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TITLE:

Cisterna Magna Injection Approach for the Treatment of Leptomeningeal Metastatic Brain Tumors

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KEYWORDS:

brain tumor, leptomeningeal metastases, medulloblastoma, CSF, cisternal magna

SUMMARY:

This protocol presents a straightforward procedure that enables direct intrathecal delivery of agents to cerebrospinal fluid without operative damage to the skull or brain parenchyma. This approach provides a solution to deliver molecules that could not otherwise cross the blood-brain barrier or blood-cerebrospinal fluid barrier to treat leptomeningeal metastatic disease.

ABSTRACT:

Leptomeningeal metastases (LM) in the setting of pediatric brain tumors are associated with particularly poor prognosis. Current therapies for pediatric brain tumors include surgery, radiation, and chemotherapy. However, LM are not amenable to surgical resection and frequently do not respond to radiation. Moreover, most chemotherapeutic drugs are unable to cross the blood-brain barrier or blood-cerebrospinal fluid (CSF) barrier to reach LM. Here, we describe a mouse model of pediatric brain tumors that develop LM and an easy, reproducible approach to deliver therapeutic agents to treat LM. We intrathecally injected a CSF tracer via the cisterna magna in mice. After a single injection, the CSF tracer influx was observed along the leptomeninges throughout the entire central nervous system and persisted for at least 6 hours. Daily injection did not result in apparent toxicity or impact animal weight. The results of this study highlight the utility of a mouse model of metastatic pediatric brain tumors as well as the potential application of a repeated intrathecal injection technique to deliver therapeutic agents to treat metastatic brain tumors.

INTRODUCTION:

Leptomeningeal metastases (LM) are common in patients with advanced brain cancer[1-3], breast cancer[4,5], lung cancer[6], melanoma[7,8], and leukemia[9]. The presence of LM uniformly confers a poor

prognosis and negatively impacts patient quality of life. Because meaningful surgical resection is impossible due to the diffuse nature of LM, the standard of care for leptomeningeal metastatic disease involves radiation and chemotherapy[10]. However, extensive irradiation of the entire central nervous system often causes severe treatment-related side effects, particularly for young patients. Moreover, many chemotherapeutic drugs cannot readily cross the blood-brain barrier and blood-cerebrospinal fluid (CSF) barrier, limiting their distribution to LM upon intraperitoneal, intravenous or oral administration[11]. For drugs that are capable of permeating these barriers, achieving effective pharmacological concentrations through simple diffusion from the blood to the LM remains a challenge[12,13]. Intrathecal injection, which allows direct drug delivery to the CSF, can circumvent these barriers and is widely used for the treatment of LM[14]. Intrathecal injection can be performed through either lumbar puncture or implantation of catheters/cannulas into the ventricles or cisterna magna[15-18]. However, the surgery that is required to perform these procedures in preclinical models is inconvenient and may cause undue stress to animals[16,18-22]. These procedures also carry the risk of post-operative injection injury[16,23]. Thus, the goal of this study was to develop a non-invasive approach to repeatedly and accurately deliver agents directly to the CSF to facilitate the treatment of LM in preclinical mouse models.

Medulloblastoma, one of the most common malignant pediatric brain tumors, is a significant cause of childhood morbidity and mortality[24,25]. Approximately one-third of patients with malignant medulloblastoma present with metastases and nearly all have metastatic disease at the relapse[26,27]. Medulloblastoma metastases are found almost exclusively along the leptomeninges on the surface of the brain and spinal cord[5,28,29]. Therefore, intrathecal drug delivery shows promise as an effective technique for the treatment of metastatic medulloblastoma. Here, we describe a protocol to generate a metastatic medulloblastoma mouse model as well as a simple and reliable intrathecal injection approach to deliver agents to the CSF in mice. Because surgery is not required, this approach is less invasive than other methods and allows animals to recover more rapidly.

PROTOCOL:

All animal procedures were performed according to the NIH guidelines and were approved by the Research Animal Facility Committee, Children's Research Institute (Protocol #30351).

1. Generation of a mouse model of metastatic medulloblastoma

NOTE: Because this is a recovery surgery, procedures should be performed under sterile conditions. All surgical tools should be sterilized by autoclaving or with a hot bead sterilizer.

1.1 Stereotactic implantation of medulloblastoma tumor cells

1.1.1 Transfect tumor cells with mCherry and luciferase reporters. To transfect cells, culture 2 x 10^5 patient-driven xenograft medulloblastoma cells in a 24 well plate with 360 µL of neural stem cell medium and proliferation supplement. Add 40 µL of lentivirus encoding both mCherry and luciferase in one vector to the culture medium without polybrene, and culture overnight at 37 ˚C.

1.1.2 After the overnight culture, collect cells into a microcentrifuge tube by pipetting.

1.1.3 Centrifuge cells at 220 x *g* for 5 min at room temperature. Remove the supernatant carefully with a micropipette and discard the supernatant into a 5% bleach solution.

1.1.4 Wash once by resuspending the pelleted cells in 500 µL of fresh neural stem cell medium and centrifuging at 220 x *g* for 5 min at room temperature.

1.1.5 Remove the supernatant and resuspend cells in 16 µL of neural stem cell medium. Keep the tube on ice for the duration of the transplantation procedure.

1.1.6 Anesthetize an immunodeficient mouse (4-6 weeks old) by placing the animal in an isoflurane induction chamber (3% isoflurane, 97% air, 250 mL/min) until the toe pinch reflex ceases.

1.1.7 Load the mouse in the prone position on a stereotactic frame over a heating pad connected to an anesthesia gas mask to keep the mouse under 1.5-2% isoflurane anesthesia.

1.1.8 Disinfect the head of the mouse with 70% ethanol and apply non-antibiotic ophthalmic ointment to keep the eyes moist while the mouse is anesthetized.

