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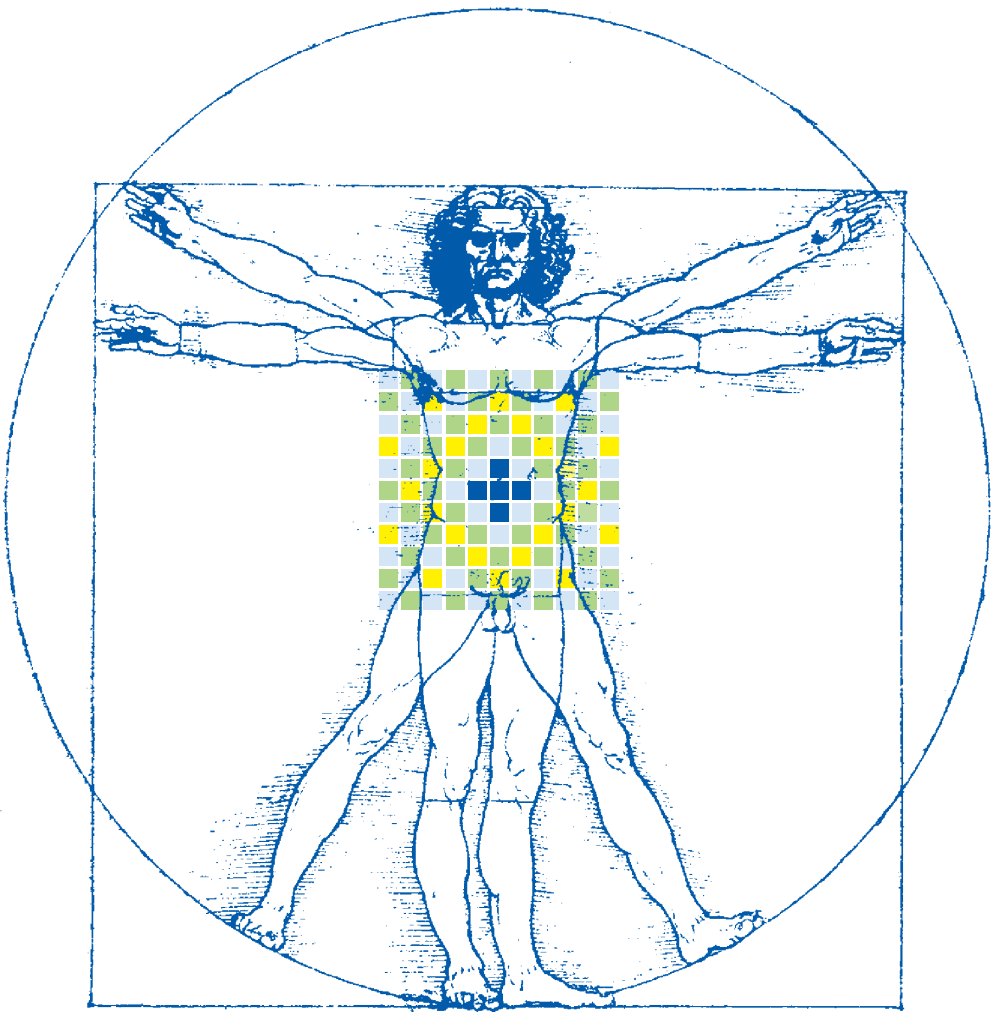

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ORIGINAL ARTICLE
PRECLINICAL IMAGING

In vivo bioluminescence imaging using orthotopic xenografts towards patient's derived-xenograft Medulloblastoma models

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ABSTRACT

BACKGROUND: Medulloblastoma is a cerebellar neoplasia of the central nervous system. Four molecular subgrups have been identified (MB_{WNT}, MB_{SHH}, MB_{group3} and MB_{group4}) with distinct genetics and clinical outcome. Among these, $MB_{group3-4}$ are highly metastatic with the worst prognosis. The current standard therapy includes surgery, radiation and chemotherapy. Thus, specific treatments adapted to cure those different molecular subgroups are needed. The use of orthotopic xenograft models, together with the non-invasive *in vivo* biolumiscence imaging (BLI) technology, is emerging during preclinical studies to test novel therapeutics for medulloblastoma treatment.

