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Laboratory-Engineered Glioblastoma Organoid Culture and Drug Screening

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Abstract

Glioblastoma (GBM) is described as a group of highly malignant primary brain tumors and stands as one of the most lethal malignancies. The genetic and cellular characteristics of GBM have been a focal point of ongoing research, revealing that it is a group of heterogeneous diseases with variations in RNA expression, DNA methylation, or cellular composition. Despite the wealth of molecular data available, the lack of transferable pre-clinic models has limited the application of this information to disease classification rather than treatment stratification. Transferring the patients' genetic information into clinical benefits and bridging the gap between detailed descriptions of GBM, genotype-phenotype associations, and treatment advancements remain significant challenges. In this context, we present an advanced human GBM organoid model, the Laboratory Engineered Glioblastoma Organoid (LEGO), and illustrate its use in studying the genotype-phenotype dependencies and screening potential drugs for GBM. Utilizing this model, we have identified lipid metabolism dysregulation as a critical milestone in GBM progression and discovered that the microsomal triglyceride transfer protein inhibitor Lomitapide shows promise as a potential treatment for GBM.

Introduction

Glioblastoma (GBM) accounts for more than 60% of all diagnosed primary brain tumors in adults, with a median survival of less than two years[1,2,3]. Despite considerable efforts in deciphering the underlying complexity of this disease, continuous attempts with targeted therapies or immunotherapies, and screening for potential anti-cancer drugs, the treatment strategy for newly diagnosed GBM patients remains to be the maximal safe surgical resection followed by radiotherapy (RT) combined with temozolomide (TMZ) and then adjuvant TMZ[4].

The highly heterogeneous mutational nature of glioblastoma within and among tumors makes it extremely difficult to dissect the molecular properties of this tumor, which

eventually leads to treatment failure. Thus, it is essential to decomplexify the disease by reducing the variety of genetic mutations to the modules of mutations that occur most often in GBM patients[5,6,7,8] and to investigate the molecular consequences of each mutational subclone individually.

Recently, organoids have emerged as a promising model for cancer research. Organoids surpass the canonical cancer models since they exhibit a human microenvironment and complex cellular components while being easy to generate and expand with a relatively low cost[9]. There were also pioneers of genetically engineered organoids with clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) to study glioma[10,11]. Following this direction, we generated a set of induced pluripotent stem cell (iPSC)-based human GBM organoid models (LEGO: Laboratory Engineered Glioblastoma Organoid) based on CRISPR/Cas9 genetic engineering of the frequent mutations identified in GBM patients. With detailed characterization of the single-cell transcriptome, DNA methylome, metabolome, lipidome, proteome, and phospho-proteome of LEGOs, we have demonstrated the high resemblance of LEGOs with human GBM and discovered major milestones during the progression of GBM, and with luciferase-based drug screen, we have identified several potential drugs for GBM treatment, the details of such results have been published previously[12]. This article describes the detailed protocol for the generation and drug screening of LEGOs (**Figure 1A**).

Protocol

1. Luciferase labeling of the iPSCs

NOTE: For detailed instructions on culturing iPSCs, refer to the protocol provided by the institutions or the companies from which the cells were obtained. In all experiments described in this protocol, the iPSCs at passages 3-10 post-recovery were used, and any cells showing signs of differentiation were discarded. For the virus production, plasmids containing an EF1α promoter driving Luc2, along with a puromycin-resistant cassette, were used.

1. When the cells reach ~70% confluency, digest iPSCs with passaging reagents A and B (see **Table of Materials**), respectively, for 4 min at 37 °C, centrifuge at 120 x *g* for 5 min, and resuspend into a single cell suspension by gentle pipetting.
2. Count the cells and seed 1 x 10^4 single cells of iPSC in each well of a basement matrix membrane (BMM)-coated 48 well plate, culture for 24 h with human embryonic cell qualified culture medium containing 10 µM rho kinase (ROCK) inhibitor (see **Table of Materials**).
3. Infect the cells with lentivirus-containing luciferase-expressing plasmids (MOI around 10) and 8 µg/mL Polybrene (see **Table of Materials**). Replace the medium with a fresh human embryonic cell-qualified culture medium 6 h later.
4. After 24 h, add 150 µg/mL Luciferase Substrate (see **Table of Materials**) to the cell culture medium and check the bioluminescence (BLI) signal with bioluminescence imaging equipment (see **Table of Materials**) with a 5 s exposure.
5. Add 2 µg/mL puromycin to the culture medium for 2 days, then change the medium every other day for 1 week to expand the cells.
6. When the cells reach ~70% confluency, digest iPSCs with passaging reagents A and B (see **Table of Materials**), respectively, for 4 min at 37 °C, centrifuge