1.1.9 Using a sterile #10 scalpel, make an approximately 12 mm incision in the skin at the midline of the scalp over the position of the cerebellum. Expose the skull by pushing aside the muscle tissue over the lambdoid suture with sterile fine forceps.

1.1.10 Determine the coordinates for injection into the cerebellum using a mouse brain atlas (1 mm lateral to the midline). Using the beveled end (sharp point) of a sterile 18 G needle to puncture the skull and create a small hole with a diameter of approximately 0.5 mm in the skull. Use a dissection microscope to visualize the injection site.

1.1.11 Load cells (5×10^4 in 4 µL of neural stem cell media) into a 5 µL microliter syringe with an unbeveled 24 G needle. Mount the syringe on a micromanipulator and introduce the needle through the hole in the skull into the brain at a depth of 2.2 mm.

1.1.12 Inject cells using an electronic pump at a flow rate of 3 µL/min. After injection, leave the needle in place for 1 min to avoid the backflow of the cells or fluid.

1.1.13 Withdraw the needle slowly and remove the mouse from the frame.

1.1.14 Close the skin with tissue adhesive or sutures.

1.1.15 Place the mouse in a new cage containing bedding. Place the cage under a heat lamp to keep the animal warm. Repeat steps 1.1.6 -1.1.15 for each mouse.

1.1.16 Monitor the animals until they have completely recovered from anesthesia and are fully mobile (approximately 2-3 min). Continue to monitor these animals for an additional 30 min post-operatively.

1.1.17 Administer 0.05-0.1 mg/kg buprenorphine subcutaneously as postoperative analgesia. Continue to administer buprenorphine every 12 h for 48 h after the procedure to manage any pain from the injection.

NOTE: Guidelines for postoperative pain management vary between institutions. Please consult with the institutional guidelines to ensure proper pain management.

1.1.18 Monitor the tumor growth once a week using a bioluminescent imaging system.

1.2 Irradiation of tumor-bearing mice

1.2.1 Irradiate the tumor-bearing animals when the luciferase signals reach ~1 x 10^6-1 x 10^7 radiance per second.

NOTE: The length of time for animals to develop tumors depends on the number of tumor cells, but typically animals develop tumors within 3-4 weeks.

1.2.2 Pre-warm the irradiator following the manufacturer's instructions.

1.2.3 Restrain the mouse in a lead shielding without anesthesia. The shielding is composed of two lead boxes with a narrow open space between, allowing radiation exposure to only the brain and spine while shielding the rest of the body.

1.2.4 Place the restrained mouse into the irradiator.

1.2.5 Irradiate the mouse with 2 Gy per dose at a rate of approximately 120 rads/min.

1.2.6 After irradiation, return the mouse to its original cage.

1.2.7 Repeat irradiation every other day for a total cumulative dose of up to 18 Gy.

1.2.8 Image mice once per week with a bioluminescent imaging system to monitor the development of metastases. Metastases typically develop within 3-4 weeks following the first dose of radiation.

1.3 Identifying metastases

1.3.1 Perfuse mice intracardially with 0.01 M phosphate buffer saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.01 M PBS at a rate of 5 mL/min.

1.3.2 Use fine forceps to remove the brain and spinal cord and place in cold PBS.

1.3.3 Use a fluorescence microscope to examine the location of $mCherry^+$ tumor cells and acquire images.

1.3.4 Fix the tissues in 4% PFA at 4 °C overnight. Then dehydrate the tissues with 25% sucrose overnight and embed in tissue embedding medium. Freeze blocks in a dry ice bath with 100% ethanol and store embedded tissue at -80 °C.

1.3.5 Use a cryostat to cut 12 µm sections of brain and spinal cord.

1.3.6 Stain the sections with 4′,6-diamidino-2-phenylindole (DAPI). Examine the metastatic tumor cells in each section under a fluorescence microscope and acquire images.

2 Intrathecal injection

NOTE: Because this is a recovery surgery, procedures should be performed under sterile conditions.

2.1 Intrathecal injection of CSF tracer

2.1.1 Prepare a 10 µL Neuros syringe by attaching a 33 G beveled needle and stopper. Set the stopper to 3 mm and wash the needle 10 times with sterile deionized water, and then 10 times with sterile PBS.

2.1.2 Anesthetize a mouse (4-6 weeks old) by placing the animal in an isoflurane induction chamber (2.5-3% isoflurane, 97% air, 250 mL/min) until the toe pinch reflex ceases.

2.1.3 Load the mouse in the prone position on a stereotactic frame over a heating pad connected to an anesthesia gas mask to keep the mouse under 1.5–2% isoflurane anesthesia.

2.1.4 Restrain the head of the mouse between the two ear bars. Use a tooth bar to align and support the front of the head.

2.1.5 Adjust the height of the ear bars and the tooth bar to make sure the line connecting the most prominent aspects of the cranium and the spina forms an angle of 15° with the horizontal line. In this position, the cisternal magna is nearly the highest point of the mouse body.

2.1.6 Shave the head and neck of the mouse, remove fur, disinfect the head with alcohol scrubs, and apply sterile ophthalmic ointment to keep the eyes moist during anesthesia.

2.1.7 Prepare CSF tracer by dissolving fluorescein isothiocyanate (FITC)-dextran-500 in artificial CSF (aCSF) at a concentration of 0.25% w/v. Load 3 µL of CSF tracer into the 10 µL Neuros syringe.

2.1.8 Place the syringe onto the syringe holder in the micromanipulator connected to the stereotactic fame at an angle of 35°.

2.1.9 Palpate the space between the occiput and C1 vertebrae with either an index finger or a cotton-tipped swab. Use these two anatomical landmarks to determine the midline of the cisterna magna. An indentation is observed, which is used to define the puncture site. Mark the puncture site with a marker.

