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Corresponding Author:	Zongjin Li Nankai University Tianjin, Tianjin CHINA
Corresponding Author's Institution:	Nankai University
Corresponding Author E-Mail:	zongjinli@nankai.edu.cn
Order of Authors:	Kaiyue Zhang
	Chen Wang
	Ran Wang
	Shang Chen
	Zongjin Li
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TITLE:

Dual Bioluminescence Imaging of Tumor Progression and Angiogenesis

AUTHORS & AFFILIATIONS:

Kaiyue Zhang[1], Chen Wang[1], Ran Wang[2], Shang Chen[1], Zongjin Li[1*]

[1]Nankai University School of Medicine, Tianjin, China
[2]State Key Laboratory of Medicinal Chemical Biology and College of Pharmacy, Nankai University, Tianjin, China

Corresponding Author:

Zongjin Li (zongjinli@nankai.edu.cn)

E-mail Addresses of Co-authors:

Kaiyue Zhang (zhangkaiyue177@163.com)

Chen Wang (wangchen_echo@163.com)

Ran Wang (546814212@qq.com)

Shang Chen (chenshang2010@163.com)

KEYWORDS:

tumor growth, tumor angiogenesis, breast cancer, tumor-bearing mice, HSV-ttk, bioluminescence imaging, Firefly luciferase, Renilla luciferase

SUMMARY:

This protocol describes the establishment of a tumor-bearing mouse model to monitor tumor progression and angiogenesis in real-time by dual bioluminescence imaging.

ABSTRACT:

Angiogenesis, as a crucial process of tumor progression, has become a research hotspot and target of anti-tumor therapy. However, there is no reliable model for tracing tumor progression and angiogenesis simultaneously in a visual and sensitive manner. Bioluminescence imaging displays its unique superiority in living imaging due to its advantages of high sensitivity, strong specificity, and accurate measurement. Presented here is a protocol to establish a tumor-bearing mouse model by injecting a Renilla luciferase-labeled murine breast cancer cell line 4T1 into the transgenic mouse with angiogenesis-induced Firefly luciferase expression. This mouse model provides a valuable tool to simultaneously monitor tumor progression and angiogenesis in real-time by dual bioluminescence imaging in a single mouse. This model may be widely applied in anti-tumor drug screening and oncology research.

INTRODUCTION:

Angiogenesis is an essential process in the progression of cancer from small, localized neoplasms to larger, potentially metastatic tumors[1,2]. The correlation between tumor growth and angiogenesis becomes one of the points of emphasis in the field of oncology research. However, traditional methods of measuring morphologic changes fail to monitor tumor progression and angiogenesis simultaneously in living animals using a visualized approach.

Bioluminescence imaging (BLI) of tumor cells is a particularly appropriate experimental method to monitor tumor growth because of its non-invasiveness, sensitivity, and specificity[3-6]. BLI technology is based on the principle that the luciferase can catalyze oxidation of a specific substrate while emitting bioluminescence. The luciferase expressed in implanted tumor cells reacts with the injected substrate, which can be detected by a living imaging system, and signals indirectly reflect the changes in cell number or cell localization in vivo[6,7].

Except for tumor growth, tumor angiogenesis (the critical step in cancer progression) can also be visualized through BLI technology using Vegfr2-Fluc-KI transgenic mice[8-10]. The vascular endothelial growth factor (Vegf) receptor 2 (Vegfr2), one type of Vegf receptor, is mostly expressed in the vascular endothelial cells of adult mice[11]. In Vegfr2-Fluc-KI transgenic mice, the DNA sequence of Firefly luciferase (Fluc) is knocked into the first exon of the endogenous Vegfr2 sequence. As a result, the Fluc is expressed (which appears as BLI signals) in a manner that is identical to the level of angiogenesis in mice. To grow beyond a few millimeters in size, the tumor recruits new vasculatures from existing blood vessels, which highly express the Vegfr2 triggered by growth factors from tumor cells[1]. This opens the possibility of using Vegfr2-Fluc-KI transgenic mice to non-invasively monitor tumor angiogenesis by BLI.

In this protocol, a tumor-bearing mouse model is established to monitor tumor progression and angiogenesis in a single mouse through Firefly luciferase (Fluc) and Renilla luciferase (Rluc) imaging, respectively (**Figure 1**). A 4T1 cell line (4T1-RR) is created that stably expresses Rluc and red fluorescent protein (RFP) to trace cell growth by Rluc imaging. To further investigate the dynamic changes of angiogenesis in the progression and regression of the tumor, another 4T1 cell line (4T1-RRT) is created that expresses suicide gene herpes simplex virus truncated thymidine kinase (HSV-ttk), Rluc, and RFP. By administration of ganciclovir (GCV), the HSV-ttk expressing cells are selectively ablated. Based on these cell lines, a tumor-bearing model in Vegfr2-Fluc-KI mice is built that serves as an experimental model bridging tumor progression and tumor angiogenesis in vivo.

