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MOUSE MODEL TO STUDY IRRADIATION EFFECTS ON BRAIN EXTRACELLULAR MATRIX *IN VIVO*

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ABSTRACT

Radiotherapy is an integral part of anti-glioblastoma treatment. However, an increase in its efficiency and protection of normal tissues against the toxic effects of ionizing radiation remain important issues. That needs more experimental studies on animal models *in vivo*. To investigate radiation effects onto glioblastoma microenvironment, we engineered a reliable and cost-effective mouse model suitable for brain tissue irradiation. Two-month-old male CBL/6Bl mice were used, and key components for the radiotherapy animal model (anaesthesia type, X-ray source, irradiation dose) were determined. Injection with zoletil/domitor combination was selected as the most suitable for brain research due to negligible short-term effects (24-72 hours) to brain tissue morphology and the expression of proteoglycans (PGs) as main extracellular components of brain tissue. X-ray irradiation of mouse brain with a 7Gy was suggested as an optimal single dose for multiple irradiation experiments corresponding to anti-glioblastoma radiotherapy regimen. Both research synchrotron and clinical linear accelerator demonstrated similar irradiation effects on brain tissue suggesting admissibility of comparative analysis of the results obtained in different irradiation experiments. Overall, this study describes a novel affordable mouse model *in vivo* to study radiotherapy effects on brain tissue and tumour microenvironment.

KEYWORDS: Glioblastoma radiotherapy, animal model, mouse brain irradiation, tissue morphology, extracellular matrix, proteoglycan expression.

1. INTRODUCTION

Up to date, multiple animal models to study different aspects of radiation effects on normal or cancer brain tissues are established [1,2]. To model radiotherapy in animals, three key variables should be determined: animal (mouse, rat or others), anaesthesia (gas or injection, choice of drug) and X-ray source (radioisotope or accelerator, conventional or microbeam radiation). Combination of these modalities creates an experimental model optimized for a specific purpose.

The optimal way could be to use specialized instruments [3], but those are not widely available. In most cases, different research or clinical accelerators are used for mouse' brain irradiation [4,5]. The published studies demonstrate different combinations of irradiation doses (10Gy-60Gy) and short-term [6,7] or long-term [8-11] effects of the radiation to mice. These studies demonstrate the functional effects of X-ray irradiation on brain tissue, although many aspects of these effects remain unknown and will benefit from new mouse radiotherapy models *in vivo*. In addition, the physical characteristics of X-ray beam from different accelerators may vary and need to be validated for their feasibility in this kind of research.

Another important issue is an anaesthetizing of the experimental animals, where gas or injection anaesthesias are the most common. Gas anaesthesia is an effective method but requires special equipment, and the used drugs (usually isoflurane or sevoflurane) possess some side-effects to various molecular parameters of brain tissue

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[12-14]. Injection anaesthesia is suggested as a good alternative to gas anaesthesia [15] although not abundant comparative evaluations of different gas or injection anaesthetic protocols resulted in no final conclusion concerning the 'best' general anaesthetic [16-18]. Controversial data are also presented for injection anaesthesia with zoletil [19,20]. Thus, there is no evident answer which anaesthesia is better, and anaesthesia type/drug/dose must be adapted to every experiment on small animal models according to the experimental design (mouse strain, age, mutation, tissue/molecules to be studied).

Our strategic goal is to investigate the molecular mechanism(s) of radiation-induced changes in normal brain extracellular matrix, which could be of importance for glioblastoma recurrence. This work aims to elaborate a mouse model for our main study including appropriate anaesthesia regimen, reliable immobilization of the animals, single irradiation dose (similar to that during anti-glioblastoma radiotherapy) and the suitability of any of the 2 accelerators available in the vicinity.

