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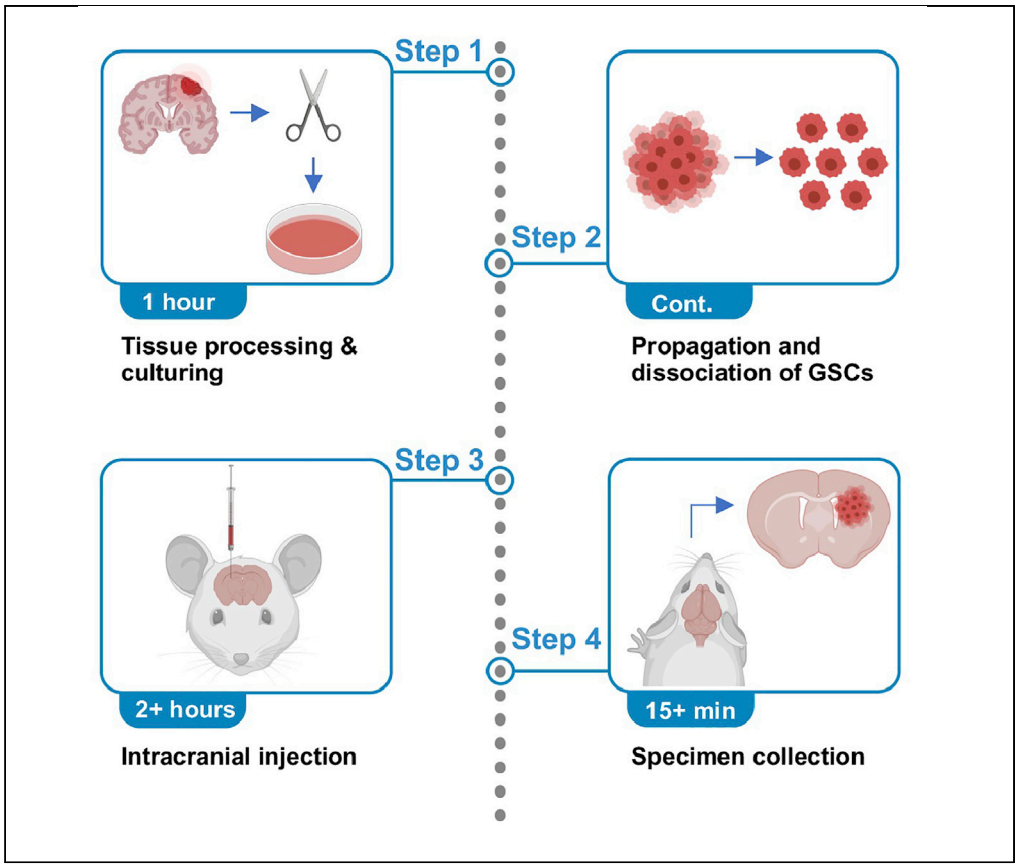
A Patient-Derived Xenograft Model of Glioblastoma



Glioblastoma (GBM) remains the most common malignant primary brain tumor in adults with a median survival of less than ~15 months. Further understanding and therapeutic development rely on the use of clinically relevant models of GBM. Here, we present our patient-derived *in vitro* and *in vivo* models that enrich for GBM stem cells (GSCs), a subpopulation of tumor cells with stem cell-like properties that recapitulate the cellular heterogeneity of its parental tumor and resist conventional therapy and seed disease relapse.

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HIGHLIGHTS

Processing of primary patient-derived glioblastoma specimens into single cells

Enrichment and propagation of patient-derived glioblastoma stem cells

Orthotopic and patient-derived xenograft model of glioblastoma

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A Patient-Derived Xenograft Model of Glioblastoma

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SUMMARY

Glioblastoma (GBM) remains the most common malignant primary brain tumor in adults with a median survival of less than ~15 months. Further understanding and therapeutic development rely on the use of clinically relevant models of GBM. Here, we present our patient-derived *in vitro* and *in vivo* models that enrich for GBM stem cells (GSCs), a subpopulation of tumor cells with stem cell-like properties that recapitulate the cellular heterogeneity of its parental tumor and resist conventional therapy and seed disease relapse.

For complete details on the use and execution of this protocol, please refer to Vora et al. (2020).

