Establishment of Stereotactic Orthotopic Brain Tumor Xenografts in Mice
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Abstract
The development of reliable, clinically relevant in vivo tumor model systems for central nervous system neoplasms is of paramount importance in the advancement of basic and translational research. Discrepancies between preclinical and clinical results of anticancer therapeutic agents demand reliable research platforms that can precisely recapitulate the behavior of such cancers. One approach that has gained great traction in neuro-oncological research is that of primary xenografts, whereby patient-derived tumors are dissociated and maintained by subsequent serial passaging prior to intracranial injection within animal lines. Herein, we describe our experiences in the development of murine models for glioblastoma (GBM), atypical teratoid rhabdoid tumor (ATRT), and medulloblastoma. This technique is a general method to introduce neoplasms through intracranial stereotactic xenograft injections in mice.

Introduction
The use of transplantation models utilizing human central nervous system malignancies has served an essential role in neuro-oncology research for decades. Prior to the advent of intracranial stereotactic injection of xenograft tissue, the most prevalent techniques to study tumorigenesis and drug development were the xenografting of human cell lines injected subcutaneously into immunocompromised mice. However, given the limitation that such subcutaneous xenografts fail to model tumors in their native tissue of origin and microenvironment, newer engraftment methods where human tumor cells are directly injected into their relevant structure mirroring behavior of the primary tumor have gained significant popularity.

The method of orthotopic xenograft modeling, whereby primary cells derived from human tumors are injected into the corresponding structure of animal brains, promotes enhanced interactions between injected tumor cells with the surrounding microenvironment to more properly recapitulate human tumor biology. The interaction of such cells with the surrounding environment of inhabitant cells, stroma, and vasculature cells has been well-documented to impact cell survival, migration, and differentiation [1-3]. The creation of such stringent models to replicate the salient features of disease is of substantial importance in the study and preclinical development of novel therapies for central nervous system neoplasms, such as glioblastoma, where current measures of surgical resection and chemoradiotherapy fall short.

Our research was overseen and protocols approved by Stanford University’s Institutional Review Board and Administrative Panel on Laboratory Animal Care (APLAC-26548). Human tissue samples were obtained from consenting patients per IRB-18672.

Technical Report
Reagents

Tumor Dissociation:
Hank’s balanced salt solution without calcium, magnesium (Cellgro)
Hank’s balanced salt solution with calcium, magnesium, and phenol red (Cellgro)
Collagenase IV (Worthington Biochemical Co.)
DNAse I (Worthington Biochemical Co.)
ACK lysing buffer (Gibco)

Injection:
Anesthetic agent (i.e., isoflurane)
Analgesic (buprenorphine)
Sterile ocular lubricant (Lacri-lube vet ointment)
Sterile phosphate-buffered saline (Cellgro)
Sterile water
Acetone
70% ethanol
Betadine ointment
Hydrogen peroxide
Bone wax (Ethicon Co.)
1% bovine serum albumin (IgG-free) in phosphate-buffered saline

**Equipment**

*Tumor Dissociation:*

Cell strainers, 40 pM and 100 pM (BD Falcon)
Serological pipettes, 10 mL (BD Falcon)
Conical tubes, 50 mL (Genemate)
Petri dishes (BD Falcon)
Nutator mixer (Model 15172-205, BD Diagnostics)

*Injection:*

Oxygen tank and apparatus
Syringes, 1 cc
Syringes, 10 microliter (Model 1701, Hamilton Co.)
Needles, 26G, 2" (Hamilton Co.)
Sterile alcohol wipes
Sterile cotton swabs
Surgical instruments
Disposable scalpels, #15 (BD Bard-Parker)
Suture, 5-0 (Ethicon Co.)
Clip applier with 7 mm clips (Model 9974, Alzet Co.)
Clip remover (Model 9976, Alzet Co.)
Small animal stereotaxic frame (Stoelting Co.)
Electric razor/clipper (Wahl Co.)
Electric microdrill (Model 18000-17, Fine Science Tools)
Microdrill bits (Model 19008-07, Fine Science Tools)
Automated injector (i.e., WPI Micro4 microsyringe pump controller)
Heating lamp or heating pad (with thermometer)
Murine brain atlas (for stereotactic planning purposes)
Immunocompromised mice (i.e., NOD scid gamma/NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, Jackson Labs)

**Procedure**

Herein, we describe our experiences with the orthotopic, intracranial injection of human tumor lines in murine models, from receipt of gross tumor specimen from the operating room to confirmation of orthotopic tumor engraftment by bioluminescence imaging.
All procedures as delineated below involving cell injection and postoperative care were reviewed and approved in full by our institutional IACUC committee.