2. MATERIALS AND METHODS

Animals and tissue samples
All experiments were performed on 2-month old male C57BL/6 mice obtained from the SPF animal house at the Institute of Cytology and Genetics (Novosibirsk, Russia). Animals were housed in polycarbonate cages with free access to food and water, 12/12h light/dark cycle. Animals were sacrificed by cervical dislocation according to AVMA Guidelines for the Euthanasia of Animals. Half of the freshly excised brain tissue was immediately fixed in 10% neutral buffered formalin, then routinely processed and embedded in paraffin using Shandon Citadel 2000 Tissue Processor and HistoStar Embedding Workstation (ThermoFisher Scientific, USA). The second half was divided on cortex and subcortex and was preserved with RNALater solution (Thermo Fisher Scientific, USA) according to the manufacturers' instruction. All procedures with the experimental animals were conducted in accordance with European Communities Council Directive 2010/63/EU and approved by the local Committee for Biomedical Ethics of Institute of Molecular Biology and Biophysics FRC FTM.

Irradiation and the radiation dose determination
For irradiation, animals were anaesthetized by a combination of drugs domitor (Orion Corporation, Finland) and zoletil (Valdepharm, France). The anaesthetized animals were placed in a special fixture elaborated by our team for stationary position and irradiated either at the research accelerator complex VEPP-4 (Budker Institute of Nuclear Physics SB RAS, Novosibirsk, Russia) or the clinical linear accelerator Elekta Axesse (Elekta Ltd., UK) (Meshalkin National Medical Research Center, Novosibirsk, Russia).

Irradiation zone included the whole mouse head for research accelerator or brain zone, which was delineated using computer tomography (CT) scans for the clinical accelerator. The dose of 7Gy was given according to the research accelerator settings or calculated using treatment planning software for the clinical accelerator, respectively.

Radiation doses were controlled by dosimetric radiochrome film Gafchromic EBT3 (Ashland Specialty Ingredients, USA). The exposed film was scanned by Canon 9000f mark II (Canon, Japan) and the optical density of the shaded areas was determined using the program ImagePro6.

Histological study
For the histological study, 3.5-µm sections were used. Routine staining with Haematoxylin and Eosin (H&E) was performed on Microm HMS740 (Thermo Scientific, USA) and photographed with magnification x400 (Axiostar Plus, Carl Zeiss). Five tissue sections from the same specimen were stained and analyzed; ten microscopic fields were estimated for each specimen. The staining was analyzed by two qualified pathologists independently.

Real-time RT-PCR analysis
Total RNA was extracted using the QIAzol Lysis Reagent (Qiagen, USA), and total RNA concentration was measured with Qubit–iT RNA Assays Kit (ThermoFisher Scientific, USA) according to the manufacturers' instructions. cDNA was synthesized from 0,5µg of total RNA using a First Strand cDNA Synthesis kit

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(Fermentas, Hanover, MD, USA). Real-time PCR for mouse PGs (syndecan-1, glypican-1, perlecan, versican, brevican, CSPG4/NG2, CD44, decorin, biglycan, neurocan) was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, USA) and the Taq-polymerase (IMCB, Russia), SYBR Green (Thermo Fisher Scientific, USA) under the following conditions: 95°C for 3min, then 95°C for 20s, 59°C for 15s, and 72°C for 30s for 40 cycles. The total reaction volume was 25 µl. Gapdh was used as the reference gene. The fold change for each mRNA was calculated by the $2^{-\Delta Ct}$ method. The PCR primers and conditions are presented in Table 1.

Table 1. Sequences of the primers used in the study

Symbol	GeneBank	Description	Sequence
Sdc1	NM_011519.2	Syndecan-1	F 5'- GGTCTGGGCAGCATGAGAC -3' R 5'- GGAGGAACATTTACAGCCACA -3'
Gpc1	NM_016696.5	Glypican-1	F 5'- CTTTAGCCTGAGCGATGTGC -3' R 5'- GGCCAAATTCTCCTCCATCT -3'
Hspg2	NM_008305.3	Perlecan	F 5'- CCGTGCTATGGACTTCAACG -3' R 5'- TGAGCTGTGGAGGGTGTATG -3'
Vcan	NM_001081249.1	Versican	F 5'- GGAGGTCTACTTGGGGTGAG -3' R 5'- GGGTGATGAAGTTTCTGCGAG -3'
Bcan	NM_012916.2	Brevican	F 5'- GTGGAGTGGCTGTGGCTC -3' R 5'- AACATAGGCAGCGGAAACC -3'
Cspg4	NM_139001.2	NG2	F 5'- TCTTACCTTGGCCCTGTTGG -3' R 5'- ACTCTGGTCAGAGCTGAGGG -3'
Cd44	NM_009851.2	CD44	F 5'- CAAGTTTTGGTGGCACACAG -3' R 5'- AGCGGCAGGTTACATTCAAA -3'
Dcn	NM_007833.6	Decorin	F 5'- CCCCTGATATCTATGTGCCC -3' R 5'- GTTGTGTCGGGTGGAAAATC -3'
Bgn	NM_007542.5	Biglycan	F 5'- GCCTGACAACCTAGTCCACC -3' R 5'- CAGCAAGGTGAGTAGCCACA -3'
Ncan	NM_007789.3	Neurocan	F 5'- CCAGCGACATGGGAGTAGAT -3' R 5'- GGGACACTGGGTGAGATCAA -3'
Gapdh	NM_008084.3	Gapdh	F 5'- CGTCCCGTAGACAAAATGGT -3' R 5'- TTGATGGCAACAATCTCCAC -3'