PROTOCOL:

Experiments must comply with national and institutional regulations concerning the use of animals for research purposes. Permissions to carry out experiments must be obtained. The treatment of animals and experimental procedures of the study adhere to the Nankai

University Animal Care and Use Committee Guidelines that conform to the Guidelines for Animal Care approved by the National Institutes of Health (NIH).

1. LV-Rluc-RFP (RR) and LV-Rluc-RFP-HSV-ttk (RRT) lentiviral packaging and production

NOTE: The pLV-RR carries the gene sequences of Renilla luciferase (Rluc) and red fluorescent protein (RFP) under the promoter EF1α, whereas the pLV-RRT carries the gene sequences coding Rluc, RFP, and herpes simplex virus truncated thymidine kinase (HSV-ttk) (**Figure 2**).

1.1. Seed 1 x 10^6 of 293T cells per well into a 6 well plate and culture overnight in a humidified incubator with 5% CO_2 at 37 °C with Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS).

1.2. Prepare the liposome suspension: mix 7.5 µL of liposome and 0.25 mL of minimal essential medium (MEM) into a 1.5 mL tube following incubation for 5 min at room temperature (RT) to disperse liposomes equally.

1.3. Prepare the DNAs solution (DNAs-RR): separately, add the pLV-RR vector and helper plasmids to 0.25 mL of MEM in a 1.5 mL tube as described in **Table 1**.

1.4. Obtain the liposome/DNAs-RR compound: gently add the DNAs-RR solution into prepared liposome suspension drop by drop and incubate for 20 min at RT so the DNA bonds to the lipid membrane.

1.5. Replace the medium of the 293T cells with 1 mL of DMEM containing 10% FBS and add the liposome/DNAs-RR compound to the medium of the 293T cells gently.

1.6. After incubating in a humidified incubator with 5% CO_2 at 37 °C for 12–16 h, replace the liposome/DNAs-RR compound containing medium of the 293T cells with 2 mL of DMEM containing 10% FBS and 100 U/mL penicillin−streptomycin.

1.7. Continue culturing the 293T cells in the humidified incubator for 48 h after transfection. Then, collect the supernatant of the 293T cells and centrifuge the medium at 300 x *g* for 5 min to pellet the 293T cells. Transfer the lentivirus-RR (LV-RR)-containing supernatant into 1.5 mL sterile polypropylene storage tubes and store at -80 °C.

NOTE: A Biosafety Level 2 (BSL-2) facility is required in order to work with recombinant lentivirus.

1.8. Repeat steps 1.1–1.7 and use pLV-RRT vector instead of pLV-RR vector in step 1.3 to obtain the lentivirus-RRT (LV-RRT). Store the LV-RRT at -80 °C.

NOTE: The non-purified lentiviral stock may inhibit cell growth in some cases. Lentiviral stock may need to be purified. The lentiviral stocks containing LV-RR or LV-RRT particles should be divided into 1.5 mL tubes (1 mL per tube) for storage to avoid multiple free-thaw cycles.

2. LV-RR and LV-RRT lentiviral transduction for gene expression in 4T1 cells

2.1. Seed 4T1 cells into a 6-well plate (5 x 10^5 cells/well) and culture with Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS overnight in a humidified incubator with 5% CO_2 at 37 °C.

2.2. Remove the medium from the culture plate and replace it with 1 mL fresh RPMI 1640 medium as well as 1 mL of lentiviral stock (LV-RR or LV-RRT) to each well. Add 8 µg/mL polybrene and gently blend the medium containing lentiviral particles by pipetting up and down.

NOTE: Please be aware that the medium contains lentiviral particles, which could transduce human cells.

2.3. Spin transduction solution in a centrifuge at 1,000 × *g* for 60 min at RT to help increase transduction efficiency. After centrifugation, culture 4T1 cells for 4–12 h and maintain in a humidified incubator with 5% CO_2 at 37 °C.

NOTE: For some cell lines, polybrene may be toxic for long-term culture. Therefore, the incubation time for transducing different cells may be changeable. Check the cell status multiple times to find appropriate incubation time.

2.4. Refresh the medium of transduced 4T1 cells with 2 mL of RPMI 1640 medium containing 10% FBS and 100 U/mL penicillin−streptomycin to remove lentiviral particles and polybrene.

