Noninvasive Visualization of Retinoblastoma Growth and Metastasis via Bioluminescence Imaging

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PURPOSE. To establish human retinoblastoma (RB) animal models that allow sensitive, noninvasive and continuous monitoring of tumor growth and metastasis in vivo.

METHODS. The human RB tumor cell lines HXO-Rb44 and Y79 were engineered to express a fusion reporter gene allowing for bioluminescence and fluorescence imaging. Intraocular and metastatic tumors were induced in immunodeficient nude mice by injection of bioluminescent RB cells into eye compartments and into the left ventricle or tail vein. The growth kinetics of intraocular and metastatic tumors was quantitatively and continuously monitored via bioluminescence imaging (BLI).

RESULTS. Intraocular injection of HXO-Rb44-GFP-luc cells resulted in 100%, 80%, and 80% successful RB tumor development in the anterior chamber, vitreal cavity and subretinal space, respectively. The subretinal injection of Y79-GFP-luc cells resulted in 100% tumor development. BLI signal intensity correlated with the number of tumor cells injected as well as the weight of the tumor-bearing eyes. After bilateral subretinal injection of HXO-Rb44-GFP-luc cells, one of six RB tumor mice developed brain metastasis. Intracardiac injection of HXO-Rb44-GFP-luc cells resulted in metastatic disease in 9 of 15 nude mice, whereas tail vein injection resulted in metastasis in 1 of 16. Metastases were developed in multiple organs, including lymph nodes, bone, and brain, resembling the metastatic profile in patients with RB.

CONCLUSIONS. BLI allowed sensitive, noninvasive, and quantitative localization and monitoring of intraocular and metastatic RB tumor growth in vivo and thus may be a useful tool to study RB biology as well as anti-RB therapies. (Invest Ophthalmol Vis Sci. 2009;50:5544–5551) DOI: 10.1167/iovs.08-3258

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Retinoblastoma (RB) is the most common primary intraocular malignant tumor in children. RB tumors are generally aggressive and occur in sporadic (mostly unilateral, unifocal), and hereditary (often multifocal and/or bilateral) forms and usually affect children younger than 5 years.1,2 Early diagnosis is crucial for successful treatment of RB.3 Enucleation is usually performed for larger RB tumors. Small tumors are treated with conservative therapeutic approaches, including chemother-apy, radiotherapy, laser therapy, and cryotherapy. For large tumors, enucleation is inevitable. The forming of distant metastases is the major cause of death.4 Therefore, early detection of intraocular tumors and distant metastases is a key prognostic factor for preservation of vision, eye retention, and survival.

RB is a prototype of human hereditary tumors, in which one allele of the RB1 tumor suppressor gene is inactivated in all somatic cells. On spontaneous inactivation of the second allele in a retinocyte, RB is formed. The gene product of RB1 is a key regulator of cell cycle progression.5,6 RB1 gene mutations are implicated in carcinogenesis of many types of cancer such as breast cancer, ovarian cancer, bladder cancer, and small cell lung cancer.7,8 Therefore, RB animal models are important tools for the study of molecular mechanisms of human cancer in general and the investigation of the efficiency of novel treatments for RB in particular.

In the past, various RB animal models have been established, of which the Y79 model and the WERI-Rb model are most widely used. The former closely resembles metastatic human RB, whereas the latter mimics nonmetastatic lesions.9 However, to obtain information about growth dynamics and metastasis, tumor-bearing animals must be killed and subjected to histologic analysis, requiring a large number of animals. In addition, micrometastases and small tumors are difficult to detect histologically, requiring labor intensive screening of thin whole body slice preparations. A variety of methods have been developed that partly overcome these limitations, and some have permitted noninvasive longitudinal studies for orthotopic tumor growth and distal metastasis. These include microcomputed tomography (microCT), magnetic resonance imaging (MRI), positron emission tomography, and fluorescence imaging. Bioluminescence imaging (BLI), a newly developed technology, presents several advantages over these methods. Advantages include high sensitivity, high-throughput, short imaging time, ease of operation, and lower cost.10,11 All these features make BLI a particularly attractive imaging strategy for monitoring intraocular tumor growth and distant metastasis.12,13