Statistical analysis

Statistical analyses were performed using ORIGIN Pro 8.5 software; a value of $p < 0.05$ was considered to indicate a statistically significant difference. Data are expressed as means ± SD.

3. RESULTS

Anaesthesia and animal fixation

Based on the literature data [19,20], injection anaesthesia with domitor/zoletil combination was tested as a simple method to anaesthetize mice under routine laboratory conditions. Dose-sleep time interdependence was determined in a number of test experiments, and domitor injection (0.125 mg/kg of mouse body weight) followed after 10 min by zoletil injection (20 mg/kg) into another part of mouse abdomen was chosen as an optimal regimen enough for a 60-80 min deep sleep sufficient for mice irradiation (Figure 1a).

To fix the sleeping animals, we elaborated a special immobilization device holding 2 mice simultaneously, unlike the previously described devices [21,22]. Our device is compatible both with research (VEPP-4) and clinical (Elekta Axesse) accelerators, allow irradiating of 2 animals and can be easily decontaminated if necessary (Figure 1b).

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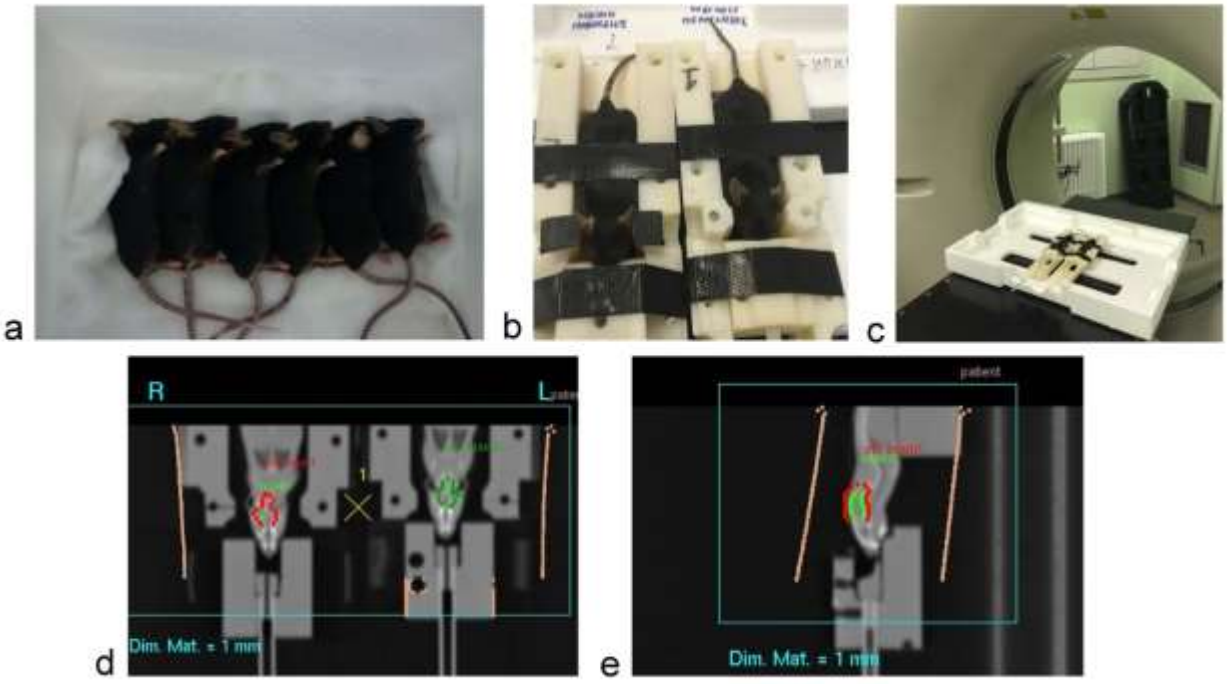