3. Drug screening and identification of LV-RR and LV-RRT transduced 4T1 cells

3.1. Select transduced cells with medium containing blasticidin (BSD) according to the BSD-resistance gene carried by LV-RR or LV-RRT as the following steps described.

NOTE: Alternatively, the transduced cells which are RFP-positive could be selected by flow cytometry according to the RFP gene carried by LV-RR or LV-RRT.

3.2. 48 h after transduction, passage 4T1 cells at the ratio of 1:3 to 1:4 with selection medium (RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin−streptomycin, and 5 µg/mL BSD). Change medium every 2 or 3 days.

NOTE: The optimal BSD concentration may vary from cell line to cell line. Therefore, a pilot experiment of kill curve should be performed to determine the optimal concentration of BSD before initial experiment.

3.3. 7 days post-drug screening, observe the LV-RR transduced 4T1 cells (4T1-RR) and LV-RRT transduced 4T1 cells (4T1-RRT) under the fluorescence inverted phase-contrast microscope. Count the number of RFP^+ 4T1 cells and all 4T1 cells in three fields of vision to estimate the RFP-positive ratio, respectively (**Figure 2**).

NOTE: Alternatively, the RFP-positive ratio of transduced 4T1 cells could be identified by flow cytometry.

3.4. Measure the renilla signals of 4T1-RR cells and 4T1-RRT cells by using a living imaging system to detect the linear relationship between cell numbers and renilla signals (**Figure 3**).

3.5. Expand BSD-screened 4T1-RR and 4T1-RRT cells with selection medium at split ratios between 1:3 and 1:4 and store the cell line stocks in liquid nitrogen.

4. Vegfr2-Fluc-KI mice and tumor-bearing mouse model

NOTE: The transgenic Vegfr2-Fluc-KI mice, 6–8 weeks old and female, are used in this experiment to non-invasively monitor angiogenesis in vivo by BLI.

4.1. Culture 4T1-RR cells and 4T1-RRT cells in 60 mm Petri dishes in a humidified incubator with 5% CO_2 at 37 °C, respectively. When the cells are at 80% confluence, remove the medium and rinse with phosphate buffered saline (PBS).

4.2. Remove the PBS and add an additional 2 mL of 0.25% trypsin-0.53 mM EDTA solution respectively. Keep the dish at RT (or at 37 °C) until the cells detach.

4.3. Add 5–10 mL of fresh medium containing 10% FBS, then aspirate and dispense cells to resuspend 4T1-RR and 4T1-RRT cells into 15 mL centrifuge tubes, respectively. Count two types of 4T1 cells using a counting chamber and prepare the cell suspensions at a concentration of 1 x 10^6 per 100 µL in RPMI 1640 medium.

4.4. Anesthetize the Vegfr2-Fluc-KI mice with 1%–3% isoflurane in 100% oxygen at anesthesia induction chamber with a flow rate of 1 L/min. Monitor the toe pinch response of the mouse to confirm the status of anesthesia. Then, apply ophthalmic ointment to the eyes of mouse to prevent dehydration.

4.5. Remove mouse from chamber and position in nosecone. Entirely remove the hair of the shoulder of mouse by using electric shaver and hair removal cream, which could provide a good view of surgical field and avoid blocking the BLI signals in following-up experiments.

4.6. Subcutaneously inject 4T1-RR cells (1 x 10^6 cells at a 100 µL total volume) and 4T1-RRT cells (1 x 10^6 cells at a 100 µL total volume) in left and right shoulders of each mouse, respectively (record as Day 0). Place mice in recovery area with thermal support until fully recovered.

4.7. After implantation of 4T1-RR and 4T1-RRT cells, touch the tumor masses to check that the mice are tumor-bearing every day (**Figure S1**). At day 7 post-implantation, intraperitoneally inject 50 mg/kg ganciclovir (GCV) to the tumor-bearing mice 2x per day until the end of experiment.

NOTE: Before this experiment, the cytotoxic of GCV on 4T1-RRT cells should be detected. The killing efficiency of GCV could be evaluated by cell counting assay with different concentration of GCV (**Figure S2**).

4.8. On the day 0, 3, 7, 14, and 21 after 4T1 implantation, monitor the tumor growth and angiogenesis of tumor-bearing mice and assess by both Rluc and Fluc imaging (**Figure 4**).

5. Dual bioluminescence imaging of tumor (Rluc) and angiogenesis (Fluc)

5.1. Open the living imaging system, initialize the living imaging software, and then initialize the system.

NOTE: The system initialization will take few minutes to cool down the charge-coupled device (CCD) camera to -90 °C before able to start imaging. The temperature will turn green when the CCD camera is cooled.