BEFORE YOU BEGIN

Timing: 1–2 h for dissociation of GBM specimens; 2–4 weeks for expansion of GSCs; 1–6+ months for orthotopic engraftment of GSCs in immunocompromised mice

Acquisition of Patient Tumor Specimens

1. Patient tumor specimens should be acquired from consenting patients as approved by local governing research ethics board(s). All *in vivo* experimental protocols should be approved by local animal research ethics board(s).
2. Patient tumor specimens should be acquired immediately after resection and stored in a sterile container at 4°C prior to processing. If tumor specimens are stored for >12 h prior to processing, it is recommended to store specimens in MACS Tissue Storage Solution (Miltenyi Biotec no. 130-100-008). This storage solution can be disposed prior to processing.
3. Prior to initiating *in vivo* trials, it is recommended that each patient-derived cell line be intracranially-injected into immunocompromised mice to determine engraftment success rates and time to endpoint.

Note: All procedures should be performed in a biological safety cabinet with sterile reagents and tools to minimize contamination. Unless stated otherwise, all processing, incubation and centrifugation steps should be performed at ambient temperature (22 to 24°C).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Cell Lines		
Patient-derived GBM specimens	n/a	n/a
Patient-derived GBM stem cell (GSC) lines: MBT06 and BT241	Dr. Sheila Singh's Laboratory	n/a
Chemicals, Peptides, and Recombinant Proteins		
Ammonium Chloride Solution	STEMCELL Technologies	07850
Antibiotic/Antimycotic Solution	Wisent Bio Products	450-115-EL
Avertin (2,2,2-Tribromoethanol)	Sigma-Aldrich	75-80-9
bFGF, Human, Recombinant	STEMCELL Technologies	78003.2
DNase Vial (D2)	Worthington Biochemical Corporation	Pennsylvania
EGF, Human, Recombinant	STEMCELL Technologies	78006
Formalin solution (10%), neutral buffered	Sigma-Aldrich	HT501128
Heparin sodium	Sigma-Aldrich	H3393
Heparin Solution	STEMCELL Technologies	07980
Iodine (10%)	Teva	PUN510685
Iodine (7.5%) with detergent	Teva	PUN104257
Isopropanol (70%)	Atlas	LAT917986
Laminin, Mouse, 1 mg	Corning	354232
Liberase™ TM Research Grade	Millipore Sigma	5401127001
MycoZapTM Prophylactic	Lonza	VZA-2031
NeuroCult™ NS-A Proliferation Kit (Human)	STEMCELL Technologies	05751
PBS pH 7.4, with calcium and magnesium	Wisent Bio Products	311-011-CL
Poly-L-ornithine solution	Millipore Sigma	P4957-50ML
Trypan blue	Sigma-Aldrich	T8154-100ML
TrypLE™ Express Enzyme (1×), phenol red	Thermo Fisher Scientific	12605028
Other		
Alcohol Prep Pads	RED Medical Supplies	211-MM-05507
Animal Heat Lamp	QC Supply	260038
CELLSTAR® Cell-Repellent Tissue Culture Dish (60 mm)	Grenier Bio	628979
CELLSTAR® Cell-Repellent Tissue Culture Dish (100 mm)	Grenier Bio	664970
Disposable Syringe (10 mL)	BD Becton Dickinson	309604
Cell strainer (70 µm)	Millipore Sigma	CLS431751-50EA
Coated Vicryl Sutures 5-0	Ethicon	J493G
Coronal brain slicing matrix	Harvard Apparatus	72-5033
Countess Chamber Slides	Invitrogen	C10228
General Surgery Pack	DRE Veterinary	13346
Insulin Syringes 0.5 mL/1.0 mL	ELI MEDICAL	U-40
Regular bevel needles (23 gauge)	BD Becton Dickinson	305145
Saline Bag	Baxter	BAXJB1323

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Saline Vials	Valuemed	HOS04888010
Sterile Cotton Swabs	Puritan	25-806 1WC
Stylo+ Versatile Craft Tool	DREMEL	2050-15
Surgical Glue	3M	1469SB
Tissue Embedding Cassettes	Kemtech America	1170Z96

MATERIALS AND EQUIPMENT

NeuroCult Complete (NCC) Medium

Store at 4°C for up to 1 week
450 mL Neurocult Basal Medium
50 mL NeuroCult Supplement
1× Antibiotic/Antimycotic solution (no preparation required; 100× stock solution)
1× MycoZap Prophylactic (no preparation required; 500× stock solution) (***Note:*** MycoZap is an antibiotic solution that prevents growth of *Mycoplasma* that are commonly found in patient-derived specimens)
0.0002% Heparin Solution (w/v)
20 ng/mL EGF
10 ng/mL bFGF

STEP-BY-STEP METHOD DETAILS

Dissociation and Culture of Primary GBM Tissue

Timing: ~1 h

This step allows you to dissociate patient-derived tumor specimens into a single cell population.