METHODS: Orthotopic MB xenografts were performed by injection of Daoy-luc cells, that had been previously infected with lentiviral particles to stably express luciferase gene, into the fourth right ventricle of the cerebellum of ten nude mice. For the implantation, specific stereotactic coordinates were used. Seven days after the implantation the mice were imaged by acquisitions of bioluminescence imaging (BLI) using IVIS 3D Illumina Imaging System (Xenogen). Tumor growth was evaluated by quantifying the bioluminescence signals using the integrated fluxes of photons within each area of interest using the Living Images Software Package 3.2 (Xenogen-Perkin Elmer). Finally, histological analysis using hematoxylin-eosin staining was performed to confirm the presence of tumorigenic cells into the cerebellum of the mice.

RESULTS: We describe a method to use the *in vivo* bioluminescent imaging (BLI) showing the potential to be used to investigate the potential antitumorigenic effects of a drug for *in vivo* medulloblastoma treatment. We also discuss other studies in which this technology has been applied to obtain a more comprehensive knowledge of medulloblastoma using orthotopic xenograft mouse models.

CONCLUSIONS: There is a need to develop patient's derived-xenograft (PDX) model systems to test novel drugs for medulloblastoma treatment within each molecular sub-groups with a higher predictive value. Here we show how this technology should be applied with hopes on generations of new treatments to be applied then in human.

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Key words: Luminescent measurements - Medulloblastoma - Drug evaluation, preclinical - Mice.

Primary brain tumors are the second most common type of pediatric cancer causing morbidity and mortality. The incidence of brain tumors for patients between 0 and 19 years ranges from 3.3 to 4.5 cases per 100,000 patients/year.[1] The therapy for pediatric brain tumors is still challenging because of the poor response to conventional cytotoxic chemotherapeutics agents and radiation therapy with the therapy-related morbidity that is a huge issue in children.[2] Medulloblastoma (MB), a primitive neuroectodermal tumor of cerebellum, constitutes 20% of all childhood primary neoplasms of the central nervous system (CNS).[3] This cerebellar neoplasm arises in the posterior fossa [4] originating from the granule neuron precursor cells (GNPs) in the external granular layer (EGL) at the surface of the developing cerebellum.[5] MB is diagnosed in patients under 15 years of age (70% of cases), with a peak age at presentation in 3-6 years old children.[6] MB exhibits a tendency

to metastasize in the subarachnoid space in about 35% of children with MB.[4] Studies of transcriptional profiling of primary MB indicated the existence of four distinct molecular clonal genetic subgroups differing from each other with respect to genetics, epigenetics, gene expression, histology, and clinical outcome: MB_{WNT}, $MB_{SHH,}$ MB_{group3} and MB_{group4}.[7, 8] Taylor *et al*.,[8] reported the current consensus correlating the biological features of the four molecular subgroups to patient demographics, tumor-cell histology and most importantly patient outcome. Within these molecular subgroups, the MB_{WNT} group have favourable patient outcome with an overall survival rates >95%. The MB_{SHH} group shows intermediate prognosis, with an overall survival rates from 60% to 80% and with low frequency to metastatize.[8] Instead, $MB_{Group3/4}$, are highly metastatic showing activation of different pathways including MYC, TGF-β and Otx2 signaling for MB_{Group3}, or mutations in CDK6 and MYCN oncogenes for MB_{Group4}.[7, 9, 10] MB_{Group3} accounts for a quarter of all MB, and carries the worst prognosis, while MB_{Group4} is the most common MB with an intermediate prognosis.[8, 11] The molecular stratification of patients with MB has not yet been routinely implemented in the clinic, and the use of targeted therapy is still at the beginning.[11, 12] To date, the standard therapy includes initial surgery followed by radiation and chemotherapy. Despite the acceptable survival rates with current multimodality treatment, patients encounter devastating morbidity, including permanent neurocognitive dysfunctions [13] and secondary malignancies.[14] Thus, new treatment options, more effective and less toxic, are needed.