Figure 1. Anaesthesia and fixation of the experimental mice. (a) Sleeping animals. (b) Animals in holding devices. (c) MRI of the animals and brain zone delineation in the frontal (d) and saggital (e) projections

Upon immobilization, brain zone was delineated by CT (Figure 1c) for both mice at the same time in the frontal (Figure 1d) and saggital projections (Figure 1e).

Irradiation mode and dose

To compare different X-ray sources, similar experiments were performed using the synchrotron VEPP-4 and clinical linear Elekta Axesse accelerators.

The VEPP-4 beamline (Figure 2a) has a high intensity of hard X-ray radiation from 9-poles wiggler. Vertical size of SR beam coming into the irradiation area is about 2 mm, and the horizontal dimension is 5 mm, the integrated radiation power equal to 65 W. The X-ray spectrum ranged from 100 keV to 250 keV. Whole mouse head was irradiated from a single-side position (Figure 2b). A dose of 7Gy was maintained by the instrument's settings.

Elekta Axesse irradiates animals with two photon beams pointed from opposite directions with energy of 6 MeV in the total dose of 7Gy (Figures 2c and d). The target volume is defined as a whole mouse brain. A dosimetric plan was created by the treatment planning system ERGO++ (Elekta Ltd, Sweden). Mean coverage of the target was defined as the absolute dose delivered to 95% of the target volume. Setup accuracy was verified by cone-beam CT imaging before dose delivery.

To calculate the absorbed dose distribution in the irradiated samples, the Monte-Carlo method (by using Geant4 toolkit) was used. In the simulation of dose distribution, the 0.9% aqueous sodium chloride solution was used as an X-ray phantom of the mouse head. To reach uniform distribution of the absorbed dose in the sample, a tungsten filter with 0.9 mm thickness (which cut off the low-energy part of the SR spectrum) was used. The rate of the absorbed dose was about 1.5 Gy/sec, and its difference along the phantom did not exceed 10%. During irradiation, the absorbed dose was measured by the radiochromic film Gafchromic EBT3 and ionization chambers.

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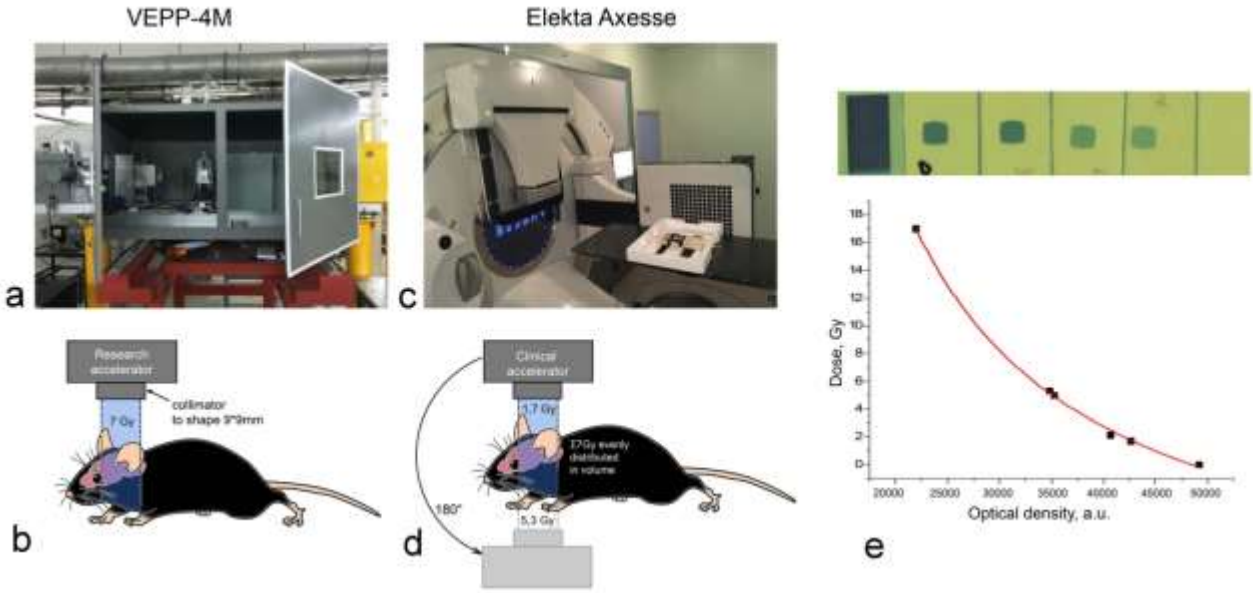