1. Mechanical dissociation of tumor
   a. Add 100–200 μL of Liberase (stock solution at 2.5 mg/mL in sterile water) to 15 mL of PBS. Warm solution to 37°C to ensure optimal enzymatic activity. Tumor specimens larger than 15 mm^3 may require a greater amount of Liberase and/or PBS for effective dissociation.
   b. Warm an aliquot of 0.8% ammonium chloride solution and NeuroCult Complete (NCC) media to 37°C. The amount of these solutions are dependent on size of the tumor specimen. A 15 mm^3 tumor specimen may require 4–12 mL of ammonium chloride solution and 7–10 mL of NCC media, largely dependent on red blood cell (RBC) abundance and number of isolated tumor cells, respectively.
   c. Place the patient brain tumor specimen in a 100 mm cell culture dish. Gently pipette 5 - 10 mL of PBS onto the sample to wash away red blood cells (RBCs) and other fluids. Discard fluids by pipetting without disturbing the specimen.
   d. Using surgical scissors and forceps, mechanically dissociate the tumor sample into a homogenous consistency. The majority of dissociated pieces should be <2 mm^3.
   e. Aliquot and snap freeze a small amount of tumor sample (~5%–10% of specimen or at least 3 mm^3) at −80°C for downstream genomic, transcriptomic, and/or proteomic analyses.
2. Enzymatic dissociation of tumor tissue
   a. Transfer the mechanically-dissociated tumor specimen into 15 mL pre-warmed PBS with Liberase (previously prepared). Incubate at 37°C for 15 min in an incubator-shaker (30 rpm). The amount of PBS with Liberase should be adjusted according to tumor size, ensuring that the amount of dissociation solution is at least double the tumor specimen volume.

b. After incubation, filter the tissue lysate through a 70 μm cell strainer into a new tube to separate and discard undigested tissue.
c. Centrifuge filtrate at 300 × *g* for 5 min. Remove supernatant carefully and resuspend pellet in 1 mL of PBS.

3. RBC lysis
   a. Add 4 - 12 mL of ammonium chloride solution and incubate for 5 min. This range is proportional to pellet size and infiltration of RBCs.
   b. After incubation, add an equivalent amount of PBS. Centrifuge at 300 × *g* for 5 min. Decant supernatant and resuspend pellet in 1 mL of pre-warmed NCC media.
4. Plating cells
   a. In a separate tube, add 10 μL of the cell suspension to 10 μL of Trypan Blue. Mix well and count cells using a hemocytometer, as per manufacturer's instructions.
   b. Transfer the cell suspension to either a 100 mm or a 60 mm ultra-low-binding tissue culture dish. If the total number of cells is < 1 million, plate cells in a 60 mm CELLSTAR® Cell-Repellent tissue culture dish with 3 mL of total NCC media. If the total number of cells exceeds 1 million, plate up to 2.5 million cells in a 100 mm CELLSTAR® Cell-Repellent tissue culture dish with 7–10 mL of total NCC media. Incubate at 37°C with 5% CO_2 for at least 48 h.
   c. When media changes color (red to orange) or 72 h have passed after plating cells, add 2 - 4 mL of NCC media to the cell culture dish. Change media completely when media color turns slightly yellow, as per instructions below.

Propagation and Dissociation of GSC Cultures

Timing: continuous

This step allows you to culture and dissociate patient-derived tumorspheres

5. Monitor the number of tumorspheres and their size over time for each GSC culture. If the number of tumorspheres and their size increases over time, the GSC culture may be cultured as spheres in low-binding cell culture dishes (see step 6). However, if the number of tumorspheres decreases over time or stays constant, the GSC culture may be grown on poly-L-ornithine and laminin coated tissue culture-treated dishes (see step 8).
6. Media replacement of tumorspheres
   a. Before you start, warm fresh NCC media to 37°C.
   b. Pipette cell culture to a sterile tube.
   c. Collect any remaining cells by rinsing the culture dish with 5–10 mL of PBS, and add it to the same tube.
   d. Centrifuge for 300 × *g* for 5 min and resuspend cell pellet in 1 mL of warm NCC media.
   e. Gently add the cell solution to the cell culture dish with additional media, as per manufacturer's recommended volumes. Cell culture dishes can be reused for up to 2 weeks.