In the present study, we used a dual optical reporter gene, encoding both enhanced green fluorescence protein (EGFP) and luciferase, to label human RB tumor cell lines, HXO-Rb44 and Y79. The EGFP reporter facilitates the selection of in vitro transduced cell populations by epifluorescent microscopy or fluorescence-activated cell sorting (FACS), whereas luciferase is a sensitive and convenient reporter for noninvasive quantitative detection of tumor growth and metastasis in vivo. The new RB-BLI models could serve as useful tools for the understanding of the early events during RB tumor growth and...
metastasis in vivo as well as for the preclinical assessment of novel anti-RB therapeutics.

METHODS

Animals
Male BALB/c nude mice were obtained from the Shanghai SLAC Laboratory Animal Co. (Shanghai, China). Animals were maintained in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research, Association for Research in Vision and Ophthalmology. All mice were 6 to 8 weeks of age at the time of tumor cell injection.

Generation of Stable Transfectants Expressing Luciferase and EGFP Fusion Protein
The human RB cell line HXO-Rb44, which was originally established by Hunan Medical University, Xiangya Hospital, Department of Ophthalmology, and therefore named HXO-Rb44, was isolated from a 2.5-year-old patient with bilateral RB with no family history.12 The right eye was enucleated because of secondary glaucoma and used for establishment of RB the cell line. The histologic examination revealed that it was poorly differentiated and no RB protein was detected by immunohistochemical staining.13,14 Y79 cells were obtained from Nanjing KeyGen Biotech. Co. Ltd., Nanjing, China. Both cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO2. Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). To obtain bioluminescent cells, we transfected HXO-Rb44 and Y79 cells with a plasmid carrying an EGFP-luciferase fusion gene, according to manufacturer’s instructions (Lipofectamine; Invitrogen). The transfectants were selected with 0.5 μg/mL geneticin (G418; Invitrogen). Positive clones were obtained by limiting dilution and detected by GFP and bioluminescence imaging. Stably transfected HXO-Rb44-GFP-luc and Y79-GFP-luc cells maintained GFP and luciferase expression when grown in RPMI-1640 medium containing 10% FBS and reduced geneticin (0.1 mg/mL). The growth rate and morphology of both HXO-Rb44-GFP-luc and Y79-GFP-luc cells were indistinguishable from their parent cells. The clones, HXO-Rb44-GFP-luc-16 and Y79-GFP-luc-F10D10, with stable and high level expression of EGFP and luciferase were selected for further studies in vitro and in vivo.

Establishment of RB Orthotopic Tumor Models in Mice
RB orthotopic tumor models were established by inoculation of bioluminescent RB cells into the subretinal space, vitreal cavity, or anterior chamber. The mice were anesthetized with intraperitoneal (IP) injection with pentobarbital (50 mg/kg body weight) in combination with topical application of 0.4% oxybuprocaine hydrochloride (Santen Pharmaceuticals, Osaka, Japan). The pupil was dilated with a 0.5%/0.5% tropicamide/phenylephrine hydrochloride eye drop (Santen Pharmaceuticals). A plastic ring filled with 2.5% cellulose was placed on the cornea to aid visualization of the fundus. Injection was performed under a binocular surgical microscope. The initial puncture was made through the sclera behind the limbus with a 28-gauge beveled needle. 1 to 2 x 106 bioluminescent RB cells (1 x 106 for HXO-Rb44-GFP-luc cells and 2 x 105 for Y79-GFP-luc cells) in 2 μL PBS were injected slowly into the vitreal cavity or subretinal space of the left eye with a 32-gauge needle attached to a 10 μL microsyringe. The right eye received no injection and served as an internal control. To mimic bilateral RB, we subretinally injected a group of six mice bilaterally with 1 x 105 HXO-Rb44-GFP-luc cells in each eye. Injection of the tumor cells into the anterior chamber was performed as described by Niederkorn et al.16 HXO-Rb44-GFP-luc cells (1 x 105) in 2 μL PBS were injected into the anterior chamber of the left eye with a 32-gauge needle. The growth dynamics of intraocular RB tumor cells in vivo were monitored by BLI. At the end of observation, the animals were imaged and the tumor-bearing eyes were enucleated, weighed, and prepared for histologic analysis.