2.1.10 Insert the needle into the puncture site to a depth of 2.8 mm at a 35° angle, and inject 3 µL of the CSF tracer at a rate of 3 µL/min using an electronic infusion pump.

2.1.11 Gently remove the needle and allow the mouse to recover in a fresh cage with new bedding under a heat lamp.

2.1.12 At 30 min or 6 h after injection, perfuse mice intracardially with 0.01 M PBS followed by 4% PFA in 0.01 M PBS at a rate of 5 mL/min.

2.1.13 Remove the entire brain and spinal cord and check for any evidence of brain tissue injury. Acquire images of the brain and spinal cord under a fluorescence microscope.

2.1.14 Post-fix the brain and spinal cord in 4% PFA overnight, and cryoprotect with 25% sucrose in 0.01 M PBS at 4 °C for 24 h.

2.1.15 Section the brain and spinal cord tissue into 12 µm thick coronal or sagittal sections using a cryostat. Stain the slides with DAPI, and examine the intracranial distribution of the CSF tracer.

2.1.16 To determine whether mice can tolerate multiple intrathecal injections, intrathecally inject aCSF for 3 consecutive days, followed by a rest period of 4 days; then inject for another 3 consecutive days.

2.1.17 Monitor the mice and record their weight daily.

REPRESENTATIVE RESULTS:

We first evaluated metastases development after irradiation in a patient-derived xenograft mouse model. Without irradiation, animals developed large tumors in the cerebellum (**Figure 1 A-E**). In contrast, irradiation ablated the majority of the tumor cells in the cerebellum but facilitated the development of metastases along the leptomeninges (**Figure 1 F-J**). We then characterized the movement of the CSF fluorescent tracer along the leptomeninges after infusion via the cisterna magna. Tracer movement within the central nervous system was evaluated by fluorescence microscopy. We observed that FITC-dextran-500 rapidly moved along the pial surface and perivascular pathways (**Figure 2**). Under the same imaging conditions, no fluorescence was observed in uninfused brain tissue (data not shown). Analysis of FITC-dextran-500 fluorescence in the whole brain and spinal cord revealed that CSF tracer influx was strong at 30 min post-infusion (**Figure 2A-D**), but declined at 6 h (**Figure 2E-H**) (n = 3 animals in each group).

This observation is consistent with previous studies showing that the influx of the CSF tracer peaks approximately 30 min post-infusion and dissipates at later time points[16,17]. To quantify the tracer movement into different areas of the brain, we evaluated four regions of interest, including the olfactory bulb, ventricle, cerebellum, and spinal cord. **Figure 2I-N** shows representative images of brain or spinal cord at 30 min after infusion. CSF tracer was observed in each of these regions, suggesting the CSF tracer movement via CSF circulation. CSF tracer circulation was observed in all 10 mice that received a single cisterna magna injection. No tissue damage surrounding the puncture site was detected in these animals, suggesting that this technique is safe and reliable. We further evaluated the effect of chronic intracisternal injections on animal weight. We performed intracisternal aCSF infusion in 6 animals on 3 consecutive days followed by a rest period of 4 days, then repeated this process for an additional week. Animal weight was recorded daily for 2 weeks. Cisterna magna injection did not affect animal weight during the two weeks of observation (**Figure 3**), suggesting that this technique can be safely used for repeat administration of agents directly to the intrathecal space.

FIGURE LEGENDS:

Figure 1: Generation of metastatic medulloblastoma mouse model. Representative images of the whole mouse (**A,F**), brain (**B,C,G,H**) or spinal cord (**D,E,I,J**) from the tumor-bearing mice treated without (**A-E**) or with craniospinal irradiation (**F-J**). Without irradiation, animals develop large tumors in the cerebellum (arrow) at 4-5 weeks post-transplantation, but rarely develop metastases. In contrast, irradiation leads to an increase in metastases along the leptomeninges (arrowheads) at 3-4 weeks after the first dose of irradiation. mCherry indicates tumor cells. Scale bars, 3 mm.

Figure 2: Injection of CSF tracer into the cisterna magna. Representative images of whole mouse brains or spinal cords at 30 min or 6 h after cisterna magna injection with the fluorescent tracer FITC-Dextran-500. Brains were imaged from dorsal (**A,E**), ventral (**B,F**) and sagittal (**C,G**) aspects. Spinal cord images (**D,H**). (**I-N**) Representative sagittal sections of brain and transverse spinal cord sections were counterstained with DAPI at 30 min after cisterna magna injection. Green indicates FITC-Dextran-500. Blue, DAPI. Scale bars, A-H, 3mm, I-N, 200 μm.

Figure 3: Change in animal weight upon multiple injections of aCSF into the cisternal magna. aCSF was injected intrathecally for 3 consecutive days, followed by a rest period of 4 days, then another 3 consecutive days. Animals that did not receive injection served as the controls. Animal weight was recorded daily for 2 weeks. CMI, cisterna magna injection.

DISCUSSION:

In this report, we present a detailed protocol to generate a metastatic brain tumor mouse model and perform intrathecal injection into the cisterna magna. This injection approach provides a straightforward method to deliver labeled molecules to CSF compartments. By injecting CSF tracer, we observed a tracer influx into different regions of the brain and spinal cord (see **Figure 2**). Agents with differing molecular sizes may have distinct diffusion properties; molecules that are smaller in size often have a faster diffusion rate than molecules that are larger in size[17,30,31].

In this experiment, FITC-dextran 500 CSF tracer was selected. With a molecular weight of 500 kilodaltons, the size of this tracer is much larger than that of most small molecule drugs, which usually have molecular weights of less than 500 daltons[32,33]. Using this approach, brain tumor metastases develop in the ventricular system (lateral, third, and fourth ventricles) (see **Figure 1**). CSF is produced predominantly by the choroid plexus in the lateral, third, and fourth ventricles and is distributed over the surface of the brain[13]. Therefore, intrathecal injection can allow agents to flow through the entire ventricular system where metastatic brain tumor cells are located. Our approach demonstrates the feasibility of a simple technique with potential clinical application to deliver therapeutic agents against brain tumor LM.