5.2. Use the following camera settings:
Check the Luminescence and Photograph.
Check Overlay.
Luminescence settings:
Exposure Time sets AUTO in normal conditions.
Binning sets to 8.
F/Stop sets to 1.
Emission Filter sets Open.
Photograph settings:
Binning sets to medium.
F/Stop sets to 8.
IVIS system settings:
Field of view: C=1 mouse view, D=5 mice view.
Subject height sets 1.5 cm.

5.3. Weigh and record the mice and calculate the volume of coelenterazine (CTZ; 2.5 mg/kg) and D-luciferin (150 mg/kg) needed.

5.4. Anesthetize tumor-bearing mouse by 1%–3% isoflurane in 100% oxygen at anesthesia induction chamber with a flow rate of 1 L/min. Monitor the toe pinch response of the mouse to confirm the status of anesthesia. Then, dispense a drop of lubricating eye ointment onto both eyes to avoid corneal damage.

5.5. Inject 2.5 mg CTZ (3.33 mg/mL) per kilogram body weight into the retrobulbar of the mouse (e.g., for a 20 g mouse, inject 15 µL to deliver 50 µg of CTZ) by using an insulin syringe needle.

5.6. Move the tumor-bearing mouse into the camera chamber with its nose in the anesthesia cone gently and acquire several pictures of the mouse dorsal immediately to get the Rluc signals from 4T1 cells until the BLI signals fade away.

NOTE: The half-life of CTZ is very short and the signals of Rluc drop precipitously ~30 s. To ensure any residual Rluc signal has dissipated and the interval between Rluc and Fluc imaging should be more than 10 min.

5.7. Intraperitoneally inject 150 mg/kg D-luciferin (30 mg/mL) using an insulin syringe needle (e.g., for a 20 g mouse, inject 100 µL to deliver 3 mg of D-luciferin). Keep the mouse at RT for 10 min before Fluc imaging.

5.8. Move this mouse into camera chamber with its nose in the anesthesia cone again and acquire several pictures of the mouse dorsal to get the Fluc signals from angiogenesis.

NOTE: The Fluc kinetic monitor should be performed for each mouse until the signals reach the maximum and then fade.

5.9. Repeat the procedures steps 5.4–5.8 for each mouse.

5.10. After imaging, maintain the mice in a warm environment until animals wake up.

5.11. At the desired time point (day 3, 7, 14, and 21), repeat above procedures (step 5.3–5.10) to detect the tumor progression and tumor angiogenesis over time.

5.12. Analyze the Rluc and Fluc signals data to investigate the relationship between the tumor growth and angiogenesis in tumor progression.

NOTE: The regions of interest (ROI) which cover the BLI signal site are used to analyze the data. Measure the total radiance (Photons) of ROI in the unit of Photons/seconds/cm^2/steradian (p/s/cm^2/sr) for every timepoint.

5.13. Analyze the Rluc and Fluc signals of ROI by using graphics software (**Figure 4**).

REPRESENTATIVE RESULTS:

In this experiment, a breast cancer mouse model was established using 4T1 cells to investigate the relationship between tumor growth and tumor angiogenesis (**Figure 1**). Firstly, two lentivirus were packaged, which carried gene sequences expressing Rluc/RFP (LV-RR) and Rluc/RFP/HSV-ttk (LV-RRT), respectively, as previously reported[7]. Then, two different

4T1 cell lines, named 4T1-RR and 4T1-RRT, were created by transducing LV-RR and LV-RRT respectively. After drug screening for 3 days, the 4T1-RR and 4T1-RRT were observed under a fluorescence microscope to detect the transduction efficiency. As shown in the fluorescence imaging, more than 99% of the of 4T1-RR or 4T1-RRT cells were RFP positive, which suggested that the 4T1-RR and 4T1-RRT cell lines were established by LV-RR and LV-RRT transduction (**Figure 2A,B**). Meanwhile, there was no differences found in cell morphology and growth between wild-type 4T1 and 4T1-RR or 4T1-RRT during the culture time. In summary, we successfully built 4T1-RR and 4T1-RRT cell lines without influencing the cellular states. Subsequently, bioluminescence imaging (BLI) of 4T1-RR and 4T1-RRT cells was captured to detect the Rluc signals. The BLI images revealed that both 4T1-RR and 4T1-RRT cells emitted strong bioluminescent signals of the same strength (**Figure 3A**). Besides, the linear relationships between Rluc signals and cell numbers were observed in both 4T1-RR ($R^2 = 0.9974$) and 4T1-RRT cells ($R^2 = 0.9989$), which suggested the Rluc signals could be used to mirror the tumor growth in vivo (**Figure 3B**).