Establishment of a Distant Metastases Model of RB
A model for systemic metastasis of RB was generated by intracardiac injection or tail vein injection of bioluminescent RB tumor cells. A cell suspension of HXO-Rb44-GFP-luc (1 x 106/100 μL PBS) was prepared immediately before injection. Mice were anesthetized by IP injection with pentobarbital (50 mg/kg body weight). HXO-Rb44-GFP-luc cells (1 x 106) in 100 μL PBS were inoculated into the left ventricle through the left-front chest according to the method described by Arguello et al.17 To maximize efficacy, we completed the nonsurgical injection procedure for each mouse within 60 seconds, interrupted by three to four short pauses in consideration of the small cardiac size of the mice. To verify whether the injection was successful, we performed BLI immediately after injection. A satisfactory injection was indicated by a thoracic/whole-body (T/WB) ratio less than 0.5 (see Fig. 3A) in accordance with Drake and colleagues’ criteria.8,9 For tail vein injection, mouse tails were placed in 40°C warm water to cause vasodilation, facilitating injection procedure. HXO-Rb44-GFP-luc cells (1 x 106) in 100 μL PBS were injected into the lateral tail vein. BLI was performed immediately after injection. Forming of metastases was evaluated by BLI on the first day and once a week for up to 5 to 6 weeks after tumor cell injection.

Bioluminescence Imaging
BLI was performed according to the manufacturer’s instructions (NightOwl LB 981 Molecular Imaging System; Berthold Technologies, Bad Wildbad, Germany). To illustrate that the luminescence is proportional to the number of cells injected and to determine the detection limit, we serially diluted HXO-Rb44-GFP-luc and Y79-GFP-luc cells into 96-well plates. D-luciferin (Molecular Imaging Products, Ann Arbor, MI) at a final concentration of 150 μg/mL was added to each well, and the plates were imaged for 10 minutes. In addition, various amounts of HXO-Rb44-GFP-luc cells were injected into the subretinal space to determine the detection limit in vivo. For in vivo imaging, the mice received an IP injection of 100 mg/kg D-luciferin in 100 μL PBS. After 5 minutes, the mice were anesthetized via IP injection of pentobarbital (50 mg/kg body weight) or 1–3% isoflurane inhalation. The exposure time ranged from 1 second to 10 minutes, depending on the intensity of bioluminescence emitting from the tumor cells. For further confirmation of tumor metastatic foci, ex vivo imaging was performed. Mice with positive metastatic bioluminescence signals were killed, the suspected metastatic tissues or organs were removed and placed in individual wells of a 24-well plate containing 150 μg/mL D-luciferin in PBS, and then imaged. Solid tissues with luminescent tumor cells, confirmed by ex vivo BLI, were subsequently fixed for histologic evaluation. The bioluminescent images were pseudocolored and superimposed on conventional photographs acquired before BLI. Regions of interest (ROI) were drawn around the tumor sites and quantified as photon counts per second.

Histologic Analysis
To confirm the presence of metastatic RB tumor cells, we excised tissues with detectable BLI emission signals and prepared them for histologic analysis. To harvest metastatic sites in the brain, we killed the mice with positive BLI signals by cardiac perfusion with 0.9% saline followed by 4% paraformaldehyde. The skull was removed and the brain was plated under a fluorescence stereoscope to locate sites with GFP expression. In addition, skull, femur and maxilla were fixed with 4% paraformaldehyde and then decalcified by immersion in 10% hydrochloride for 24 hours. Other soft tissues including lymph nodes were fixed with 4% paraformaldehyde. Other procedures, including paraffin embedding, sectioning, hematoxylin and eosin (H&E) staining, and immunohistochemical staining for GFP, were performed accord-
ing to standard protocols. For immunohistochemistry a mouse anti-GFP monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a concentration of 1:25.

**Statistical Analysis**

Regression plots were used to describe the relationship between bioluminescence intensity and the number of tumor cells or the weight of tumor-bearing tissues. The \( r^2 \) values were presented to assess the quality of the regression model. For orthotopic RB models, the growth kinetics of RB tumors were analyzed by ANOVA followed by the post hoc Dunnett’s \( t \)-test. \( P/\alpha < 0.05 \) was considered statistically significant (SAS Institute, Cary, NC).