Figure 2. Irradiation of the mice. Animals were irradiated on the research (a) and clinical (c) accelerators from one (b) or two (d) sides. The obtained irradiated doses calculated by the instruments' software were controlled using radiochromic film and appropriate dose curve (e)

Anaesthesia effects on brain tissue morphology and ECM components expression

To evaluate the suitability of zoletil/domitor as anaesthetic drugs for studies of brain ECM, histological and molecular characteristics of the brain tissue were analysed.

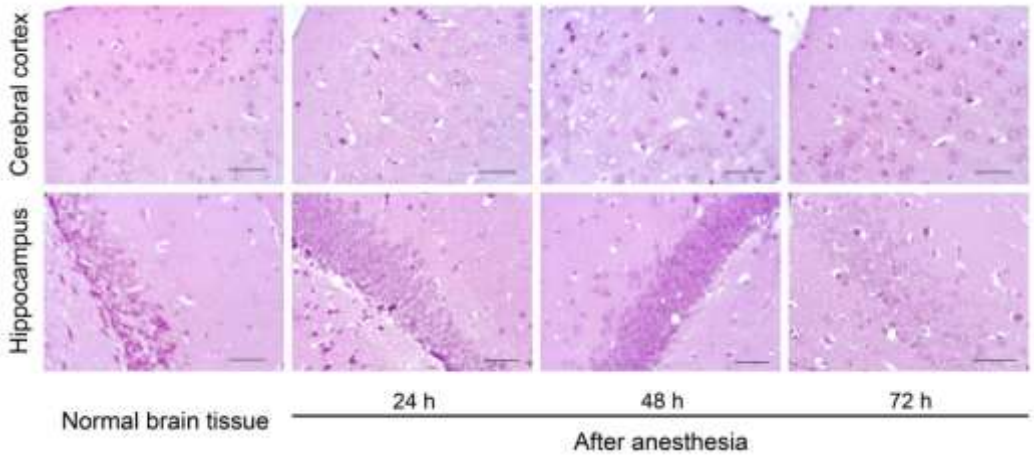

Figure 3. Anaesthesia effects on mouse brain morphology. Morphology of cerebral cortex and hippocampus after 24, 48 or 72 hours after anaesthesia. Staining with Haematoxylin-Eosin. Magnification *400, scale bar size 50 µm

Zoletil/domitor injection resulted in some morphological changes in the hippocampus but not cerebral cortex, which were diminished by 72 h after injection (Figure 3). However, heterogeneity of both cellular structure and tinctorial properties of neurons and brain ECM in the control brain tissues suggests an unspecific nature of the observed changes.

A single administration of these drugs in the dose sufficient for 60-80 min sleep did not result in significant changes in overall transcriptional activity of PG-coding genes for 24-72 hours after injection, although the expression levels of some low-expressed PGs (brevican, CSPG4/NG2) were attenuated (Figure 4).