7. Dissociation of tumorspheres
   a. Transfer cell culture to a sterile tube.
   b. Collect any remaining cells by rinsing the culture dish with 5–10 mL of PBS, and add it to the same tube.
   c. Centrifuge cell suspension at 300 × *g* for 5 min and resuspend cell pellet in 1 mL of PBS.
   d. Add 3–5 μL of Liberase (stock solution at 2.5 μg/μL in sterile water) and 5–10 μL DNase I (stock solution at 2000 U/mL in PBS) to the cell suspension. Incubate in a 37°C water bath for 5 min or less.

⚠ CRITICAL: If tumorspheres are still visible after incubation, incubate the cell solution for an additional 2–5 min. Do not exceed 15 min of total incubation time to avoid significant cell death.

e. After incubation, pipette up and down gently using a micropipette to aid complete cell dissociation.
f. Add 5–10 mL of PBS to the cell suspension. Centrifuge at 300 × *g* for 5 min. Remove supernatant and resuspend cell pellet in 500–1,000 μL of PBS for use in assays or in 1 mL of NCC media for replating.
g. For use in assays, filter the cell suspension into a 12 × 75 mm tubes with 35 μm cell strainer cap.
h. Determine cell number and viability using Trypan blue solution. Adjust cell count as required for specific assays or replating. For replating, cell density should be between 25%–50% as per dish size and depends on cell doubling time, cell size, and media consumption. Dissociated tumorspheres can be plated on tissue culture-treated or cell-repellent dishes, and should not be plated on poly-L-ornithine and laminin coated dishes (see step 8).

8. Plating tumorspheres on coated dishes
   a. Add poly-L-ornithine and sterile water in a 1:3 ratio to the dish, as required to completely coat the surface. Incubate at 37°C with 5% CO_2 for 1 h.
   b. Remove poly-L-ornithine solution and wash the culture dish with 2–5 mL of sterile water.
   c. Add PBS with 4 μg/mL laminin to the dish, as required to completely coat the surface. Incubate at 37°C with 5% CO_2 for up to 12 h. A longer laminin coating incubation period may improve cell adherence.
   d. Tumorsphere or dissociated adherent cells can be plated on coated dishes. Tumorspheres should not be dissociated prior to plating on coated dishes.
   e. Resuspend desired cell solution in warm NCC media. This media volume should equal 5%–10% of the cell culture dish's working volume.
   f. Remove laminin solution and wash the culture dish with sterile PBS immediately before plating cells. Do not allow the coated dish to dry.
   g. Add the cell solution to the coated dish in a drop-wise manner and place gently into an incubator. Incubate at 37°C for 5 min to 1 h.

⚠ CRITICAL: After plating tumorspheres and incubating cells for 5 min at 37°C and 5% CO_2, determine if the tumorspheres have become adherent by gently rocking the plate and observing movement of tumorspheres using a microscope. If tumorspheres have not adhered, incubate cells for up to 4 h 37°C and 5% CO_2. Increasing the amount of laminin while coating dishes may increase adherence of tumorspheres.

   h. If the tumorspheres are visibly adherent, top up media with an appropriate volume as per culture dish size.
   i. Subsequent media top up and replacement can be performed as required, taking care not to disturb the adherent cells.
   j. At 70%–80% confluency, cells can be dissociated by removing NCC media and adding an amount of TrypLE that is 5% of the total media volume. Incubate at 37°C for 5 min.
   k. Transfer dissociated cells to a sterile tube. Collect any remaining cells by rinsing the culture dish with 5–10 mL of PBS, and add it to the same tube. Follow appropriate instructions above for replating on coated or low-binding dishes, or for use in assays.

Intracranial Injection of GSCs into Mice

Timing: 2+ h

This step allows you to intracranially-inject patient-derived tumor cells into mice.

9. Preparation of GSCs
   a. Adherent GSCs should be grown as tumorsphere for 72–96 h prior to xenograft experiments (Figure 1).