Despite new insight into the biology of cancer, novel therapeutic strategies fail. This is mostly due to the poor clinical predictive power of the model systems used to test the novel antitumorigenic drugs. When evaluating our approach to target discovery, we should consider if the drugs are being tested on model systems that have a clinical predictive power.

Orthotopic MB xenograft murine models, consisting in human MB tumorigenic cells transplanted into the cerebellum of immunocompromised mice,[15] have been shown to have advantages in evaluating the responses to a drug [15] with respect to those genetically engineered MB mouse (GEM) models.[5, 15-20] In fact, "orthotopic" tumor xenografts retain the genetic and epigenetic abnormalities found in MB patients and reproduce the tumor microenvironment with the exception of T-cell populations due to the use implantation in athymic nude mice.[15]

However, the use of murine xenograft using human tumorigenic cell lines has a variable predictive power during the translation of cancer therapeutics into clinics. A more clinically predictive models of human cancer is obtained when primary tumors are used as an orthotopic xenograft (also known as Patient-Derived Xenografts or PDXs).[16] Orthotopic PDX models are based on the transfer of primary tumors directly from the patient into the organ of choice of an immunodeficient mouse.[16] This orthotopic PDX models accurately mimic the human tumors from which they are derived in terms of histology and gene expression profiles from mice to patients. The major challenges of these orthotopic models (xenograft and PDX) for brain tumors are due to the surgical technique for tumor implantation and the difficulty for following tumor growth, during the early stages of tumorigenesis.

Therefore, the use of *in vivo* imaging technologies is of importance to follow tumorigenesis using these orthotopic xenograft and PDX models during preclinical studies. Several tecniques have been recently described: X-ray, computed tomography (CT), ultrasound (US), magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT), positron emission tomography (PET) and bioluminescence imaging (BLI).[21]

Among these, *in vivo* BLI technology is emerging as a "non-invasive" detection method that allows disease-specific treatment strategies to be evaluated during preclinical studies.[22] BLI is a technique based on the detection and quantification of the light emitted from the conversion of chemical into photon energy. Bioluminescence signal is produced by the ATP-dependent conversion of Luciferin by Luciferase enzyme into the light-emitting product oxyluciferin, that is then externally captured by the highly sensitive charge-coupled device (CCD) camera.[23] In details, *in vivo* bioluminescent imaging (BLI) detects bioluminescent light produced by several luciferase enzymes, such as Firefly, Renilla, Gaussia, Metridia, Vargula, or bacterial Luciferase in the presence of Luciferins. Among these, Firefly Luciferase (Luc) has been widely used. It catalyzes the oxidation of Luciferin (D-(−)-2-(60-hydroxy-20 benzothiazolyl)-thiazone-4-carboxylic acid) in presence of molecular

oxygen and adenosine triphosphate to generate CO_2, AMP, PPi, oxyluciferin emitting a yellow-green light at a wavelength of 562 nm. The spectrum of the emitted light can be revealed and quantified with ultra-sensitive cooled CCD cameras.[23, 24] Indeed, BLI, exhibiting minimal background signals, is a highly sensitive technique for studying cell proliferation and migration *in vivo* in a specific anatomical site.[25, 26] Moreover, the luminescence is due to an ATP-dependent reaction, and therefore, only the metabolically active cancer cells provide bioluminescence production. For the above reasons, BLI offers an effective and valuable preclinical instrument to investigate distinct biological processes and to explore human diseases, including tumors growth and metastases. Importantly, due to the lack of invasiveness, this BLI approach can reduce the number of animals during the experiments. Moreover, after mice sacrifice, the experiments can be also performed *ex vivo* using BLI, thus eliminating pain, suffering or distress in the experimental protocols. This is in agreement with the ethical use of animals in testing (3RS).[27] This technology has been applied to study the potential therapeutics in several brain tumors, including MB.[28-31]

Here, we focus our attention on the role of BLI method to study MB using orthotopic xenograft mice models. This technology is of importance to study MB progression and to evaluate the potential antitumorigenic effects of novel and less toxic compounds. This method will also allow novel drugs, including natural agents with a mean of chemo-adjuvant therapy, to be tested for pediatric MB treatment.