On this basis, using the transgenic Vegfr2-Fluc-KI mice, a tumor-bearing mouse model was established to investigate the angiogenesis as the breast cancer grows. As a result of knocking Fluc sequence into the first exon of the Vegfr2 sequence in murine, the Fluc was expressed (which appears as bioluminescent signals) in a manner identical to the angiogenesis in mice during the tumor progression. After subcutaneous injection of 4T1-RR and 4T1-RRT cells, cell growth was monitored by Rluc signals in the presence of CTZ at days 0, 3, 7, 14, and 21 (**Figure 4A**). At the same time, angiogenesis induced by tumor growth was evaluated by Fluc signals in the presence of D-luciferin in the same mouse. At day 7 post-implantation of 4T1-RR and 4T1-RRT, GCV was administered to the tumor-bearing mice, which led the 4T1-RRT cells to die. The BLI images revealed that Rluc signals of 4T1-RR and 4T1-RRT cells increased at the same rate before GCV treatment; however, Rluc signals of 4T1-RRT cells sharply decreased post GCV treatment. while the Rluc signals of 4T1-RR still increased gently. Obviously, a significant relativity existed between Rluc signals and the tumor size (**Figure S1**).

Meanwhile, according to the Fluc images, the Fluc signals increased in accordance with the Rluc rise and decreased following the Rluc decline (**Figure 4B**). These results suggest that there was a direct correlation between tumor angiogenesis and tumor growth. The death of tumor cells induced by drug GCV may lead to inhibition of tumor angiogenesis (**Figure 4C**). To demonstrate that the Fluc signal was indeed detecting the angiogenesis within the tumors, the animals were sacrificed after finishing imaging at day 21 to obtain histological evidence of vasculature. According to the images of anti-VEGFR2 immunostaining, the microvascular structures in 4T1-RR tumor tissue were significantly more evident than in 4T1-RRT tumor tissue, which were consistent with the Fluc signals (**Figure 5**). In summary, this dual bioluminescence imaging strategy can be used to monitor tumor progression and angiogenesis as well assess anti-tumor effects of different drugs on tumor growth and angiogenesis in the tumor microenvironment.

FIGURE & TABLE LEGENDS:

Figure 1: Schematic map of dual bioluminescence imaging of tumor growth and angiogenesis. The 4T1 cells transduced by LV-RR and LV-RRT were implanted in Vegfr2-Fluc-KI transgenic mice. During tumor growth, BLI of Rluc and Fluc were simultaneously performed in a single mouse to reflect tumor growth and angiogenesis status, respectively.

Figure 2: Transduction efficiency of 4T1-RR and 4T1-RRT cells identified by fluorescence imaging. (A) The diagrammatic drawing of pLV-RR showed that Rluc and RFP sequences were expressed under the promoter EF1α. The bright and fluorescent images of one field of view revealed that 4T1-RR cells were RFP-positive. **(B)** The diagram drawing of pLV-RRT showed that the single promoter EF1α activated Rluc, RFP, and HSV-ttk genes. The bright and fluorescent images of one field of view revealed that 4T1-RRT cells were RFP positive. Scale bar = 200 μm.

Figure 3: Bioluminescence imaging of transduced 4T1-RR and 4T1-RRT cells. (A) Bioluminescence imaging of 4T1-RR and 4T1-RRT cells in the presence of CTZ. **(B)** The measured Rluc signals of 4T1-RR and 4T1-RRT cells maintained a linear relationship with cell numbers.

Figure 4: Visualization of the dynamic processes of tumor growth and angiogenesis in a living animal. (A) Flow diagram of the experiment and dual BLI detection of Rluc and Fluc. **(B)** Representative Rluc images of tumor progression and Fluc images of angiogenesis during tumor development in a transgenic mouse. **(C)** Measurement of Rluc signals demonstrated that the implanted tumor cells grew fast, while 4T1-RRT cells were significantly regressed after GCV administration. **(D)** Quantification of Fluc signals showed that angiogenesis occurred after tumor cell implantation, following a parallel trend with tumor growth and death induced by GCV.

Figure 5: VEGFR2 immunostaining of 4T1-RR and 4T1-RRT tissues at day 21. Representative images of tumor tissues sections stained for VEGFR2 (green) at day 21. The nuclei were counterstained with DAPI (blue). Scale bar = 100 μm.

Figure S1: Curve of tumor size during tumor progression in vivo. The tumor size of 4T1-RR and 4T1-RRT cells increased after implantation, but the tumor size of 4T1-RRT cells started decreasing post-GCV treatment.

Figure S2: Cytotoxic effect of GCV on 4T1-RRT cells. The 4T1-RRT cells died with the elevated concentration of GCV.