Prior to the performance of any procedures, technicians should ensure the surgical area is clean and organized with all necessary tools readily available. Tools should be sterilized prior to use.

**Tumor Dissociation:**

Dissociate tumor cells maintained prior to intracranial injection to ensure continued patency of injection syringe and to ensure successful engraftment. In our experience, the number of cells to be prepped for injection varies on a case-to-case basis across tumor types and subgroups, from counts as low as $3.0 \times 10^4$ to as great as $5.0 \times 10^5$. While a variety of dissociation protocols exist within the literature, our experience is documented as below.

a. Plate tumor sample into a petri dish, suspend in Hank’s buffered salt solution (HBSS) with calcium and magnesium, and mince tissue with aid of sterile scalpel into fine particulate matter. Add 1:50 collagenase IV (1 mg/mL) and 1:100 DNAse I (250 units/mL) to solution to aid in dissociation.

b. Transfer solution to 50 mL conical tube and place on nutator for 30 minutes at 37°C, 5% CO2.

c. Following incubation, continue to mechanically dissociate tumor with use of 10 mL serological pipette in conical tube. Continue this step until solution is well-homogenized.

d. Once completed, filter contents through 100 uM and 40 uM cell strainers, respectively.

e. Centrifuge sample at 300g for 10 minutes.

**NOTE:** Centrifuge should be set to run at maximum acceleration/deceleration at room temperature (20°C).

f. Wash cells with HBSS (without calcium and magnesium), recentrifuge at same settings, and aspirate supernatant. Perform this step twice.

g. After removing supernatant, add 30 mL of 0.9 M sucrose buffer and resuspend pellet gently.

h. Centrifuge solution at 700g for 10 minutes.

**NOTE:** Change centrifuge settings to zero deceleration to allow for sufficient separation of pellet contents.

i. Aspirate supernatant carefully and add 5 mL of ACK lysing buffer (0.15 M NH4Cl, 1.0 mM KHCO3, 0.1 mM Na2-EDTA) and incubate on ice for five minutes.
j. Fill conical tube to 50 mL with HBSS (without calcium and magnesium) and centrifuge at 300g for 10 minutes (same settings as step e above).

k. If pellet remains red, repeat ACK lysis step as above.

NOTE: A portion of cells dissociated should be saved for the protocol as described below. The remainder of the cells may be plated for neurosphere formation in tumor-stem media consisting of neurobasal media (-A, Invitrogen), E27 (-A, Gibco), human FGF (20 ng/mL, Shenandoah Biotech), human EGF (20 ng/mL, Shenandoah Biotech), and heparin (10 ng/mL).

**Preparation of Cells:**

a. Following dissociation, the viability of cells should be validated by Trypan blue staining.

b. Wash dissociated cells with sterile phosphate-buffered saline (PBS) and resuspend in solution of 1% bovine serum albumin (IgG-free) in PBS. Viable cells should be kept on ice prior to being loaded for subsequent intracranial injection.

NOTE: Other published protocols recommend the use of Matrigel to aid in the engraftment of transplanted cells. In our experience, we do not recommend such use, as the substance seems to restrict cell viability.

**Equipment and Specimen Setup:**

a. Assemble and calibrate small animal stereotaxic frame (Stoelting Co., Wooddale, IL) per manufacturer’s instructions to ensure most accurate intracranial engraftment of cells, dependent upon the particular region of interest targeted. Minor inaccuracies in calibration may result in unnecessary murine morbidity and mortality given the proximity of elegant brain structures to regions targeted for injection.

b. Heating pad or lamp necessary for post-procedural care must also be turned on with continued monitoring of temperature with thermometer.

c. Program automated injector with regard to syringe size and desired rate of injection.

NOTE: We recommend a slow rate of infusion (i.e., 3 microliters over two to four minutes).

d. Ensure sufficient oxygen and isoflurane anesthetic and appropriate flow of agents to stereotaxic frame setup with suitable tubing.