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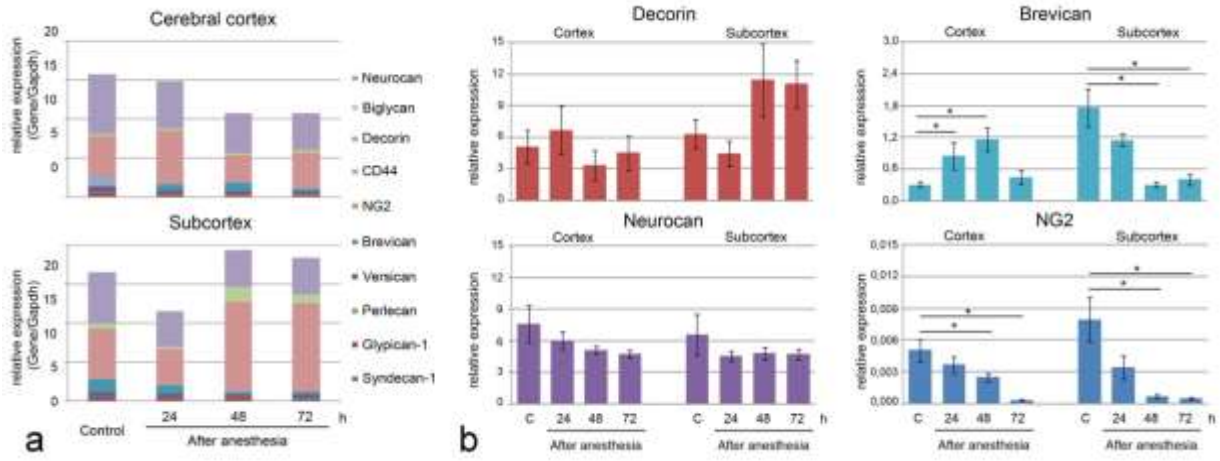

Figure 4. Anaesthesia effects on PGs expression in mouse brain tissue. (a) Overall transcriptional activity of PGs in mouse brain tissue before and after anaesthesia. PGs profiling was performed at 24, 48 and 72 hours after zoletil/domitor injection. Stacked column compares the contribution of each value to a total across categories. (b) Expression levels of individual PGs. Real-time RT–PCR analysis, expression normalized to Gapdh, bars represent the mean ± SD from triplicate experiments (OriginPro 8.5). A value of p<0.05 was considered statistically significant (*- p<0.05)

The contribution of these changes to the overall PG expression is weak, and their functional significance needs to be investigated further.

Overall, injection anaesthesia with zoletil/domitor combination does not change histological and molecular characteristics of brain tissue and looks to be suitable to study irradiation effects on brain extracellular matrix.

4. DISCUSSION

Small animal models remain an important option for preclinical investigations of negative radiotherapy effects towards the brain tissue, in spite of the development of new methodological approaches based on 3D cell culture systems [23]. Here, we elaborated an affordable mouse model to study radiation effects on brain tissue using either research synchrotron X-ray source or clinical linear accelerator, subject of availability.

Our results extend the previously published controversial data on zoletil effects [19,20] and suggest a combination of zoletil/domitor as a suitable anaesthetic agent to study ECM-related genes in brain tissue. This anaesthesia regimen is relatively neutral to the expression of brain PGs, whereas gas anaesthesia with sevoflurane inhibits expression of CD44 in off-spring rats from the sevoflurane-exposed mothers [13] and reduces glycocalyx shedding through the washout of syndecan-1 and heparan sulfate in the postischemic coronary bed of isolated guinea pig hearts [14]. Also, it gives preference in routine experimental practice because the injection anaesthesia does not require expensive gas anaesthesia apparatus and can be performed at any laboratory.

According to our results, both single injection with zoletil/domitor and single irradiation of mouse brain with 7Gy dose do not significalty affect the morphology of the brain tissue or transcriptional activity of brain ECM-related genes, and observed moderate changes (if any) are diminished by 72h after irradiation. It confirms the similarity of this mouse single-irradiation protocol to that used for clinical irradiation during anti-glioblastoma radiotherapy and suggests its suitability for multiple-irradiation radiotherapy experiments.

5. CONCLUSION

Here, we suggest a new mouse model *in vivo* to study radiation effects on brain extracellular matrix. The setup conditions for irradiation regimen include injection anaesthesia with zoletil/domitor combination, single irradiation dose 7Gy and confirm the suitability of synchrotron or clinical linear accelerators for this kind of research. The elaborated mouse model can be used to perform multiple-fraction irradiation of brain tissue, modelling adjuvant anti-glioblastoma radiotherapy in small animals and facilitate new radiotherapy-related studies.

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