This study represents substantial modifications of previous methods[34,35] to allow accurate and replicable drug delivery into the cisterna magna in mice. This approach uses direct visualization of the puncture site through removal of the hair as opposed to transcutaneous blind skin incision and injection. This technique also improves upon previous methods by placing the injection needle in the stereotaxic apparatus and controlling the injection rate using an electronic pump instead of injecting by hand. Furthermore, the stopper on the needle allows improved control of the puncture distance. The placement of the animal in the stereotaxic apparatus is crucial to control the puncture direction. If the animal is not placed correctly, the injection coordinates will not correspond to the area of interest. Importantly, the electronic pump connected to the Neuros syringe maintains a constant desired injection rate. A slow rate of infusion is necessary to reduce the risk of damage to the brain caused by the high pressure of rapid injection. The control of the puncture distance is also important. If the needle tip enters the cerebellum, CSF tracer will be retained in the tissue and fail to distribute throughout the subarachnoid space. Damage to the medulla is frequently fatal, whereas cerebellar damage from chronic injection can result in prostration and general animal behavior abnormalities.

Compared to more invasive procedures, such as lumbar puncture or cannula insertion, this modified intrathecal injection method does not require surgery. Surgery often results in operative damages to the skull and brain parenchyma. This approach entails transient puncture of the dural membrane such that the intracranial pressure is only transiently perturbed and is quickly restored. Cannula insertion into the cisterna magna is often associated with nervous tissue damage during insertion and scarring due to the material and size of the cannula. The complex methods to fix the cannula by drilling holes into the occipital bone also cause the surgical procedure to be more complex and time-consuming[15,16]. However, with practice, a single intrathecal injection procedure as described here can be completed in 10 minutes, allowing treatment of larger cohorts of animals. Although intracisternal infusions have potential for iatrogenic complications, including traumatic tissue injury, we did not observe intracranial hypertension resulting from CSF tracer injection at the cisterna magna at an infusion rate of 3 µL/min. However, some animals did not survive when the infusion rate was increased to 5 µL/min (data not shown). Based upon these findings, we further performed intracisternal aCSF infusion at 3 µL/min for 3 consecutive days each week for a total of 2 weeks. All animals (n=6) survived, and animal weight was not significantly impacted by the chronic infusions. In addition, using this approach, animals do not need to be single housed after injection, thus minimizing confounding factors due to the effects of stress. Overall, this approach circumvents difficulties associated with

delivery of chemotherapeutic agents to the central nervous system. Intrathecal delivery via the cisterna magna could, therefore, be routinely employed to administer therapeutic agents to treat metastatic brain tumors in preclinical mouse models. It is noted that direct injection into the CSF may result in limited drug penetration to the brain parenchyma due to the slow diffusion rates. Intrathecal drug delivery may, therefore, be limited in its capacity to treat brain tumors in the parenchyma.

In conclusion, intrathecal injection via cisternal magna is a fast, easy, and reliable procedure to deliver drugs that could not otherwise cross the blood-brain barrier directly to the CSF compartment. Our non-invasive approach has promising clinical potential to deliver long-term therapy against leptomeningeal metastatic disease arising from brain tumors and other types of cancers.

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DISCLOSURES:

The authors have nothing to disclose.

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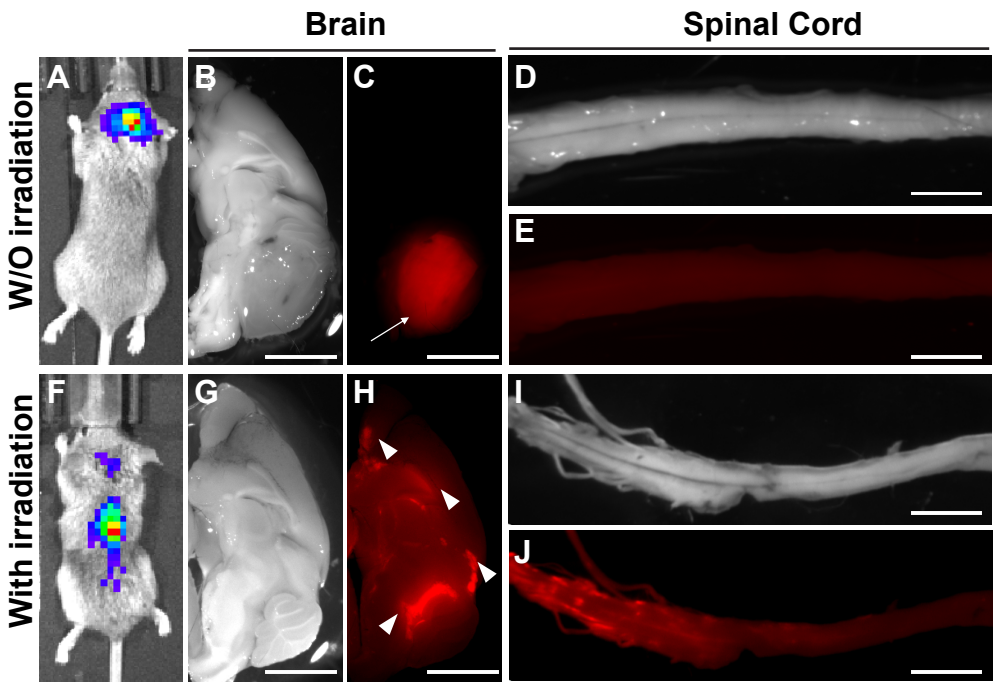