**Surgical Procedure:**

- **Preoperative Animal Preparation**

a. Anesthetize mouse by isoflurane gas (induction: 2%-4%, maintenance: 1%-2%) through use of induction chamber or alternative means (i.e., ketamine/xylazine 100 mg/kg and 5 mg/kg intraperitoneally).

b. Place a lubricating ophthalmic ointment (i.e., Lacri-lube) in the mouse’s eyes to prevent drying of the cornea.

c. Remove hair from surgical site at dorsum of murine skull with electric clipper or razor. Depilatory cream should be avoided, as it can result in burns. Collect loose hair with adhesive tape to minimize risk of surgical site infection.

d. Disinfect surgical site by alternating between cotton tipped swabs soaked in Betadine (or similar surgical scrub) for a minimum of three cycles.

e. Position anesthetized mouse appropriately in stereotactic apparatus. Nose and teeth should be appropriately positioned to ensure adequate flow of oxygen and anesthetic agent during procedure. Once the mouse in the correct position with occiput of head level, tighten ear holders firmly.
FIGURE 2: Appropriate positioning of mouse within stereotaxic frame.

NOTE: Ensuring the animal skull is as flat as possible in the dorsoventral plane is essential to ensure accurate accuracy of injection sites and to minimize animal morbidity and mortality.

- Preoperative Cell Preparation
  
a. Clean Hamilton syringes and needles (26G) by rinsing in 70% ethanol, sterile water, and acetone in a sequential fashion prior to installing onto stereotactic frame.

b. Gently resuspend cells in microcentrifuge tube with pipette 5-10 times prior to each injection to prevent cell clumping.

c. Draw 3-5 microliters of cells into Hamilton syringe with great care to avoid aspiration of bubbles. Confirmation of successful loading into syringe is critical, as variance in volumes injected between animals can lead to significant variability in bioluminescent imaging and time-to-event results.

NOTE: Reload cells for each individual injection between procedures to prevent clumping and ensure a consistent number of cells injected across animal cohort.

NOTE: We recommend use of a cell concentration approximating 5.0x10^4-7.5x10^4 cells/microliter. Too dilute of a suspension prevents successful engraftment, and excessively concentrated cells become excessively clumpy and often clog syringes.
d. Following loading, clean exterior of syringe needle with alcohol swab to wipe clean any adherent cells that might promote extracranial tumor seeding.

- Procedural Care

a. Make a 1.0-1.5 centimeter midline sagittal incision with a sterile disposable scalpel along the superior aspect of the cranium from anterior to posterior, reflecting skin downwards to external auditory meati.

b. If field is bloody, apply one to two drops of hydrogen peroxide to surgical site to improve visualization of landmarks.

c. Identify bregma (conjunction of coronal and sagittal sutures) anteriorly and lambda (conjunction of sagittal and lambdoidal sutures) posteriorly to serve as landmarks for stereotactic localization prior to injection.

![Exposed mouse skull with (A) bregma and (B) lambda landmarks used to orient site of injection intraprocedurally.](image)

**FIGURE 3:** Exposed mouse skull with (A) bregma and (B) lambda landmarks used to orient site of injection intraprocedurally.

**Injection of Cells:**

a. Using microdrill, make a small burr hole with sterile drill bit at predetermined coordinates with care to remain superficial in order to avoid traumatic injury to brain.

b. Insert 10 microliter syringe (Hamilton Co., Reno, NV) snugly into drilled burr hole, maintain needle perpendicular to skull, and slowly advance needle prior to injecting cells.

NOTE: In our experiences, for supratentorial injections (i.e., glioblastoma), coordinates of 2 millimeters posterior to the bregma, 1 millimeter laterally, and 2 millimeters deep to the dura ensure highest rates of successful engraftment. For infratentorial injections (i.e., medulloblastoma), coordinates used are 2 millimeters posterior to lambda in a midline fashion 2 millimeters deep. Ensure appropriate angle of needle insertion at all times to prevent intraventricular injection of cells and damage to critical brain structures.

c. During injection, repeatedly dry the skull with sterile swabs to remove any cell suspension that may have refluxed out of the burr hole.

d. Keep syringe in place for one to two minutes before slowly withdrawing upon completion of injection.

e. Plug site of burr hole with bone wax, and close skin with 5-0 suture or clips.

f. Following removal of animal from stereotaxic device, immediately clean syringes and injection equipment to ensure patency of needle for future use.
Postoperative Care and Follow-up:

a. Following injection and closure of surgical site, reapply Betadine to wound.
b. Transfer anesthetized mouse to heating pad set to 37 °C or heating lamp with attention to maintenance of appropriate temperature of pad to avoid instances of hypo- or hyperthermia. Keep mouse positioned on its back to prevent asphyxiation as it recovers from anesthesia.
c. Observe mouse to ensure recovery from anesthesia. Once recovered, mice may be transferred to their respective cages.