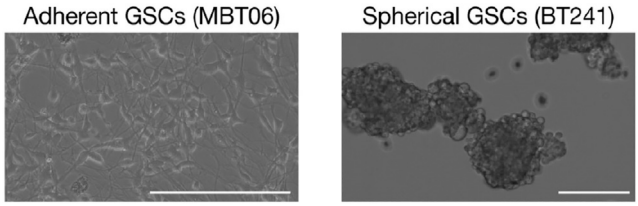

Figure 1. GSCs Grown in Adherent or Spherical Conditions

Microscopic images of GSCs grown in adherent (MBT06) or spherical (BT241) conditions. Scale bar represents 200 μm.

b. Dissociate tumorspheres and prepare a single cell solution of GSCs as detailed above. Resuspend GSCs in 1–2 mL of PBS. Count cells using Trypan Blue as described above.
c. The number of cells to be injected should be resuspended in PBS but the volumes should not exceed 10 μL per mouse. The number of injected cells can range from 50,000 to 1,000,000 cells, depending on cell line tumorigenicity and engraftment ability.
d. Cell concentrations are adjusted as desired in a volume of PBS between 5 and 10 μL. Store tubes on ice.

10. Preparation of mice for injection
    a. All *in vivo* procedures are performed according to Animal Research Ethics Board approved protocols. All injection procedures are performed aseptically in a BSL2 hood (Figure 2), with all equipment sterilized before placing it inside the hood.
    b. Set up the anesthetic machine as required. Place one mouse at a time in the induction chamber (Figure 3) until the mouse in unresponsive (determined by no response by lightly pinching the toes with tweezers). Mice are anesthetized using Isoflurane, induced at 5% and maintained at 2.5% for the surgical procedure (Figure 4A).
    c. Weigh and record each mouse prior to placing it on the injection platform.

11. Intracranial injection of GSCs
    a. Cut away hair approximately by a 1 cm × 1 cm above the right side of the frontal lobe to expose the skin region for incision (Figure 4B).
    b. Using three sterile cotton swabs, scrub the incision site with 10% iodine solution, followed by 70% isopropanol and finally 7.5% iodine solution. Using surgical scissors, cut a 0.5–1 cm opening along the middle of the disinfected area.
    c. Using scissors cut a 1 cm opening into the disinfected area.
    d. Using the Dremel drill with a fine tip for etching, avoiding visible veins and arteries, slowly apply pressure to the skull until the brain is reached, no further. The landmark is 2–3 mm anterior to coronal suture, 3 mm lateral to midline. Angle needle 30 degrees anterior to avoid hitting ventricle.
    e. Using a 23-gauge Hamilton syringe, draw up and expel 70% ethanol to clean the syringe, followed by a rinse with PBS to ensure remaining ethanol does not kills cells.
    f. Draw up an appropriate amount of cell suspension, as determined from your calculations beforehand. Prepare an additional 10% of cell suspension to account for pipetting error.
    g. Insert the needle tip no more than 2 mm into the brain through the drill hole and slowly inject the cells. Hold steady for 2 min to allow the pressure to dissipate within the brain before withdrawing the syringe at 2 mm/min. This will avoid backpressure causing the cells to come out of the brain.

⚠ CRITICAL: When injecting cells intracranially, inject slowly (about 0.5 μL/s) and hold steady for 2–5 s when fully expelled to allow cells to dissipate into the tissue. Removing the syringe too quickly will cause negative pressure causing the cells to escape out of the cavity. Slow injections will ensure more consistent engraftments among mice.

Figure 2. Surgical Area Setup

Surgery is performed in a BSL2 hood with adequate airflow and sterilization of all equipment before entering the surgical area.

h. Carefully suture the incision with a double knot of dissolvable vicryl stitches and apply a small dab of surgical glue to the knot to prevent mice from reopening the site.
i. Post-injection, using single use insulin syringes provide the mice with 1 mL of saline and painkiller according to site specific animal protocols.
j. Place mice back in their cage and provide them with a source of heat (heat pad or lamp) to aid in recovery.
k. Mice should be regularly monitored until they reach endpoint according to your experimental and institutional instructions. Endpoint typically consists of >10% loss in body weight, ruffled fur, and hunched back.

Mouse Specimen Collection and Processing

Timing: 15–30 min/mouse

This step allows you to collect patient-derived xenografts for downstream analysis.

12. Collection and processing for immunohistochemistry
    a. When mice reach endpoint, anesthetize the mice with intraperitoneal injection of 25 mg/mL Avertin (18 μL/g of mouse weight). Wait until mice are unresponsive to toe pinching.
    b. While the mouse is unresponsive, lay it on its back and tape each limb to the surface. With scissors, remove the rib cage and cut the diaphragm to expose the heart without rupturing the lungs or any major circulatory components near the heart.