Figure 1

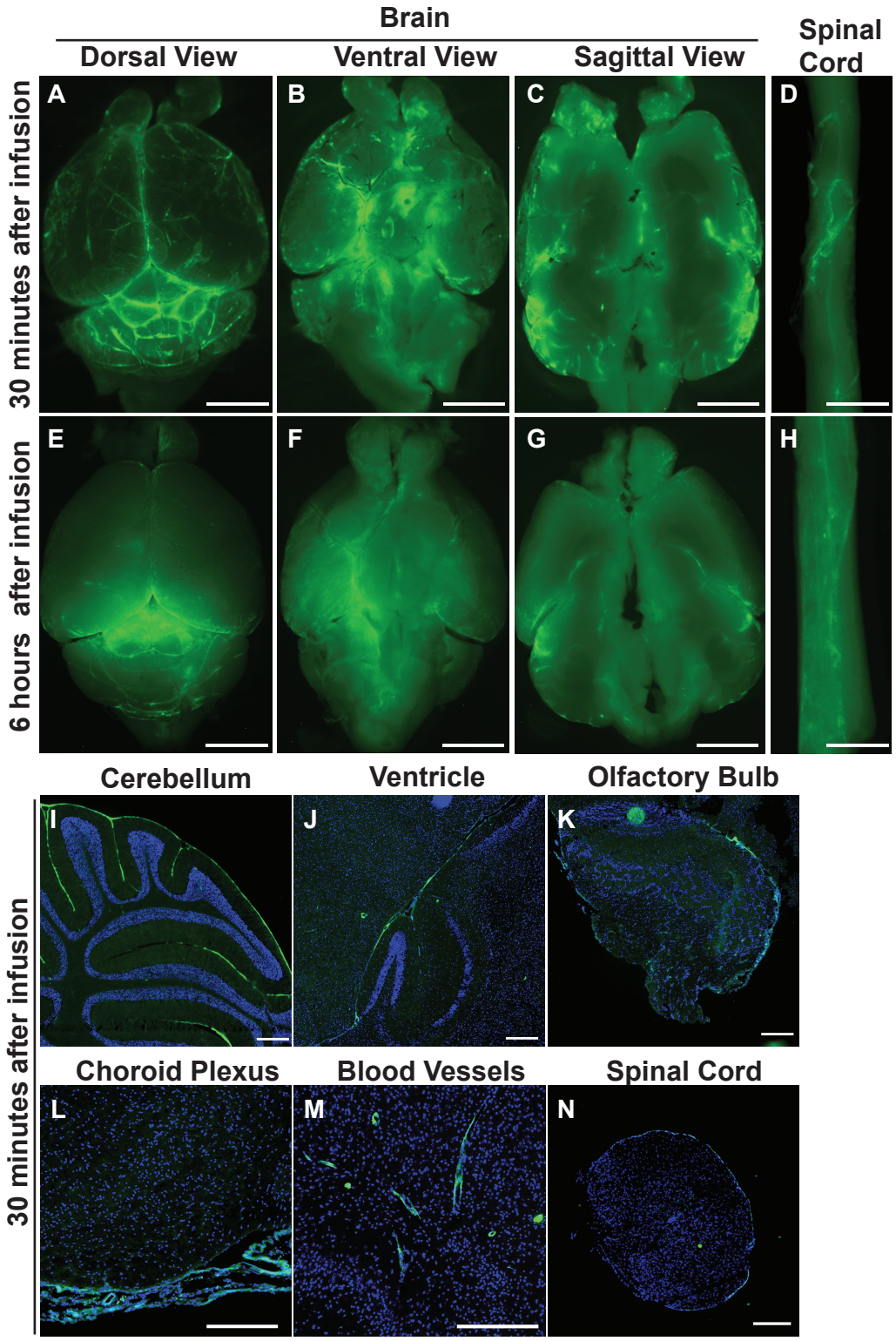

Figure 2

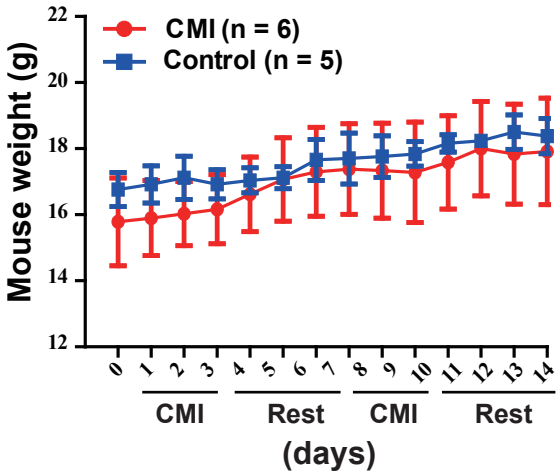

Figure 3

Name of Material/ Equipment	Company	Catalog Number
33-gauge Neuros syringe, 10uL	Hamilton Company	65460-05
4% Paraformaldehyde solution in PBS	Alfa Aeser	J19943
5 µL Microliter Syringe Model 7105 KH, Knurled Hub Needle, 24 gauge, 2.75 in, point style 3	Hamilton Company	88000
aCSF	Tocris Bioscience (Fisher Scientific)	35-252-5ML
Bard-Parker Sterile Disposable Scalpels #10	VWR	89176-380
Buprenorphine HCL injectable, 0.3MG/ML, C3	Covetrus	059122
Butler Schein Paralube Non-antibiotic opthalmic ointment	Fisher Scientific	NC1865385
Cryostat	Leica	CM1950
DAPI, FluoroPure grade	Thermo Fisher Scientific	D21490
Deltaphase Isothermal Pad	Braintree Scientific	BP-PAD
Digital 3-axes Manipulator Arm, left	Stoelting	51904
Digital Conversion for Stoelting Manipulator Arm, left	Stoelting	51914
Digital Just for Mouse Stereotaxic	Stoelting	51730D
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	14190250
Dumont #5 Forceps	Fine Science Tools	11295-00
Dumont #7 Forceps	Fine Science Tools	11297-00
Ethanol	Fisher Scientific	04-355-226