**RESULTS**

**Stable GFP and Luciferase Expression in HXO-Rb44 and Y79 Cells**

HXO-Rb44 and Y79 cells stably expressed GFP and luciferase after transfection with an EGFP-Luc dual reporter gene construct (pCMV-EGFP-Luc) and selection by G418. The proportion of GFP-positive cells in a selected clone (denoted HXO-Rb44-GFP-luc-16 and Y79-GFP-luc-F10D10), determined by flow cytometry analysis, consistently exceeded 98% during in vitro culturing for 2 months. There were no obvious changes in either growth rate or morphology in comparison with the parental cells.

**Correlation of Bioluminescent Intensity with the Number of Bioluminescent RB Cells In Vitro and In Vivo**

The ability to detect bioluminescent tumor cells in vitro and in vivo depends on the following three major factors: (1) intensity of luminescent signal from tumor cells (i.e., light emission per cell and the total numbers of tested cells), (2) sensitivity of the instrument, and (3) signal attenuation through tissue. To determine the efficiency and limitation of BLI both in vitro and in vivo, we plated a series of different numbers of cells into black 96-well plates and injected a series of different numbers of cells into the subretinal space. As shown in Figure 1, the luminescent emissions from HXO-Rb44-GFP-luc cells and Y79-GFP-luc cells, propagated in black 96-well plates, were efficiently detected by the molecular imaging system (NightOwl LB 981; Berthold Technologies) allowing detection of as few as 157 cells per well for HXO-Rb44-GFP-luc cells and 3125 cells per well for Y79-GFP-luc cells. Light emission per cell was 12.7 ± 4.4 photon counts/s for HXO-Rb44-GFP-luc cells and 5.05 ± 0.74 photon counts/s for Y79-GFP-luc cells.
FIGURE 2. Intraocular RB models of HXO-Rb+i-GFP-luc cells. Mice received intraocular injections of HXO-Rb+i-GFP-luc cells in the anterior chamber, vitreal cavity, or subretinal space. After inoculation, the mice were observed with BLI. At the end point of the observation period, the tumor-bearing eyes were enucleated and weighed, and the eyes and brains were subjected to histologic examination. (A) Serial images at different time points from a representative mouse in three different intraocular models. (B) Tumor growth curves obtained by quantification of bioluminescent signals in the eyes of individual mice. (C) On day 40, the tumor-bearing eyes were enucleated and weighed. The weight of the tumor-bearing eye (in grams) was correlated with the bioluminescence signal in each subject. (D) A representative photograph of H&E sections from three different intraocular RB models. (E) GFP immunohistochemical staining of three different intraocular RB models. (F) Image of mouse with bilateral RB and metastasis in the brain (arrow: metastatic focus). (G) H&E section from of brain tissue with metastatic RB tumor cells. T, tumor; C, cornea; R, retina; L, lens.
cells, calculated based on the ratio of detected photons per well to the total number of cells. Linear regression analysis showed a good correlation between the number of cells and photon counts per second (Fig. 1).

In vivo, similarly encouraging results were obtained for HXO-Rb44-GFP-luc cells, as shown in Figure 1C. BLI allowed detection of as few as 10,000 cells immediately after injection into the subretinal space. Linear regression analysis showed a significant correlation between the bioluminescence intensity of the eye and the number of cells in the subretinal space ($r^2 = 0.877$; Fig 1D).