Materials and methods

Generation of Daoy-Luc cells

MB_{SHH} Daoy cell lines were grown in Minimum Essential Medium (Euroclone, Milan, Italy) supplemented with 10 % (v/v) fetal bovine serum (Euroclone), 2 mM L-glutamine (Euroclone), and 1 % (v/v) antibiotics (10,000 U/ml penicillin, 10 mg/ml streptomycin (Euroclone). The cells were grown at 37° C in a humidified atmosphere of 95% air and 5% CO_2 (v/v).

For lentiviral infection, Daoy cells (20^4) were plated in complete medium into 1 well of a 24 well-plate for 24 hr; the medium was then replaced with 500 µl of fresh complete medium containing hexadimethrine bromide (Polybrene) at a final concentration of 4 µg/mL; the appropriate amount of viral particles (50 multiplicity of infection [MOI]/cell) encoding Firefly Luciferase gene (RediFect Red-Fluc-Puromycin, CLS960002, by Perkin Elmer, Waltham, MA, USA) was added to the medium. After 3 days, the medium was replaced and the Puromycin Dihydrochloride (A1113802, Thermo Fisher) was added at a final concentration of 0.5 ug/mL to select only the transduced cells.

Ethical approval for mouse use: *Ministero della Sanità* 546/2015 PR released to the Director of Studies, Massimo Zollo, 19/06/2015, art. 31 D.lgs. 26/2104.

Ortotopic xenograft implantation into fourth right ventricle of cerebellum of nude mice

To establish intracerebellar DAOY-Luc xenograft models, 6 weeks old nude mice were anesthetized with Tribromoethanol (Avertin®) [Sigma-Aldrich, Saint Louis, MO, USA #T4, 840-2] (50 mg/kg); after this, the mice were placed onto a stereotactic frame by hooking its incisors onto the frame hold. A small skin incision (1 mm) and a burr hole (0.7 mm in diameter) were created with a microsurgical drill (Fine Science Tools, Foster City, CA, USA). Daoy-Luc cells (10^5) were suspended in 5 µL PBS and injected slowly, through the burr hole, into the right cerebellar hemisphere (stereotactic coordinates from bregma, anteroposterior 5.5 mm; right lateral 2.1 mm; dorsoventral 5.0 mm), with a steady force in a 30 second time frame of the recipient, using a 10-AL, 26-gauge Hamilton Gastight Model 1701RN (Sigma-Aldrich) syringe needle that was inserted perpendicular to the cranial surface. The needle was left in its place after completion of injection for additional two minutes and then the needle and syringe were removed, and the incision was sutured using Safil polyglycolic acid synthetic absorbable suture (6/0). Later, mice were kept on a warming blanket after the surgery to help maintain its body temperature. Mobility and respiratory patterns were observed continuously and once the mouse had recovered from anesthesia, were place in a sterile housing cage. The animals were then monitored daily for development of potential neurological deficits.

Acquisition of BLI

Seven days after the implantation of the cells, the mice were imaged, and tumor growth was evaluated by

acquisitions of BLI using IVIS 3D Illumina Imaging System (Xenogen/Caliper).

For the acquisitions, the mice were anesthetized by inhalational of Isofluorane and D-Luciferin (122799, Perkin Elmer) (15 mg/ml stock) was intra peritoneally injected (100 µl per 10 g body weight) after 5 minutes from Luciferin injection, they were imaged for 1.30 min. Several acquisitions were made per mouse till each mouse exhibits its peak of photon emission. Then n. 5 total acquisitions (weekly for a total of 28 days of analysis), near to the peak value, for each mouse were analyzed.

To quantify the bioluminescence, the integrated fluxes of photons (photons per sec) within each area of interest were determined using the Living Images Software Package 3.2 (Xenogen-Perkin Elmer).