Extra Narrow Scissors	Fine Science Tools	14088-10
Fisherbrand Sterile Alcohol Prep Pad	Fisher Scientific	22-363-750
Fluorescein isothiocyanate–dextran	Sigma Aldrich	FD500S
Fluorescence microscope	Olympus	BX53
Fluorescence stereo microscope	Leica	M205 FA
Gas Anesthesia Mask for Stereotaxis	Stoelting	51609M
Germinator 500	Fisher Healthcare	NC9956482
Graefe Forceps, 10.2 cm	Stoelting	52102-60P
Heat Lamp with table clamp	Braintree Scientific	HL-1 120V
Induction chamber, anesthesia accessories, small animlal, 5.25 liter	Medvet International	93805107
Isoflurane solution	Covetrus	029405
IVIS Lumina III In Vivo Imaging System	Perkin Elmer	CLS136334
Liquivet (Rapid) Tissue Adhesive	World Precision Instruments	504561
Neurocult stem cell medium and proliferation supplement	Stem Cell Technologies	Cat# 05751
Quintessential Stereotaxic Injector (QSI)	Stoelting	53311
Restrainer & Split Shield	Braintree Scientific	MHS2-HE set
RS 2000 Biological System Irradiator	Rad Source Technologies	
Sterile Cotton Tipped Applicators	Medline Industries	MDS202000
Sucrose	VWR International	EM-8510
Surgical Clipper, Cordless, Rechargeable w/#40 blade	Stoelting	51465
Tissue-Plus OCT Compound	Fisher Scientific	23-730-571

XenoLight D-Luciferin Potassium Salt	Perkin Elmer	1227799

	Comments/Description
10uL glass syringe with 33-gauge beveled needle and stopper	
4% PFA	
Microliter syringe	
artificial cerebrospinal fluid	
Scalpels	
eye ointment	
heating pad	
Manipulator	
Digital Display for manipulator	
Stereotaxic frame	
Phosphate buffered saline	
forceps	
forceps	

scissors

FITC dextran 500

Dissection microscope

Hot bead sterilizer
forceps

induction chamber

IVIS Imager

Tissue adhesive

cell culture medium

electronic pump for injection

Restrainer

Irradiator

Electric shaver
Tissue embedding medium

Luciferin

Rebuttal Letter

We appreciate the reviewers' and editor's assessment of our work and the constructive feedback regarding our manuscript. We have addressed each of the concerns and revised the manuscript accordingly, which we believe has substantially improved the quality of the manuscript.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have reviewed the manuscript to ensure that there are no spelling or grammar errors.

2. Please provide an email address for each author.

Response: We have provided an email address for each author in the manuscript.

3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "This protocol presents …"

Response: We rephased the Summary as "*This protocol presents a straightforward procedure that enables direct intrathecal delivery of agents to cerebrospinal fluid without operative damage to the skull or brain parenchyma. This approach provides a solution to deliver molecules that could not otherwise cross the blood-brain barrier or blood-cerebrospinal fluid barrier to treat leptomeningeal metastatic disease*."

4. Please ensure all abbreviations are described during the first-time use.

Response: We have reviewed the manuscript to ensure that all abbreviations are defined upon their first introduction.

5. Please ensure the Introduction include all of the following with citation:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application

Response: We have revised the Introduction to incorporate the goals, rationale, advantages, and broader context and applications of this method.

6. Please move all equipment to the Table of Materials. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.

Response: We have moved all equipment information to the Table of Materials.

7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: (Stem 86 Cell Technologies Cat# 05751), NeuroCult, Eppendorf, Vetbond, Neuros, etc.

Response: We have removed the commercial language in the manuscript. Specifically, we removed the section titled "Equipment for Implantation, Irradiation, and Injection," which contained many trade names.

In the protocol, we have removed the terms Neurocult and Stem Cell Technologies #05751 in section 1.1.1. We replaced Eppendorf with microcentrifuge in section 1.1.2. We replaced Hamilton with 5uL glass in section 1.1.8 (now 1.1.11). We removed 3M Vetbond from section 1.1.11 (now 1.1.14). We replaced IVIS imager with *in vivo* imaging system in section 1.1.18. We removed OCT in section 1.3.4 and replaced with tissue embedding medium. In section 2.1.1, we replaced 33-gauge Neuros syringe with 10 µL glass syringe, 33-gauge beveled needle and stopper. We removed the words Sigma Aldrich and Tocris Bioscience in section 2.1.5 (now 2.1.7).

8. Please revise the following lines to avoid overlap with previously published work: 17-18, 35-36, 51-55, 191-195, 230-237, 250-254, 256-258.

Response: We have rephased the indicated lines.

9. Please ensure each step contains all the actions associated with the step.

Response: We provided additional details to ensure that each step fully explains all associated actions. For step 1.1.2, we added more information on how to collect and prepare cells for transplantation. We clarified how to make an incision in step 1.1.6. We added a description for how to determine coordinates for injection in step 1.1.7 (now 1.1.10). We also added details for removing the brain and spinal cord in step 1.3.2.

10. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Response: We have provided additional details to the protocol steps. For step 1.1.2, we added more information on how to collect and prepare cells for transplantation. We clarified how to

make an incision in step 1.1.6 (now 1.1.9). We added a description for how to determine coordinates for injection in step 1.1.7 (now 1.1.10). We also added details for removing the brain and spinal cord in step 1.3.2. We also added more details to the protocol for section 1.2.

11. How do you introduce mCherry?

Response: We transfected tumor cells with lentivirus encoding an mCherry-Luciferase reporter prior to transplantation. Viral supernatant was added into the culture medium without polybrene and incubated overnight. This information is provided in step 1.1.1. The mCherry-luciferase lentivirus typically has 90% transfection efficiency.