**Monitoring Orthotopic Tumor Growth via In Vivo BLI**

As bioluminescent signals correlated well with the number of cells, we suggested that longitudinal quantification of bioluminescent signals emitted from tumor-bearing eyes could be used to determine tumor growth kinetics. Intraocular tumors, formed by inoculation of $1 \times 10^5$ HXO-Rb44-GFP-luc cells into the anterior chamber, vitreal cavity, and subretinal space, showed generally similar growth dynamics (Figs. 2A, 2B). A substantial initial reduction in signal intensity was observed in all three intraocular RB models, indicating death or clearance of a large proportion of tumor cells soon after intraocular injection. Thereafter, a steady increase in photon emission was observed, reflecting proliferation of the surviving tumor cells. Successful engraftment of transplanted HXO-Rb44-GFP-luc tumor cells was recorded in 100%, 80%, and 80% of recipients in the anterior chamber, vitreal cavity, and subretinal space of nude mice, respectively. After subretinal injection of $2 \times 10^5$ Y79-GFP-luc cells, we suggested that longitudinal quantification of bioluminescent signals emitted from tumor-bearing eyes could be used to determine tumor growth kinetics. Intraocular tumors, formed by inoculation of $1 \times 10^5$ HXO-Rb44-GFP-luc cells into the anterior chamber, vitreal cavity, and subretinal space of nude mice, respectively. After subretinal injection of $2 \times 10^5$ Y79-GFP-luc cells, luciferase signals were not detected until 2 weeks after injection, when the tumors had already grown to a significant size. However, tumors developed in 100% of mice injected with Y79-GFP-luc cells (Fig 3). At the end of the observation period, the tumor-bearing eyes were enucleated and weighed. In all intraocular models, the eyeball weight correlated with photon counts per second per eye obtained from in vivo BLI. The presence of human RB tumors was confirmed by histologic analysis and immunochemistry staining, and almost all RB tumor cells showed positive GFP expression (Figs. 2, 3).

**Detecting Systemic Metastases**

To establish a model of systemic metastases common in patients with RB, we injected HXO-Rb44-GFP-luc cells into the left ventricle or the tail vein. Satisfactory intracardiac injection was indicated by a thoracic/whole body (T/WB) ratio of less than 0.5 (Fig. 4A) as recommended by Drake et al.18 BLI was performed immediately after intracardiac injection, where the luminescent RB cells appeared as diffuse photon accumulations throughout the body of the animal, including the abdomen and testes. Photon emission appeared to be completely abolished 24 hours after injection. Metastatic tumor growth was tracked by serial imaging at weekly intervals. Nine of 15 mice that had received intracardiac injection subsequently developed metastases (Table 1, Figs. 4A, 4B).

Although direct invasive spread to the orbital tissue or the surface of the cornea had been observed in both orthotopic HXO-Rb44-GFP-luc and Y79-GFP-luc RB models, only one of six mice with bilateral RB tumors developed brain metastasis 10 weeks after subretinal injection of HXO-Rb44-GFP-luc cells, as detected by in vivo BLI and further confirmed by histologic examination (Figs. 2F, 2G). The growth pattern and metastasis of orthotopic RB tumor models from HXO-Rb44-GFP-luc and Y79-GFP-luc cells is summarized in Table 2.

Luminescence signals from metastatic foci were first detected on day 22 after intracardiac injection and steadily increased until the end of the observation period (Fig. 4C). Metastatic sites, detected by BLI on day 36 after injection, were located in the head (brain, maxilla, and skull), hind leg femur, and thoracic sites (Figs. 4A, 4B). Four mice had multiple metastatic foci. None of these metastatic foci was superficially palpable. The signal intensities of each separate metastatic focus increased with time (Fig. 4C). Ex vivo BLI or fluorescence stereoscope imaging was conducted with tissues excised from sites where in vivo BLI emission signals had been detected (Fig. 4B). The presence of RB tumor cells in these metastatic lesions was further confirmed by H&E and immunochemistry staining, and almost all RB tumor cells were GFP positive (Figs. 4D, 4E). The most common sites of metastatic lesions were the lymph nodes (7/15, 47%), bone (6/15, 40%), and brain (2/15, 13%) (Table 1). The intensity of in vivo bioluminescence from living mice and ex vivo bioluminescence from excised tissues correlated with the size of the metastatic lesions as detected by histologic analysis.

The distribution of tumor cells and the rate of metastatic foci formation after tail vein injection was quite different from that after intracardiac injection. Immediately after tail vein injection, bioluminescence signals accumulated in the thorax, indicating that the majority of injected cells became stuck in the lung during their first pass through its circulation (Fig 5A). After 24 hour, photon emission appeared to be completely abolished. Only 1 of 16 mice that had received a tail vein injection showed metastatic signals within the 6-week observation period, appearing on day 29 after injection and steadily increasing thereafter (Figs. 5B, 5D). Histologic analysis confirmed that RB tumor cells were present in the mandible (Fig 5E).