Histological analyses with hematoxylin-eosin stainings

Following sacrifice, fresh brain was removed from the skull, washed in iced phosphate buffer saline (PBS; ECB4004L, Euroclone, Pero, Milan, USA), fixed with 4% paraformaldehyde (PFA, 387507, Carlo Erba, Milan, Italy) and embedded into paraffin (Lab-O-Wax, R0040, Hysto-line laboratories, Milan, Italy). Sections of 3 µm in thickness were cut through the cerebellum using a microtome (RM2125RT, Leica, Wetzlar, Germany). The sections were then deparaffinised using Bioclear (06-1782D, Bio-Optica, Milan, Italy) and placed in a solution consisting of absolute Methanol (32213, Sigma-Aldrich) and 0.3% hydrogen peroxide (216763, Sigma-Aldrich) for 15 min. They were then washed in PBS and processed with hematoxylin-eosin staining for microscopic examimation. Subsequently, the slides were washed in PBS, dehydrated with alcohol andxylene, and mounted with cover slips using a permanent mounting medium (Permount, ProSciTech, Kelso, Australia). Micrographs were taken with a Leica DFC320 digital camera.

Results

MB tumorigenesis is followed in vivo *through BLI in orthotopic xenografts*

The use of BLI technology is of importance to unravel MB biogenesis and to test the clinical response to novel drugs in different MB mice models. For this purpose, MB_{SHH} Daoy cells were engineered to express Firefly Luciferase (Luc) gene by using Lentiviral particle (Redifect Red.Fluc-Puro, CLS960002, Perkin Elmer). This allowed us to select only Firefly Luciferase positive cells (Daoy-Luc), which were then implanted into the cerebellum of ten nude mice (as described in details in Material and Methods section). Daoy-Luc cells were directly injected into the fourth right ventricle of cerebellum using the following stereotactic coordinates from Bregma: anteroposterior 5.5 mm, right lateral 2.1 mm, dorsoventral 5.0 mm (as described in "Materials and methods" section).

Tumor growth was monitored from the time of implantation (T0) up to 4 weeks (T4) through *in vivo* bioluminescent imaging (BLI), as shown in Figure 1A, B. *BLI* images were captured every week, as clearly shown in Figure 1B. Subsequently, the number of photons/seconds in the Region Of Interest (ROI) was also measured to quantify the BLI signals (see Table I for BLI values) in order to perform statistical analysis using the analytics software SPSS, as shown in Figure 1C. Our statistical analyses (N. 10 mice group dataset) demonstrated that BLI signals gradually increase from the second week (T2) up to the fourth week (T4) after the implantation. In details, statistically significant differences in tumor growth were found in the comparison between those groups of mice analysed between T2, T3, T4 and T0 ($P<0.017$, $P<0.000$, $P<0.000$). There were no differences when BLI values taken in the first week (T1) compared with those from T0. This is due to a temporary loss of engraftment and some cellular deaths observed of those cells implanted in early phases of tumorigenesis in the recipient cerebellum.

After four weeks, mice were sacrificed due to evident signs of illness and pain for the tumor burden evaluated by photon/sec emission data. The brain and the cerebellum were collected and tumor sections were used to perform histopathological analysis. The presence of tumorigenic cells within the cerebellum was confirmed through hematoxylin/eosin staining, as shown in Figure 1D.

Discussion

Our data present a non-invasive technology "BLI" as an efficient method to follow the tumor progression *in vivo*. Importantly, the use of BLI imaging technology, applied to these orthotopic xenograft models, permits to