Figure S3: BLI image of 4T1-RR cells in the lung. After tail vein injection of 4T1-RR cells, the Rluc signal of cells was detected by BLI.

Table 1: **Transfection conditions of lentiviral packaging system for producing LV-RR and LV-RRT viral stocks in 293T cells. (A)** The pLV-cDNA vector was pLV-RR and pLV-RRT,

respectively. **(B)** The Gag-Pol + Rev expression vector can be either pCMV-deltaR8.91 (TRC) or psPAX2 (Addgene). **(C)** Gag-Pol expression vector can select any one of pMDLg/pRRE (Addgene), pLP1 (Invitrogen), and pPACKH1-GAG (SBI). **(D)** Rev expression vector should be pRSV-REV (Addgene), pLP2 (Invitrogen), or pPACKH1-REV (SBI). **(E)** VSV-G expression vector can select pMD.G (TRC), pMD2.G (Addgene), pCMV-VSV-G (Addgene), pVSV-G (SBI), or pLP/VSVG (Invitrogen). In this protocol, the three-plasmid system was used, including psPAX2, pMD2.G, and pLV-RR or pLV-RRT.

DISCUSSION:

In this protocol, a non-invasive dual BLI approach is described for monitoring tumor development and angiogenesis. The BLI reporter system is first developed, containing the HSV-ttk/GCV suicide gene for tracking tumor progression and regression in vivo by Rluc imaging. Meanwhile, tumor angiogenesis is assessed using Vegfr2-Fluc-KI mice via Fluc imaging. This tumor-bearing mouse model is able to provide a practical platform for continuous and non-invasive tracking tumor development and tumor angiogenesis by dual BLI in a single mouse with high relevance, reproducibility, and translatability.

Angiogenesis concerns long-term tumor progression and is thereby of high importance[1]. It is necessary to study the relationship between tumor progression and angiogenesis. An increasing number of anti-angiogenesis strategies have been investigated for cancer treatment, which rely on visualized monitor approaches for accurately assessing the treatment outcomes. Further, the neovascularization of tumor tissue after traditional radiotherapy and chemotherapy is another popular area of oncology research[12-14]. These studies require an animal model that allow monitoring of tumor growth and angiogenesis in real-time. The pathological changes of tumor tissues in traditional animal models are usually dependent on histopathological examination, which requires animal sacrifice. These dual BLI mouse models help address the problems of larger error ranges and higher costs from the sacrifice of animals.

In this dual BLI mouse model, the most critical step is using two types of luciferases, including Fluc and Rluc, to respectively trace cells and angiogenesis at the same time. The substrate specificity of these two luciferases makes it possible to perform two types of BLI in a single host. Besides, the half-life of coelenterazine (the substrate of Rluc) is very short, which results in the Rluc signals fading away quickly without influencing the next Fluc signal detection[15]. Hence, in the operating process, the Rluc imaging should be implemented before Fluc imaging on account of the longer half-life of D-luciferin (the substrate of Fluc). In addition, figuring out the incubation time of the substrates is the key to acquiring perfect BLI images. The metabolism of substrates can change the concentration of substrates in vivo, leading to variation in BLI signal intensity.

Owning to advancements in technology, other imaging modalities for in vivo tracking of certain cellular and subcellular events have been applied in preclinical and clinical researches, such as fluorescent imaging, magnetic resonance imaging (MRI), and positron emission tomography (PET)[16,17]. Compared with these imaging strategies, bioluminescence imaging has high sensitivity, strong specificity, and accurate measurement, showing its unique superiority in the field of living imaging studies[15]. The Rluc imaging employed allows for tumor growth and anti-tumor effects of the HSV-ttk/GCV prodrug system to be visualized dynamically in a living animal. Except for monitoring subcutaneous tissues, Rluc has been used to trace cells in lungs by BLI technology in other research. After tail vein injection of 4T1-RR into a mouse, we have moved this mouse into the living imaging system to detect the Rluc signals after administration of CTZ. The image of Rluc signal showed that injected cells were mainly located in the lung (**Figure S3**). As mentioned above, the Rluc report gene can trace various cancer cells in different locations, which encourages the full utilization of this mouse model in cancer biology research.

In addition to these advantages, BLI technology can be used to sense the expression levels of specific molecules. Previously, fluorophores reporter genes, which are expressed under relevant promotors, have been used to measure vessel development in subcutaneous tumors. During tumor progression, the vascular structure and molecules can be observed through the surgically implanted window chambers in mice. However, this method still has limitations, including unavoidable invasion, fluorophore quenching, and strong background noise. The tumor-bearing mouse model established in the Vegfr2-Fluc-KI mouse creates a non-invasive observation of the expression level of Vegfr2, which is the most important molecule in tumor angiogenesis. Meanwhile, the BLI images display great specificity without noise. The dual BLI mouse model may have broader applications in studying the potential molecular mechanisms in tumor progression and regression.