Figure 3. Induction Chamber Setup
The induction chamber administers vaporized isoflurane to the mouse.

c. Inject 20 μL of Heparin sodium directly into the left ventricle of the heart to prevent blood clotting.
d. Administer 10–50 mL of saline using a 22 gauge needle into the left ventricle of the heart to flush blood from the circulatory system.
e. Inject 10 mL of 10% formalin using a 22 gauge needle into the heart to perfuse the body; limbs should almost immediately begin embalming.
f. Carefully harvest the brain with scissors and forceps, removing the skin and skull while keeping the brain as untouched as possible. Place the brain in a tube with 5 mL (or enough to submerge the brain) 10% formalin. Store for at least 48 h in a 4°C fridge.
g. After at least 48 h, place the brain in a coronal brain slicing matrix, slice the brain into 2 mm sections with razor blades, place each 2 mm section into a cassette (Figure 5A).
h. When all sections are cut, place the cassette into 50% ethanol. After 5 min, transfer the cassette into 70% ethanol. These sequential steps will progressively remove water from the specimens without rupturing cells or damaging tissue.
i. After at least 24 h of storage in the 70% ethanol, samples are ready to be sent for paraffin embedding and immunohistochemistry accordingly.
j. Tumor xenografts after successful hematoxylin and eosin (H&E) staining (Figure 5B) can be scanned, visualized, and quantified for tumor volume and further histological examination when needed.

EXPECTED OUTCOMES

Outcomes will vary depending on the cell line and number of cells injected per mouse (50,000 to 1,000,000 cells/mouse). Any additional protocols such as therapeutic regimens or genetic modifications to cell lines will need to be reviewed by researchers on a case by case basis. It is recommended each cell line be tested in a non-experimental manner to determine engraftment rates

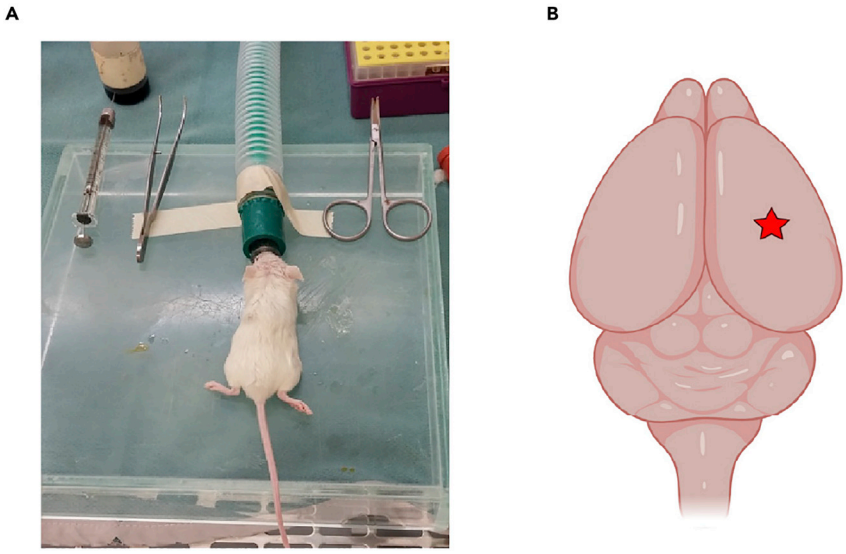

Figure 4. Mouse Positioning under Anesthesia and Surgical Site

(A) A mouse placed on a surgical platform is administered gaseous anesthesia via nose cone during the procedure.

(B) Overview angle of mouse brain; the injection site is marked by the star, 2 mm into the right frontal lobe.

and time until endpoint prior to initiating larger more valuable experiments to determine critical timepoints.

LIMITATIONS

Human-mouse brain tumor xenografts provide valuable platforms to conduct brain tumor research but for a variety of reasons, limitations are inevitable. Depending on the brain tumor type and specific cell line being examined, variation will occur. The mouse species utilized will also play a critical role in how results should be interpreted for a variety of reasons, particularly the immune system of the host. A mouse with an intact immune system will experience graft-versus-host disease, obscuring the role of immune systems in oncology. On the other hand, if mice lacking an immune system are used, a major component of the tumor microenvironment is not present.