**DISCUSSION**

In this study we describe a new orthotopic and metastatic RB model that combines dual reporter gene luciferase-GFP-labeled human RB tumor cells with BLI technology and allows sensitive, continuous and noninvasive monitoring of intraocular tumor growth and metastasis in vivo.

In the past, efforts have been made to develop animal models for RB. Xenograft models have been created by injecting human RB tumor cells subcutaneously or into the eye

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**TABLE 1. Summary of Metastatic Foci after Intracardiac Injection in Nine Nude Mice**

<table>
<thead>
<tr>
<th>Location of Metastatic Foci</th>
<th>Metastatic Foci (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>2†</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>7 (axillary 2†; submandibular 2†; paraaortic 3†)</td>
</tr>
<tr>
<td>Bone</td>
<td>6 (maxilla 3†; skull 1†; femur 2†)</td>
</tr>
</tbody>
</table>

* Metastasis confirmed by histologic analysis (H&E staining).
† Metastasis confirmed by ex vivo BLI.
compartments of immunodeficient mice or rats. The widely used Y79 cell line is capable of developing metastasis in the brain parenchyma after injection into the vitreous. However, it fails to develop distant metastases in other organs commonly affected by human RB. Another widely used model cell line for human RB is WERI-Rb; however, this cell line does not form metastases as well.

We chose HXO-Rb44 and Y-79 cell lines to generate GFP and luciferase reporter carrying model cell lines for RB. Both of these cell lines are Rb protein negative and exhibit similar morphology and growth dynamics. As the luciferase detection limit for HXO-Rb4 cells compared to Y79 cells was 20 times higher in vitro, we chose to use the HXO-Rb44 cell line over these cell lines are Rb protein negative and exhibit similar morphology and growth dynamics. As the luciferase detection limit for HXO-Rb4 cells compared to Y79 cells was 20 times higher in vitro, we chose to use the HXO-Rb44 cell line over the better established Y79 cell line for our models for systemic metastases, allowing us to detect very early metastatic foci. We do not know why the rate of brain metastases with the HXO-Rb44 cell line was lower than that of Y79 model described by Chevez-Barrios et al., but we suspect that it may be due to different recipient mice. It has been reported that the Rag-2-knockout mice, used by Chevez-Barrios et al., lacked NK cells and therefore allowed more efficient metastasis from primary tumors. In contrast, the nude mice used in this study have been shown to have residual innate immune cells and therefore exhibit a reduced rate of metastasis.

Human retinoblastomas exhibit four patterns of invasion and metastasis: (1) direct invasive spread along the optic nerve to the brain, the orbital tissue, and adjacent bone; (2) spread via the circulating subarachnoid fluid tumor cells to the spinal cord, distant sites of the brain, and the contralateral optic nerve; (3) hematogenous dissemination causing widespread metastasis to the lungs, bones, brain, and other viscera; and (4) lymphatic spread, if the tumor is located anteriorly or massive extraocular invasion has occurred, tumor cells can spread via the lymphatic system. Because our RB cell line failed to form distal metastases after intraocular injection, we injected bioluminescent RB cells directly into the systemic circulation. Systemic injection of tumor cells resulted in metastatic disease with a pattern similar to human RB metastatic diseases. However, it has to be noted that, with the injection of tumor cells into the circulation, early steps of metastases such as localized invasion and invrasation are omitted in our models. As mentioned in the results, the majority of mice receiving intracardiac injection developed systemic metastatic disease (9/15) whereas of those mice receiving tail vein injection, only 1 mouse (of 15) actually developed metastasis. The most common site of metastases was the lymphatic system (47%), bone (40%), and brain (13%).

