSCs that have been differentiated into neuroprogenitors and will thus give neurons and glial cells, and then the different types. So we can start from neuroprogenitors to make our spheroids.

What is the difference between neurosphere and spheroid, if there is one?

Aurélien Roux

"Neurospheres are a type of spheroid. For example, it is also possible to make gliospheres if we select only the glial cells. In our laboratory the neuroprojenitors give the different cell types neurons and glial cells."

What are the derivation steps ?

Aurélien Roux

"Here we start with primary cells. Having access to human neurons is very hard so we can use embryonic (ES) cells which have their advantages and disadvantages. Now there are iPSCs, which are better because they allow us to have human cells and to have different associated pathologies, for example Zika, or to enter into personalised medicine by taking stem cells from patients. Any laboratory can cultivate iPSCs for cell culture. However, differentiating iPSC cells to a particular cell type is very difficult. Now there are starting to be protocols. It

takes a lot of time, we have to check each time that we are going in the right direction. We say we are working with iPSCs but in reality we are working with iPSC-derived cells: neuroprojenitors. It's hard to direct them to make sure they are pure neuroprogenitors. In the beginning we differentiated them ourselves. We got neurons but also cartilage and sometimes heart. They were a mixture and almost embrioids. “

How are neurospheres made ?

Luc Soppini

"We start with cells that are iPSCs, we dissociate them, we put a cocktail of products that will orientate them with a culture medium to obtain only neural type cells, neuroprojenitors that will survive. The difficulty is to have a pure population. Then it is possible to amplify them and what we do is to differentiate them to give neurons or glial cells with a very precise cascade.

"Some people only make neurospheres with neurons. Our choice was to take neurons and glial cells from neuroprojenitors. So we get our neuroprojenitors that have differentiated between iPSCs, oriented with a cocktail of molecules to give neuroprojenitors. When we decide to make cultures, we amplify the neuroprojenitors and we make cultures. So we create our own neurosphere cells and we now have thousands of them of different ages where we can do toxicology studies. We have another model where we do brain engineering trauma in vitro. Transplantation of neurospheres and dissociated cells to restore the neurons of patients is currently being used for Parkinson's disease. In this case, neurospheres enriched with dopaminergic neurons could be used. Potentially it will be possible to restore the neurons of patients, whereas before it was taken from human embryos. So there are various applications, some remain in vitro and some do not, so there are two big families.”

What are the applications?

Aurélien Roux

"The applications depend on what you have. For example, spheroids are small and simple, so they're used for toxicology studies when there are lots of compounds to test. Brain organelles are more suitable for observing mechanisms of action. So there are always advantages and disadvantages. Cerebral organoids are bigger and more complex, there is more organisation but a lot of variation. Size is important, when it's big there are oxygenation problems creating a lot of necrosis in the centre. The last papers that Lancaster published were done with the air-liquid interface technique if I am not mistaken. So the methods of maintaining the culture are

important. So there's the air-liquid interface and the agitation method both of which allow for high oxygenation for example using bioreactors.

How do you generate 3D?

Luc Stoppini

"There are three main techniques. The first is hanging drops, which consists of a drop that is turned upside down, where the cells will settle at the bottom of the drop and aggregate. We can use a conical tube and put them at the bottom, we let them aggregate by gravity. The other method is by centrifugation, so by agitation. The third is aggregation by high concentration at the bottom of a well on plates. "