12. How do you collect cells, centrifugation speed, etc? Please ensure all centrifugation speed is in x g.

Response: We have added a description of how the cells are collected and centrifuged, including the centrifuge speed, in steps 1.1.2 – 1.1.4.

13. How do you make the incision in the scalp?

Response: We use a sterile, disposable #10 scalpel to make the incision. This information has been added to the manuscript.

14. How long does it take for the cells to metastasize?

Response: Metastases are observed 3-4 weeks after the first dose of radiation. We have added this information to the manuscript.

15. Please include a single line space between each step, substep, note and highlight 3 pages of the protocol section including headings and spacings. This will be used for generating scripts for the video and hence ensure that it makes a cohesive story for the protocol section.

Response: These formatting changes were made to the manuscript.

16. Please place all the Figure Legends together at the end of the Representative Results in the manuscript text.

Response: All Figure Legends are now placed after the Representative Results section.

17. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique

d) The significance with respect to existing methods
e) Any future applications of the technique

Response: We revised the Discussion section as the editor suggested.

18. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Response: A scale bar is now included.

19. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sort the table in alphabetical order.

Response: We have removed trademark (™) and registered (®) symbols from the Table of Equipment and Materials and sorted the table in alphabetical order.

Reviewers' comments:

Reviewer #1:

The manuscript entitled "A reliable and reproducible approach to deliver agents through cisterna magna injection for the treatment of leptomeningeal metastatic brain tumors » by Xu Zhenhua et al. reports the methodology to inject drugs into the cerebrospinal fluid of mice.
The manuscript is quite easy to follow. It is divided into two parts: i) the first part describes the protocol of implantation into mouse brains of tumor fragments of a patient-derived medulloblastoma; ii) the second part described a protocol of intra-thecal administration of drugs into the cisterna magna after a single injection.
Overall, the two protocols are well detailed and clear enough to be reproduced.

Main concerns:
- There is no real originality. Implantation of human tumors into mouse brains has been previously reported in details, even in protocols with stereotactic coordinates. As an example, the recent publication by Oshi et al [Cancers 2020, 12, 444; doi:10.3390/cancers12020444] is particularly interesting, describing three different procedures to generate cancer brain metastases orthotopic xenograft models with the percentage of grafting and specific death for each procedure.

Response: Novelty is not a requirement for publication. Additionally, the publication by Oshi et al. described a protocol for injecting tumor cells into the forebrain. In our protocol, we inject tumor cells into the cerebellum, which is where medulloblastoma tumors occur in patients.

The lack of originality is also true for the second protocol. A similar procedure is reported by Ineichen et al. (Nature Protocols), except that Xu Zhenhua et al. propose to shave the animal to have better anatomical landmarks.

Response: Novelty is not a requirement for publication. Additionally, the procedure reported by Ineichen et al. (Nature Protocols) inserted a catheter into the cisterna magna or lumbar spinal cord, which may cause stress to the animals. In our manuscript, we developed a non-invasive protocol that can repeatedly and accurately deliver drugs to the leptomeningeal metastases without operative damage to the skull, brain parenchyma or spinal cord.

- The authors should detail more precisely the time required to obtain their PDX orthotopic brain models. This point should also be specified in the legend of Figure 1.

Response: The animals usually exhibit detectable luminescent signal at 1 week and show symptoms, including weight loss and ataxia, at 4-5 weeks post-transplantation. Animals develop metastases approximately 3-4 weeks after the first dose of irradiation. We have incorporated this information into in text and the legend for Figure 1.

- Line 101-104: How is opened the skull? Do the authors use a drill or some specific technique to soften the bone?

Response: We did not use a drill to open the skull or soften the bone for tumor cell transplantation. Instead, we made a small hole with a diameter of approximately 0.5 mm in the skull by puncturing the skull with the beveled end (sharp point) of a sterile 18-gauge needle.

- Line 154-156: The authors should give more precision on the prone position. What is the exact angle of the head? Indeed, tilting the animal's head down opens the space between the occiput and the first vertebra and provides a better access to the cisterna magna. However, tilting the head makes it difficult to hold and maintain the isoflurane mask.

Response: The head of mouse was restrained in two ear bars, and a tooth bar was used to align and support the front of the head. The height of the ear bars and the tooth bar were adjusted so that the line connecting the most prominent aspects of the cranium and the spine formed a 15-degree angle with the horizontal line. In this position, the cisternal magna is nearly the highest point of the mouse body.

- Line 163-166: Even if the animal is shaved, this transcutaneous approach is risky. Did the authors blunt the needle before the procedure, to avoid damages to the central nervous system?

Response: We did not blunt the needle. There is an adjustable stopper on the needle that helps to control the puncture distance. With the stopper placed 3 mm from the tip of the needle and the mouse positioned appropriately, we have never observed any damage to the central nervous system caused by the injection.

- Line 279-281: The authors propose some clinical applications for their procedure, with repeated injections into the cisterna magna. This does not seem appropriate for patients, for

whom reservoirs like Ommaya are safe, easy to put, and highly required to administer drugs repeatedly over time, but also to sample the cerebro-spinal fluid for pharmacokinetic studies.

Response: We agree with the reviewer that Ommaya reservoirs are commonly used in patients to administer drugs to the CSF in the clinic. However, the Ommaya reservoir may cause some morbidity. The procedure carries a small risk of intracerebral hemorrhage along the track of the ventricular catheter and there is a small risk of infection. While our approach will primarily be used to deliver drugs to the CSF in preclinical animal models, it does not cause any operative damage to the skull or brain parenchyma and thus may have potential clinical applications.

Minor points:
- There are too many abbreviations.

Response: We have reduced the number of abbreviations used in the manuscript.

- English language should be revised.

Response: We have revised the manuscript.