at 120 x *g* for 5 min, and resuspend into a single cell suspension by gentle pipetting.

7. Count the cells and seed the single cells at a concentration of 50 cells per 10 cm dish for 2 dishes. Change the medium every other day until the clones reach 3-5 mm in diameter (**Figure 1B**).
8. Check the BLI signal as described above and pick the clones with strong BLI signals under the microscope with sterilized 10 µL pipette tips.
9. Expand the cells for further usage.

2. gRNA design and plasmid cloning

NOTE: These steps can be carried out simultaneously with Section 1. CRISPR/Cas9 plasmids with a proper selection system should work. These specific plasmids were used because they enable simultaneous knockout of several target genes. This section demonstrates how to clone a plasmid with two gRNA scaffolds; more gRNA scaffolds can be cloned in one plasmid following the same principle. The original protocol was previously published[13].

1. Design gRNAs targeting the respective genes of interest with online web tools (see **Table of Materials**). Select the gRNAs targeting the exons presented in all splicing variants and locate the closest to the transcription starting site.
2. Anneal gRNA oligos (95 °C for 5 min, cool down to 25 °C at a speed of 1 °C/s) and linearize pX330A-1x2 (see **Table of Materials**) and pX330S-2 (see **Table of Materials**) plasmids with Bbs1 enzyme.
3. Ligate one of the gRNAs with pX330A-1x2 or pX330S-2, respectively, with T4 ligase at room temperature (RT) for 4 h.
4. Digest the two resulting plasmids with BsaI enzyme (37 °C for 1 h) and purify the products by gel extraction.
5. Ligate the gRNA scaffolds from the pX330S-2-gRNA plasmid to the linearized pX330A-1x2-gRNA plasmid with T4 ligase at RT for 4 h.
6. Clone puromycin resistance gene (PuroR) from the pX459 (see **Table of Materials**) plasmid to the resulting plasmids by EcoRI digestion (37 °C for 1 h) and subsequential T4 ligation at RT for 4 h.
7. Transform the plasmids in DH5alpha-competent cells and extract the plasmids after expanding.

3. Knockout generation and validation on iPSCs

NOTE: Follow the standard CRISPR/Cas9 knockout protocols published before[13,14]. This section describes the method used to perform the knockouts via electroporation.

1. Digest the iPSCs with passaging reagents A and B (see **Table of Materials**) for 4 min at 37 °C each, and spin down the cells for 5 min at 120 x *g* and resuspend in human embryonic cell qualified culture medium (see **Table of Materials**) to generate single-cell suspensions.
2. Count the cells and divide them into several tubes to achieve 1.2 x 10^6 cells per sample and spin down the cells at 120 x *g* for 5 min.
3. Meanwhile, mix 15 µg of the plasmids in 135 µL of resuspension buffer from the transfection kit (see **Table of Materials**).
4. Use 120 µL of the mixture to resuspend the cell pellet.
5. Electroporate the cells for two pulses at 1200 V for 20 ms with an electroporation system (see **Table of Materials**) and plate the resulting cells in one 6 well plate well with a human embryonic cell qualified culture medium

containing reagent to facilitate single-cell survival (see **Table of Materials**).