Consider administration of post-procedural analgesic per institutional APLAC protocols and dosing regimens (i.e., buprenorphine, carprofen).

Special considerations

Pup Injections:

The protocol as delineated above is specific to the injection of adult murine animals. However, adjustments may be made to the protocol to study the impact of orthotopic xenografts in the setting of younger animal models. To best model slow growing childhood brain tumors (i.e., medulloblastoma and diffuse intrinsic pontine glioma), P1 to P5 old mice pups are usually used. Anesthesia is achieved by cooling the internal body temperature of the pups by wrapping them in napkin and placing them on ice for two to five minutes. Prior to injecting tumor cells in locations consistent with the origin of primary tumors, pups are positioned on the stereotaxic frame accordingly to acquire the best setting for proper coordinates. In our experience, we inject DIPG and MB cells 1 millimeter posterior to lambda and 3 millimeters deep in a midline fashion. The needle in itself pierces the skull and dura, replacing the need for any incision to be made. Once the needle is maneuvered into the appropriate position we recommend a cell infusion rate of 3 microliters over two to four minutes, identical to the protocol as delineated above. Following completion, pups should be kept warm under a heating lamp for several minutes until the resumption of spontaneous activity. At that point, they may be returned to their mother until weaning time.

In some cases of in vivo experiments on pups, it is recommended that parental mice are anesthetized prior to the manipulation of their offspring to prevent cannibalization; however, in our experience, this has not proved to be a concern. As recommended, injected pups are monitored individually for any potential post-procedural symptoms and complications. 1,000-3,000 MB cells and 50,000-80,000 DIPG cells tend to give rise to tumors in two months and six months, respectively.

Termination of Animal Studies:

Endpoints for such procedures with a potentially high degree of morbidity and mortality should be predetermined. In the setting of undue morbidity and mortality, performing humane euthanasia to terminate an animal may be appropriate, as preemptive euthanasia of moribund animals can prevent further pain and distress. Conditions that may suggest need for termination in our experience include, but are not necessarily limited to, any injury interfering with eating and drinking (i.e., inability to ambulate following injection), evidence of marked dehydration, inability to maintain an upright position, and unresponsiveness to noxious external stimuli (i.e., toe-pinch withdrawal test) [4-5].

Bioluminescence Imaging:

The use of bioluminescence imaging using luciferase-expressing tumor cells has been extensively documented [6-8]. Prior to orthotopic injection, cells are transduced with GFP and luciferase-encoding lentivirus. Specifically, the pCDH-CMV-MCS-EF1-puro HIV-based lentiviral vector (System Biosciences) contains a ubiquitin promoter driving the expression of the luciferase-eGFP fusion product. Viral content integration with the host genome enables the cells to be monitored through bioluminescence monitoring software, such as the Xenogen IVIS 100 imaging system (Caliper Life Sciences, Hopkinton, MA). Following intraperitoneal injection of 150 mg/kg of D-luciferin, mice are anesthetized with 2% to 4% isoflurane (Baxter Inc., Deerfield, IL). Serial images are taken on a weekly basis using identical imaging settings to measure total flux values obtained from the anatomic region of interest. Bioluminescence intensity is quantified using Living Image 4.0 software (Caliper Life Sciences, Hopkinton, MA).
FIGURE 4: Bioluminescence imaging following intraperitoneal injection of D-luciferin demonstrating tumor engraftment in mice injected with medulloblastoma. Note spine metastasis in animal 1.

Discussion
In the setting of the central nervous system neoplasia, with a particularly complex associated inherent environment of cells, stroma, and vasculature, an orthotopic stereotactic model of engraftment is of particular importance in the in vivo study of systemic function and dysregulation. Human glioblastoma and other tumor xenografts have been shown to retain many of the genetic alterations present in the original parent tumor [9-11].
Figure 5: Histologic H&E images of (A) ATRT, (B) medulloblastoma, (C) U87 glioma cell line, and (D) primary GBM samples successfully engrafted orthotopically in NOD scid gamma murine model.