BLI imaging has some distinct advantages over histopathologic analysis for monitoring tumor growth dynamics. These advantages are as follows: (1) Noninvasive in vivo monitoring: BLI allows continuous noninvasive monitoring and precise quantification of in vivo tumor growth and metastasis. Tumor xenografts can be continuously followed up in the living mouse by using multiple imaging time points. In contrast to non-imaging-based methods that require killing the mice for each assessment time point, BLI imaging experiments require fewer animals per experimental group. (2) High sensitivity: BLI is very sensitive and detects a small number of luciferase reporter carrying cells. The metastatic lesion detected in this study was as small as 0.138 × 0.088 mm. Lesions of this size are impossible to detect with other imaging techniques such as CT or MRI. Further improvement in sensitivity and resolution of future BLI devices might allow the detection of smaller micrometastases in the micrometer range. (3) Good correlation between the signal and the number of cells: There is a good correlation between number of cells and the BLI signal. Changes of the BLI signal over time precisely reflect tumor growth or regression. As only living cells possess luciferase activity, necrotic parts of the tumor will not contribute to the overall BLI signal. (4) High throughput, fast, and easy to use:

**Table 2. Summary of Intraocular RB Models**

<table>
<thead>
<tr>
<th>Site of Injection</th>
<th>Number of Mice</th>
<th>Cell Line and Number of Cells Injected</th>
<th>Initial Occurrence of Luc Activity (Signal Intensity)*</th>
<th>Observation period (Signal at End of Observation Period)*</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unilateral subretina</td>
<td>5</td>
<td>HXO-Rb44-GFP-luc 1×10⁶</td>
<td>Day 0 (1e+4.70) ± (1e+0.63)</td>
<td>6 weeks (1e+6.38) ± (1e+1.59)</td>
<td>None</td>
</tr>
<tr>
<td>Unilateral intravitreous</td>
<td>5</td>
<td>HXO-Rb44-GFP-luc 1×10⁶</td>
<td>Day 0 (1e+5.00) ± (1e+0.67)</td>
<td>6 weeks (1e+6.08) ± (1e+1.45)</td>
<td>None</td>
</tr>
<tr>
<td>Unilateral anterior chamber</td>
<td>5</td>
<td>HXO-Rb44-GFP-luc 1×10⁶</td>
<td>Day 0 (1e+5.18) ± (1e+0.92)</td>
<td>6 weeks (1e+6.90) ± (1e+0.45)</td>
<td>None</td>
</tr>
<tr>
<td>Bilateral subretina</td>
<td>6</td>
<td>HXO-Rb44-GFP-luc 1×10⁶</td>
<td>Day 0 (1e+5.00) ± (1e+0.44)</td>
<td>10 weeks (1e+7.73) ± (1e+0.90)</td>
<td>1 (Brain)</td>
</tr>
<tr>
<td>Unilateral subretina</td>
<td>7</td>
<td>Y79-GFP-luc 2×10⁵</td>
<td>Day 14 (1e+3.68) ± (1e+0.54)</td>
<td>7 weeks (1e+6.56) ± (1e+0.36)</td>
<td>None</td>
</tr>
</tbody>
</table>

*Signal intensities are expressed as photon count/second ± SD.

**Figure 5.** Systemic metastasis model after tail vein injection with HXO-Rb44-GFP-luc cells. HXO-Rb44-GFP-luc (1 × 10⁶) cells were injected into the tail vein and followed up by BLI. (A) BLI images taken immediately after injection. (B) Serial images at different time points from the same mouse. (C) Ex vivo BLI of excised tissues to confirm the presence of metastatic lesions. (D) Growth curve of metastatic lesion generated by quantification of bioluminescent signals of various time points. (E) The metastatic lesion identified on H&E section. T, tumor; B, bone.
Commonly used BLI devices are able to examine five mice at a time in a few seconds to minutes.

Of course, BLI has its limitations. Compared with other imaging modalities such as CT or MRI, it has a much lower spatial resolution and can therefore not provide information about the shape or morphology of the tumor. Clearly, it cannot substitute for histologic analysis, but it is a very fast and sensitive method of screening for metastatic lesions, which then can be subjected to biopsy and further histologic analysis.

In conclusion, we think that the method described herein is a powerful tool for sensitive, continuous, and noninvasive monitoring of intraocular tumor growth and metastasis in vivo, which can be used to study the molecular mechanisms of tumor growth and engraftment of distal metastases as well as the efficacy of new therapies.

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**References**