6. Add 2 µg/mL puromycin to the culture medium every 12 h for 2 days. Then, expand the cells for 1 week before harvest.
7. Check the efficiency of the knockout by western blotting or webtool analysis (see **Table of Materials**) and select the knockout population with the highest efficiency.
8. Digest the iPSCs with the highest knockout efficiency with passaging reagents A and B for 4 min at 37 °C each, and spin down the cells for 5 min at 120 x *g*. Resuspend in human embryonic cell qualified culture medium to generate single-cell suspensions.
9. Count the cells and seed approximately 100 cells in two 10 cm dishes (50 cells in each dish) containing pre-warmed human embryonic cell qualified culture medium supplied with a reagent to facilitate single-cell survival.
10. Change the medium every other day and culture the cells for 2 weeks.
11. When the single-cell colonies are 3-5 mm in diameter, pick the colonies with 10 µL pipette tips under the microscope and expand the cells in two wells of 48 well plates.
12. When the cells reach 30%-50% confluency, extract the DNA from one well of the 48 well plate and perform PCR.
13. Sequence the PCR products and analyze the results with the web tool (see **Table of Materials**).
14. Select 5 positive clones to expand and further validate the knockout results with TA cloning and western blots.

4. Organoid Culture

NOTE: The organoids were generated following previously published protocols with minor adaptations[15,16,17]. The optimal age of the organoids (the culture duration) should be determined based on the specific purpose of the study. For instance, when investigating genotype-to-phenotype associations or observing organoid growth patterns, analyzing multiple time points ranging from 1-4 months or longer is recommended. Organoids aged 2-2.5 months were used for drug screening, as they tended to develop a necrotic core in the center due to nutrient limitations at larger sizes, which could impact the accuracy of drug screening results.

1. On day 0, digest iPSCs with passaging reagents A and B (see **Table of Materials**) respectively for 4 min at 37 °C, centrifuge at 120 x *g* for 5 min, and resuspend into a single cell suspension using low basic fibroblast growth factor (bFGF) hES media (**Table 1**).
2. Count the cells and add an appropriate number of cells to reach a concentration of 6×10^4 cells/mL.
3. Add 50 µM ROCK inhibitor and 6 ng/mL bFGF to the low bFGF hES medium-containing cells.
4. Seed 150 µL of cell suspension (9000 cells) into each well of the 96-well ultra-low attachment plate for both control (WT organoids) and experimental (knockout organoids) groups.
5. On day 3, carefully remove 80 µL of old culture medium from each well and add 150 µL fresh low bFGF hES medium.
6. On day 5, when embryonic bodies (EBs) begin to brighten and have smooth edges, remove as much old

culture medium as possible from each well and add 150 µL to 200 µL of fresh neural induction medium (**Table 1**).

7. On day 7, remove as much old culture medium as possible from each well and add 150-200 µL of neural induction medium.

8. On day 9, observe the EBs under the tissue culture microscope and select the EBs that are brighter around the outside.

9. Thaw BMM at 4 °C or on ice for 30 min before starting the following steps.

10. Use a cut 200 µL wide pipette tip to transfer the EBs to the organoid embedding sheet (see **Table of Materials**), remove the excessive medium, drop 30 µL of BMM on the organoids, position the EBs in the middle of the droplet with a 10 µL pipette tip, and let it solidify in the 37 °C incubator for 20 min.

11. Wash off the BMM droplets to a 6-well plate containing 3 mL of NeuroDMEM - A medium (**Table 1**) and 3 µM CHIR99021 (see **Table of Materials**).

12. On day 11, remove as much of the old culture medium from each well and add 3 mL of fresh NeuroDMEM -A medium containing 3 µM CHIR99021.

13. On day 13, remove as much of the old culture medium from each well as possible and add 3 mL of NeuroDMEM + A culture medium (**Table 1**). Place the 6-well plate on a shaker rotating at 75 rpm in the incubator.

14. Refresh NeuroDMEM + A medium every 3 days until organoids are used.

15. Use the organoids for multi-omic analysis following the standard protocols or drug screen as performed in the previous publication[12].

5. Drug screen with LEGOs

NOTE: The exposure time for iPSCs or organoids BLI should be evaluated using various equipment or laboratory environments.

1. Transfer organoids at the desired age to a 24-well plate containing 1 mL of NeuroDMEM + A medium, one organoid per well, 2 days before the experiment.