Tumor microenvironments are well-known to dictate the biological behavior of xenograft tumors [1, 3]. Antunes, et al. demonstrated such an impact when they showed that the characteristic infiltrative pattern of GBM was frequently attenuated when the graft was established in murine flanks [12]. As such, orthotopic brain tumor models, as the one described above, may prove to be of greater relevance in the study of tumor biology than conventional subcutaneous xenografts and other heterotopic approaches.

The techniques delineated above are specific to neuro-oncologic efforts but may be extrapolated for use in the modeling of other human cancers. There have been numerous efforts documented within recent literature on the creation of xenograft tumors derived from patient cancer cells to serve as surrogates for therapeutic purposes [2, 9, 13-18]. Our model, which is orthotopic in nature, may increase the validity of our preclinical platform. However, caveats with use of such a model do exist, as differences exist between the murine brain microenvironment in comparison to their human counterparts. For example, human tumors grow preferentially in media containing human specific growth factors, and the lack of these factors may limit full growth potential in the mouse brain.

While proper technique and experimentation with individual protocols is essential for engraftment success, verified by subsequent bioluminescence imaging or histopathologic analysis, a number of other factors must be actively considered that are likely to influence the frequency of successful xenograft establishment. These considerations, particularly when dealing with primary human tissue versus established cell lines, include the age and strain of immunodeficient mice being used [19-20]. In our experience, immunocompromised mouse strains (i.e., NOD scid gamma) six to eight weeks serve as optimal hosts for engraftment, with decreasing rates of success with increasing age. The NOD scid gamma (NSG) murine strain we choose to utilize (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, Jackson Labs) is deficient in mature lymphocytes and NK cells due to homozygous mutations in the common gamma chain of IL-2R. Severe combined immunodeficiency (SCID), X-linked in humans, is deficiency in the gamma chain of IL-2R blocks NK cell development and results in additional defects innate immunity in comparison to other immunocompromised murine strains that have been extensively studied, such as NOD-scid and NOD-Rag1 [21-22]. Also meriting consideration, given anecdotal observations that in vitro growth rates of particular cells correlate directly with time between injection and onset of neurological symptoms ranging from two weeks to six months, care should be taken to select cells that show evidence of stable growth and likely engraftment.

Our model of intracranial brain tumor xenografting relies upon a small animal stereotactic frame to ensure consistent, reliable injection location but at the expense of procedural time. Procedural time should be taken into account, particularly when injecting large cohorts of animals. In our experience, two experienced technicians can inject approximately 15-20 mice per hour whereas previously cited alternatives for intracranial tumor cell implantation, such as that of an unassisted free-hand approach, is more expedient with a trade-off of diminished surgical precision [23]. Another limitation of our model is that given such intracranial xenograft tumor volumes cannot be directly monitored with calipers or upon external assessment, monitoring of engraftment and growth relies upon the use of noninvasive imaging, such as bioluminescence imaging as aforementioned. Magnetic resonance imaging and positron emission tomography are also viable alternative, but neither approach rivals bioluminescent imaging in terms of cost effectiveness and speed.

Conclusions

The establishment of an intracranial brain tumor xenograft model provides an appropriate environment for modeling the behavior of many central nervous system malignancies in addition to possible therapeutic agents prior to advancement to human clinical trials [24-25]. The techniques outlined within this text to stereotactically implant tumor cells in an orthotopic fashion reliably generate in vivo models that reasonably recapitulate the behavior of GBM, medulloblastoma, and other central nervous system malignancies. Such techniques are generally reproducible by many translational laboratories for characterization of tumor growth, response to anticancer agents, and for related endeavors with a low degree of associated animal morbidity and mortality.

Additional Information

Disclosures

Human subjects: Consent was obtained by all participants in this study. Stanford University's Institutional Review Board issued approval # IRB-18672. Human tissue samples were obtained from consenting patients.
per IRB-18672. Animal subjects: Stanford University’s Administrative Panel on Laboratory Animal Care issued protocol number # APLAC-26548. Conflicts of interest: In compliance with the ICMJE uniform disclosure form, all authors declare the following: Payment/services info: All authors have declared that no financial support was received from any organization for the submitted work. Financial relationships: All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. Other relationships: All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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