BLI technology, based on expression of Rluc (emission 480 nm) and Fluc (emission 562 nm), has been adopted in a number of in vivo disease models. The widespread use of BLI technology in vivo has been restricted because of the low sensitivity of bioluminescence at wavelengths below 600 nm in detecting deep tissue. This is caused by the absorption and scattering of light, which decreases the detectable signal up to ten-fold per centimeter of tissue. To address this question, some researchers have focused studies on the red-emitter variants of Fluc that emit light above 600 nm[18]. Because the absorption and scattering of light can be remarkably reduced by using these variants of Fluc[18,19], the applications of luciferase variants will extend this protocol to a larger field of oncology research.

DISCLOSURES:

The authors have nothing to disclose.

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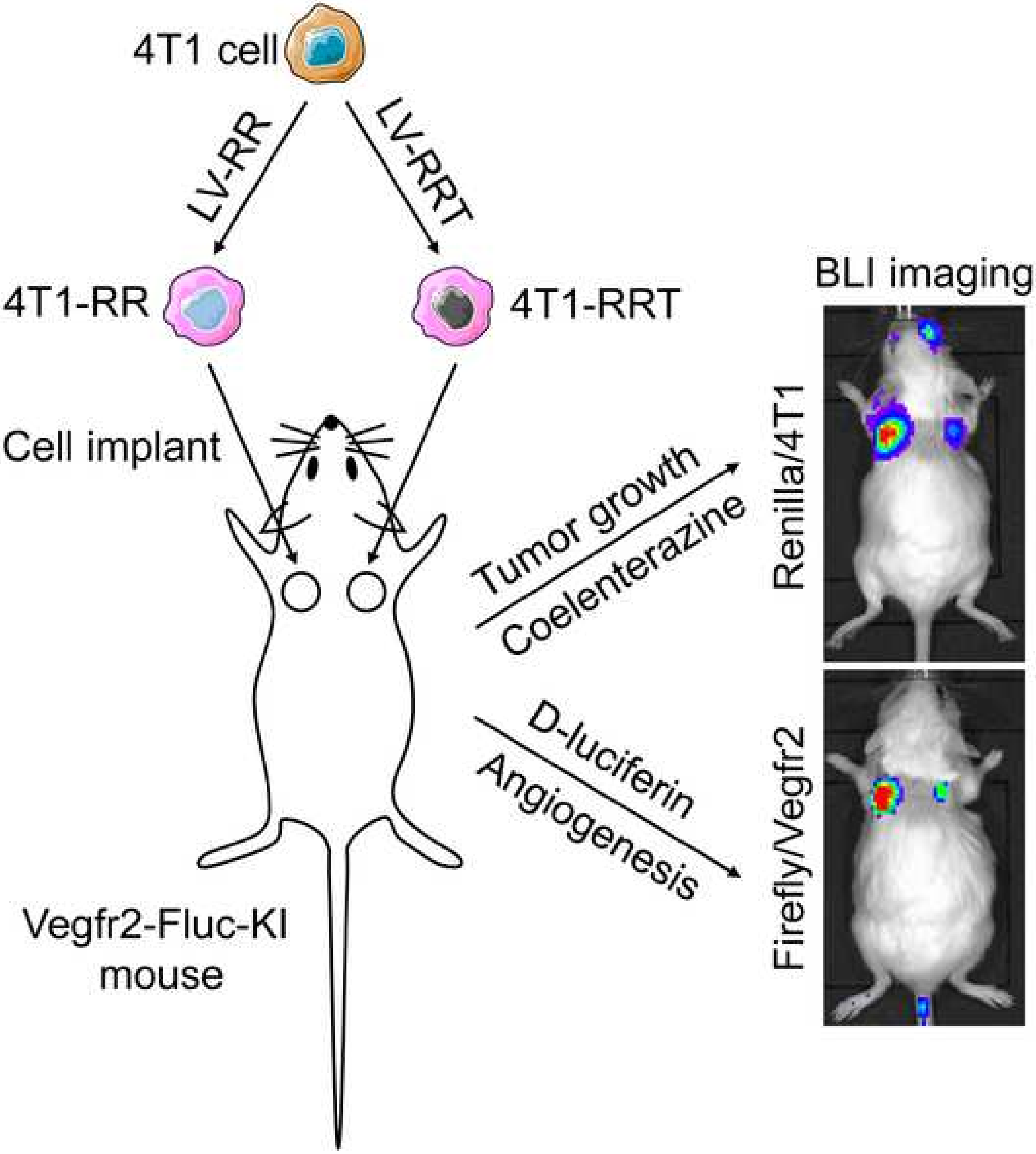