2. Add 150 µg/mL D-luciferin and incubate on the shaker for 15-30 min.

3. Perform BLI as described before with an exposure time of 1 s-5 s.

4. Select the organoids with similar signal strengths for subsequent experiments (at least 3 organoids for each group).

5. On day 0, repeat steps 5.2 and 5.3. Then apply dimethyl sulfoxide (DMSO) or interested drugs to the culture medium of the organoids. Start with an initial drug concentration of 10 µM, then perform an IC50 assay or adjust the concentration further according to different purposes.

6. Repeat step 5.5 every 2 days. Treat the organoids for 6-10 days.

   NOTE: The duration can be changed according to different purposes or the different nature of the drugs.

7. Add 100 µM BrdU to the medium and incubate it in a CO_2 incubator for 2 h before harvesting the organoids, if needed, for subsequent proliferation analysis.

8. Normalize the BLI signal to the DMSO control measured on the same day and then compare it with before drug treatment to evaluate the drug treatment effect. Consider

drugs with a *P* value less than 0.05 and a signal decrease of more than 50% effective.

Representative Results

In our hands, LEGOs derived from mutant iPSCs displayed increased expansion compared to the WT isogenic control (**Figure 1C**) and showed atypical nuclear after 4 weeks of culture (**Figure 1D**), which supports the potential malignant transformation of the cells in mutant organoids[18]. The tumorigenic potential of the organoids can be further verified with xenografted experiments if required, as we have shown previously[12].

The LEGOs with different mutations could harbor different phenotypes; this can be assessed with different approaches, such as staining, single-cell RNA sequencing, metabolomics, lipidomics, etc. We have shown that LEGOs with *NF1* mutation highly simulate the mesenchymal subtype of GBM[12].

Ideally, with appropriate steps, the BLI signal of the organoids would increase as the LEGOs grow larger. When reaching the desired age, drug screening can be performed. Normally, effective drugs would have simultaneous signal drops in all the organoids in the experimental group compared to the control (**Figure 1E**).

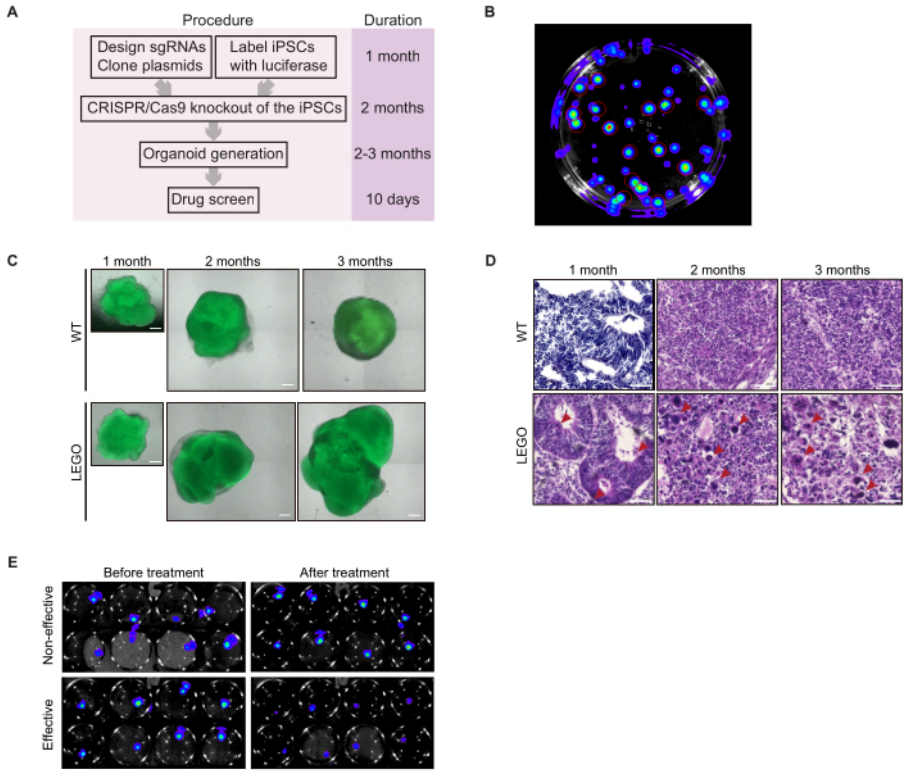

Figure 1: Representative results. (**A**) Overview of the protocol. (**B**) A representative image illustrates the appearance of the single-cell colonies under bioluminescence imaging in a 10 cm plate. (**C**) Representative images[12] showing the morphology of organoids at indicated time points in different groups. Scale bars, 500 µm. (**D**) Representative hematoxylin and eosin (HE)