Reviewer #2:
Manuscript Summary:
The authors describe a method to produce animal models of medulloblastoma using intracerebellar injection of human tumor cells, as well as a method to deliver agents through the CSF using a non-invasive cisternal injection. They track the spread of the infusion using a fluorescent tracer and discuss that the ability to reach the leptomeninges is relevant for the treatment of leptomeningeal metastases typically observed with this kind of tumor.

Major Concerns:
1. Many materials and compounds are omitted in the list of equipment. The authors should make sure each material mentioned in the description of the procedure is included in the list of equipment (e.g., everything needed for the establishment of the animal model).

Response: We have carefully reviewed the manuscript and added any materials or reagents that were missing to the Table of Materials.

2. The authors mention in the discussion that their method avoids alterations to the intracranial pressure and astrogliosis (lines 255-257). However, they do not run any experiments to confirm that this is actually the case. The authors could consider including a few panels in their figures to support these statements or remove the comments from the discussion. For instance, they could stain with GFAP the sections of brains subjected to CMI to detect signs of astrogliosis.

Response: We have removed the description of the intracranial pressure and astrogliosis from the Discussion because we do not currently have evidence to support the statement.

3. Step 1.1.3: does this mean multiple animals are placed in the chamber at once? Prolonged induction of anesthesia affects body temperature and can decrease breathing. To minimize animal suffering, the authors should be placing in the chamber one animal at the time and only add the new animal after the first has been operated.

Response: We placed mice individually in the induction chamber to anesthetize before surgery. We have edited the text in the protocol (step 1.1.3 and 1.1.4, now 1.1.6 and 1.1.7) to reflect this.

4. If feasible, it would be very relevant to see how the tracer spreads in the brain of mice with tumors to see if the molecule can actually reach the LM.

Response: The purpose of this manuscript is to develop a non-invasive technique that can repeatedly deliver agents directly to the CSF and evaluate the accuracy of the agent delivery using the temporal and spatial intracranial distribution of the dye. We have tested this technique for treating metastatic brain tumors with a small molecule inhibitor and found that the metastatic tumor cells are completely eliminated by the inhibitor. The manuscript describing these results will be submitted for publication soon.

Minor Concerns:
1. Abstract: the authors could consider avoiding using abbreviations that are not repeated throughout the paragraph (e.g., CNS, BBB) and should be careful to introduce the meaning of the abbreviations, if they intend on using them (e.g., BBB, CSF, CMI).

Response: We have removed unnecessary abbreviations and introduced the meaning of the necessary abbreviations in the abstract.

2. Figure 2N: the grey matter in the spinal cord seems to be infused with the tracer. Though it may just be an imaging artefact? The authors could consider adding a section of figure showing the uninfused brain and spinal cord for comparison.

Response: Tracer influx was only observed on the surface of the spinal cord along the leptomeninges. The green color in the grey matter of the spinal cord in Figure 2N is artificial. We have adjusted the figure with the reduced non-specific fluorescent background.

3. It would be relevant in the discussion to add a word about molecular size of the fluorescent tracer used in this experiment compared to that of the agents one may intend to infuse to treat the LMs in patients. This is because agents that have markedly different molecular size may differ in their diffusion properties - e.g. spread throughout the ventricular system and penetrating of the underlying parenchyma (see for instance Iliff et al., 2012).

Response: We agree with the reviewer that agents of different molecular sizes may have distinct diffusion properties with smaller molecules having a better diffusion rate than larger

molecules. In our experiment, we selected FITC-dextran 500 CSF tracer with a molecular weight of 500 kilodaltons, which is much higher than most small molecule drugs that usually have a molecular weight of less than 500 Daltons. We have added this description to the Discussion Section with citations.

4. The authors might be more specific about ways to sterilize instruments prior to surgery (line 80).

Response: We have added a sentence describing how instruments are sterilized for surgery in the note under the section title "Generation of a mouse model of metastatic medulloblastoma."

5. Step 1.1.6: use metric system as done for the rest of the manuscript.

Response: We have changed ½'' to 12 mm.

6. Step 1.1.7: the authors could briefly describe how to find the coordinates for the injection (e.g., using an atlas) and mention what coordinates they used for their experiment.

Response: We have added that we used an atlas in step 1.1.10.

7. Step 1.1.13: the authors could add, as note, that postoperative pain relief strategies may change in different institutes and that consultation with the vet unit is important to ensure proper pain management

Response: We have added a note regarding differences in postoperative pain management between institutions in step 1.1.17.

Reviewer #3:
Manuscript Summary:
The manuscript presents techniques for modeling radiation therapy and drug administration into the subarachoid space, via a cisterna magna approach. The techniques are versatile and will allow other investigators to perform new experiments. As such the manuscript will be a significant contribution to the field. The writing is clear and I have few suggested changes. I hope that my concern about anesthesia, described below, will not unduly delay publication, as I think the work is important.

Major Concerns:
Line 122 The restraining of mice without anesthesia may not be consistent with minimizing distress to experimental animals. The use of isoflurane anesthesia administered by vaporizer or drop method should be considered.

Response: We understand and appreciate the reviewer's concern for the well-being of the animals. The animals are kept in restraint without anesthesia during irradiation for less than 5

minutes. The short restraint time helps to minimize any distress to the animals. We have also edited the language in the protocol to reflect that the mice are irradiated individually.

Minor Concerns:
Line 27 change "are not responsive to radiation" to "may not be responsive to radiation"- some patients with LM do well after radiation and it is important not to state otherwise.

Response: We have revised the statement the reviewer suggested to reflect that some patients may respond to radiation.

Line 42 It is problematic to state "The functional importance of these locations makes meaningful surgical resection of such metastases impossible." The diffuse nature of LM is the primary obstacle to resection. It would be better to state "The diffuse nature of LM makes meaningful surgical resection impossible."

Response: We have revised the statement the reviewer suggested to reflect that surgical resection is impossible due to the diffuse nature of LM.