TROUBLESHOOTING

Problem

Extensive death of tumor cells during tumorsphere dissociation.

Possible Solution

Incubating tumorspheres for a prolonged time in the presence of Liberase can lead to excessive cell death and reduce the overall health of the population. With this in mind, we recommend that incubation time of tumorspheres with Liberase should not exceed 5–7 min. Alternatively, a lower amount of Liberase can be used if a longer incubation is necessary. Please note that this does not apply to patient tissue specimen dissociation using Liberase.

Problem

Dissociated cells begin to clump together after enzymatic dissociation with Liberase.

Possible Solution

Variability in Liberase sensitivity is observed among different patient-derived tumor cell lines. Whereas some tumor cell lines are stable as single cells post-dissociation, some dissociated tumor

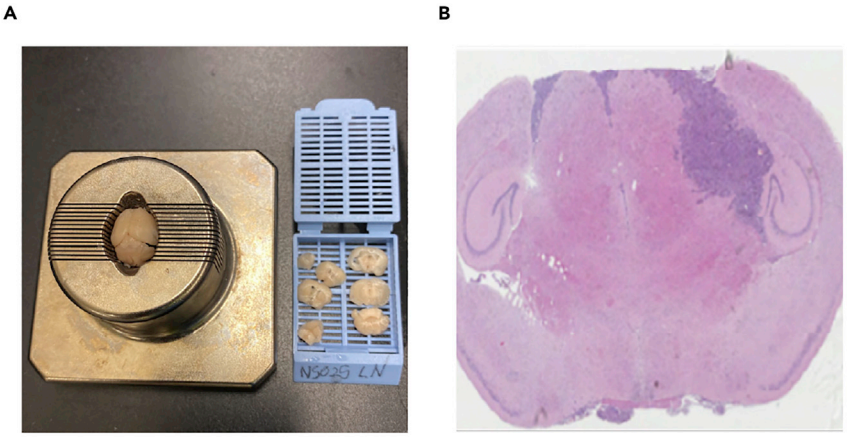

Figure 5. Mouse Brain Histology

(A) Formalin-fixed mouse brain in a coronal brain slicer matrix (left) and 2-mm-thick brain slices in tissue embedding cassettes (right).

(B) Successful H&E staining of a human-mouse glioblastoma xenograft. Tumor cells (purple) appear infiltrative into healthy brain tissue (pink), indicative of GBM's invasive morphology.

cells readily clump together and cause issues during downstream assays. As such, adding additional DNase to the tumorsphere dissociation step is encouraged for such samples.

Problem

Tumorspheres are still visible after incubation.

Possible Solution

Incubate the cell solution for an additional 2–5 min. Do not exceed 15 min of total incubation time to avoid significant cell death.

Problem

Tumorspheres are not adherent after plating.

Possible Solution

After plating tumorspheres and incubating cells for 5 min at 37°C and 5% CO_2, determine if the tumorspheres have become adherent by gently rocking the plate and observing movement of tumorspheres using a microscope. If tumorspheres have not adhered, incubate cells for up to 4 h 37°C and 5% CO_2. Increasing the amount of laminin while coating dishes may increase adherence of tumorspheres.

Problem

Inconsistent engraftment in mice.

Possible Solution

When injecting cells intracranially, inject slowly (about 0.5 μL/s) and hold steady for 2–5 s when fully expelled to allow cells to dissipate into the tissue. Removing the syringe too quickly will cause negative pressure causing the cells to escape out of the cavity. Slow injections will ensure more consistent engraftments among mice.

RESOURCE AVAILABILITY

Lead Contact

Dr. Sheila K. Singh, Professor, Department of Surgery, Division of Neurosurgery, Faculty of Health Sciences, Email: ssingh@mcmaster.ca

MATERIALS AVAILABILITY

Requests for cell lines and information of all other pertinent information can be directed toward Dr. Sheila K. Singh.

Data and Code Availability

No exclusive data or code was used or generated for this method.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

C.C. drafted the protocol for tissue dissociation, culturing, and propagating cell lines, and helped with other materials associated with the protocol. N.S. drafted the protocol for intracranial injections and specimen collection, and helped with all materials associated with the protocol. C.V. and S.K.S. contributed to integration of the writing sections and final edits.

DECLARATION OF INTERESTS

Dr. Sheila K. Singh is a scientific advisor for Century Therapeutics Inc. and her role in the company has been reviewed and is supported by McMaster University.

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