staining images[12] of the organoids at different stages. The red arrows indicate the cells with atypical nuclei. Scale bar, 50 µm. (**E**) Representative bioluminescence images (BLI) of effective and non-effective drug treatments on LEGOs. Please click here to view a larger version of this figure.

Table 1: Homemade cell culture medium composition. Please click here to download this Table.

Discussion

The lack of personalized treatment in human GBM could largely be attributed to the fact that many GBM models, such as human cell lines or mouse models, cannot faithfully recapitulate the human GBM. Consequently, the treatment strategies selected based on these model systems cannot be transferred into clinical applications. Instead, organoids can tackle these translational problems with the presence of human physiological conditions. To this end, we have generated LEGO and shown that LEGOS can faithfully recapitulate many aspects of the molecular and cellular pathology of human GBM[12]. With LEGO, we can identify the major molecular consequences driven by genetic mutations and perform genotype-specific drug screens to enable personalized treatment of human GBM[12].

In this protocol, we generated and conducted a GBM drug screen using gene-mutated organoids. The first critical step involved ensuring the pluripotency of the iPSCs after knockout generation and thoroughly verifying the knockouts. We observed that *NF1* knockout leads to increased signs of differentiation in the iPSCs. To maintain the pluripotency, we applied 3 µM of CHIR99021 and 1 µM of PD0325901[19] to these clones during iPSC culture and the embryonic body culture stages. It is essential to frequently check the pluripotency of the iPSCs using staining or flow cytometry.

Controls are crucial in this protocol. Mutations are generated on the isogenic controls and can be incrementally increased. Therefore, the controls included not only WT controls but also those with fewer mutations. These controls should be carefully assessed before initiating the protocol.

The organoid drug screen relies on BLI. Given that organoids are 3D structures requiring more time for luciferase penetration, we tested different incubation times. A trial experiment is recommended to determine the optimal incubation time for different facilities. In our experience, the BLI signal increases during the first 15 min of incubation, reaching its maximum and stabilizing for about 1 h. This step is crucial for obtaining a stable signal for drug effect comparison.

Before the approach described in this protocol, previous studies have utilized electroporation to deliver plasmids to healthy brain organoids[10,11] or co-culture healthy brain organoids with GBM cells[10,20,21] to induce tumors. These approaches would be ideal for studying tumor invasion, migration, and interaction with surrounding normal brain cells. However, in these GBM organoid models, the tumor cells only occupy a small portion of the entire tumor-bearing organoid, which leads to a limited ability to study tumor intrinsic properties. Hence, constructing the mutational spectra at the iPSC level and inducing organoids from the knockout iPSCs would be preferable to enrich the tumor cells and allow for more detailed characterization.

GBM is a highly heterogeneous disease. The approach described here decreases complexity by studying each subclone individually. However, the complexity can easily be increased by assembling pieces of different mutant organoids

as a concept, like building blocks, as in the concept of LEGO. Also, the heterogeneity of GBM is also underlined by the presence of stromal cells, such as brain resident microglia, blood-derived immune cells, or myeloid cells. These cell types could be incorporated into the present model by adding cells to the culture medium or by co-embedding them in the matrigel to allow investigation of genetic heterogeneity determining immune cell behaviors. Once the experimental parameters are set, organoids with stromal cells can also be used for evaluating immune therapy responses. Further experiments should also aim to improve the general growth conditions of the organoids, for example, with the constant addition of fresh medium by bioengineering[22] or by co-culture with blood vessel organoids[23].

Disclosures

The authors have no conflicts of interest to disclose

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