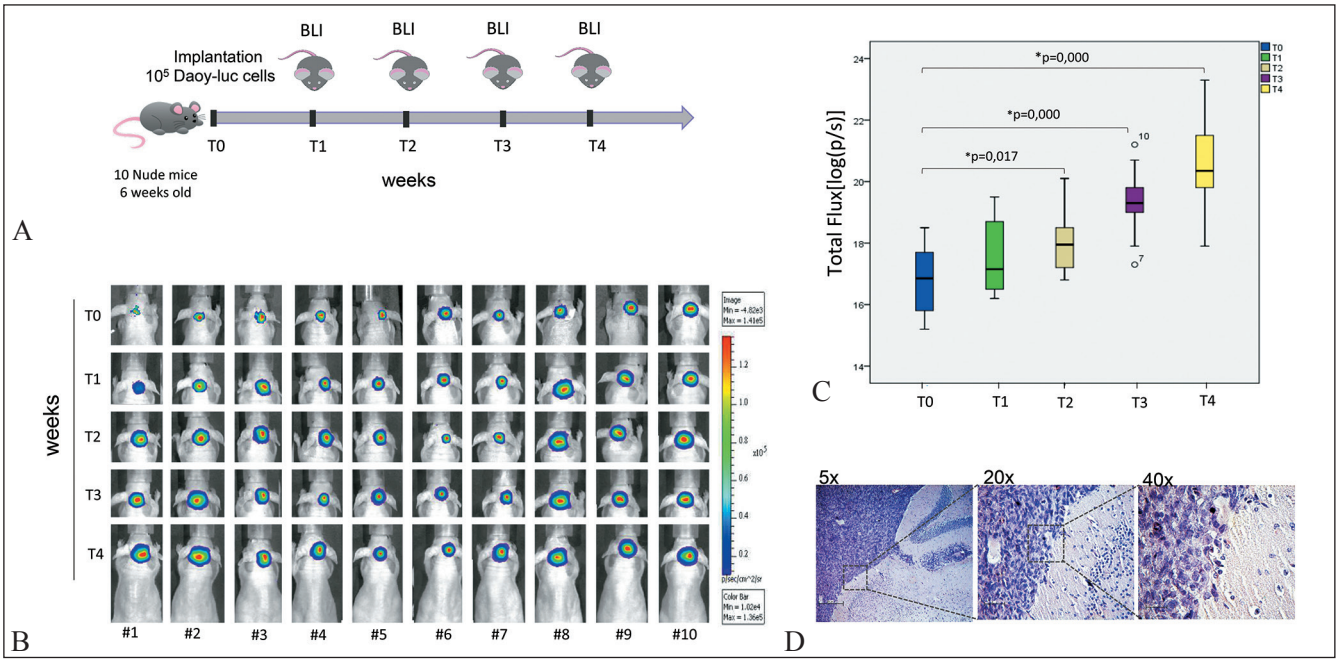

Figure 1.—Orthotopic xenograft of MB_{SHH} model. A) Representative scheme for the orthotopic xenograft experiment. MB_{SHH} Daoy-Luc cells (10^5 cells) were implanted in the cerebellum of nude mice at T0. Starting after 7 days from the implantation, the mice were imaged weekly using IVIS Spectrum In Vivo Imaging System. After 28 days, the mice were sacrificed due to the tumor burden; B) Representative orthotopic xenograft experiment, following the injection of ten nude mice with MB_{SHH} Daoy-Luc cells (10^5 cells), previously infected with a lentiviral particle (100 MOI) encoding Firefly Luciferase gene (Redifect Red.Fluc-Puro, CLS960002, by Perkin Elmer), within the fourth ventricle of nude mice (ten mice were used); C) BLI analysis of photon emission from the cerebellum of the mice orthotopically injected with MB_{SHH} Daoy-Luc cells was performed using the statistic software SPSS. BLI images were taken weekly from the time of implantation (from T0 to T4). The trend of the tumor growth is presented in the Box-Plot generated with SPSS software. The differences in Total Flux (photon per second) are statistically significant from the second week from the implantation with respect to T0 (P-value <0.05). In details, the values from T2, T3 and T4 were compared with T0; D) Hematoxylin/Eosin staining of cerebellar tumors generated by implanting Daoy-Luc cells into the fourth ventricle of nude mice was shown (5X, 20X and 40X). The staining detects the presence of tumorigenic cells and the tumor burden within the cerebellum of the implanted mice.

TABLE I.—*Total flux values from $_{MBSH}$H DAOY orthotopic xenograft models.*

Mice number	Mean log2 [tot Flux (p/s)]				
	T-0	T-1	T-2	T-3	T-4
1	1.52E+01	1.65E+01	1.75E+01	1.98E+01	2.33E+01
2	1.55E+01	1.62E+01	1.75E+01	1.90E+01	2.09E+01
3	1.58E+01	1.76E+01	1.84E+01	1.93E+01	1.98E+01
4	1.63E+01	1.80E+01	1.84E+01	1.93E+01	1.98E+01
5	1.68E+01	1.67E+01	1.72E+01	1.90E+01	2.07E+01
6	1.72E+01	1.65E+01	1.68E+01	1.79E+01	1.79E+01
7	1.69E+01	1.64E+01	1.68E+01	1.73E+01	1.87E+01
8	1.77E+01	1.87E+01	1.85E+01	1.93E+01	2.00E+01
9	1.79E+01	1.94E+01	2.01E+01	2.07E+01	2.15E+01
10	1.85E+01	1.95E+01	1.98E+01	2.12E+01	2.17E+01

The quantified bioluminescence values (photons per sec) are reported in the table. Mice (1-10) were imaged weekly (from T0 to T4), and tumor growth was evaluated by acquisitions of bioluminescence imaging (BLI) using IVIS 3D Illumina Imaging System. The integrated fluxes of photons (photons per sec) within each region of interest (ROI) were determined using the Living Images Software Package 3.2 (Xenogen-Caliper, Alameda, CA, USA).

study the response to novel therapeutic regimens *in vivo* in terms of tumor growth/regression by quantifying the BLI results (ROI).

BLI technology has been applied to several orthotopic MB xenograft models to study *in vivo* the response to novel therapeutic regimens, including natural agents,

in terms of tumor growth/regression. The introduction of natural agents into cancer treatments is emerging a helpful strategy that meets the need to develop novel antitumorigenic drugs with low toxicity.[32] Recently, the antitumorigenic action of Norcantharidin, a natural compound belonging to the most emergent antitumorigenic agents,[32] has been found to inhibit the growth of intra-cerebellum tumors *in vivo* using orthotopic MB xenograft mice by measuring bioluminescence emission (BLI) levels.[33] This preclinical study, using BLI method, enhances the potential for the future use of this natural compound to treat children affected by MB.

BLI technologies have been also used to study the synergistic combination of novel antitumorigenic drugs and radiotherapy. Through the use of BLI, the role of Celecoxib, a selective COX-2 inhibitor, in enhancing the effects of ionizing radiotherapy (IR) on tumor growth has also been described in orthotopic MB xenografts.[34]

BLI tecnique is also of importance for translational research on MB metastases, which are the primary cause of treatment failure in MB. To this purpose, BLI method has been applied to generate an experimental mouse model simulating the leptomeningeal dissemination (seeding) of MB by intra-cisternal inoculation of human MB cells.[35] Importantly, through BLI, these "metastatic MB cells" can be monitored *in vivo* during the spreading and the seeding. The establishment of this MB seeding model [35] through the use of BLI, is a valuable resource for future translational research to test novel antimetastatic drugs on the extent of tumor spread in live condition, especially for the treatment of metastatic $MB_{group3/group4,}$ as recently demonstrated.[31]

Conclusions

In vivo BLI technology in GEM, orthotopic xenografts and PDX models, is useful for enhancing our understanding of MB development and treatment. GEM are useful models for examining the role of specific genes during tumor development and progression, while the orthotopic xenograft and PDX models are more suitable for predicting drug response to novel anticancer therapeutics.

However, most novel anticancer therapeutics fail, upon reaching Phase III clinical trials. Because MB subtypes are clinically, transcriptionally and genetically distinct, they should be targeted individually. It should be important to have murine models representative of all the molecular subtypes to test novel targeted therapies in appropriate animal models. Human gene expression signatures faithfully replicate the biology of primary tumors from which they are derived.[36] This supports the use of subgroup specific orthotopic MB PDX models for preclinical drug screenings for MB treatment.

In conclusion, orthotopic MB PDX models could overcome the limits of GEM and orthotopic xenograft models. The challanges of PDX are the difficulties for the orthotopic primary tumors implantation and for the following of tumorigenesis *in vivo*. Their use requires molecular imaging studies to verify the location of tumor grafts after implantation. The growing development of molecular imaging will allow us to develop novel drugs, specific for MB molecular subgroups using these MB PDX orthotopic models, to obtain a very high predictive clinical response.

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Conflicts of interest.—The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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