4T1 cell
LV-RR
LV-RRT
4T1-RR
4T1-RRT
Cell implant
Vegfr2-Fluc-KI
mouse
Tumor growth
Coelenterazine
D-luciferin
Angiogenesis
BLI imaging
Renilla/4T1
Firefly/Vegfr2

Figure 2

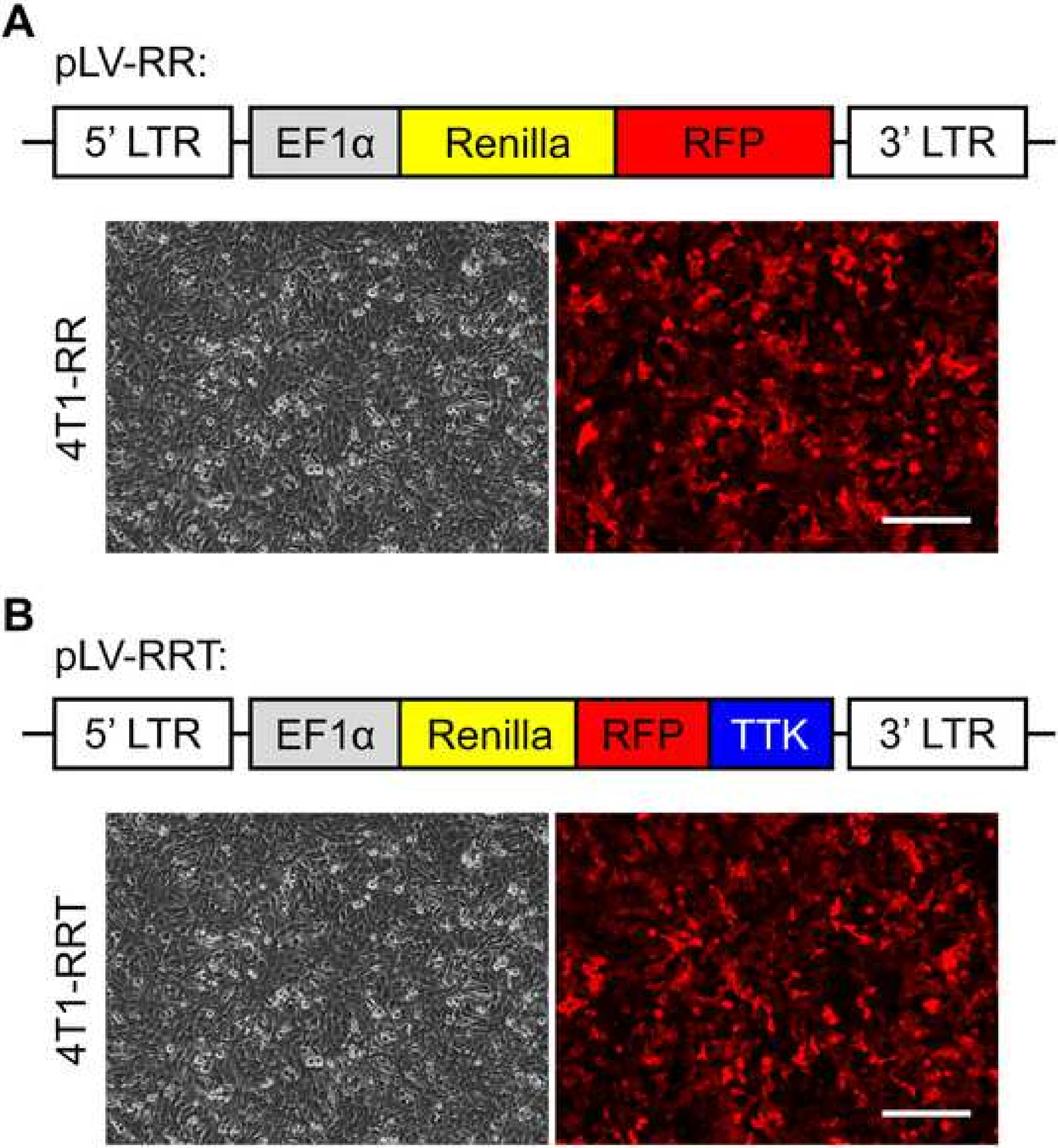
A
pLV-RR:
5' LTR
EF1α
Renilla
RFP
3' LTR
4T1-RR
B
pLV-RRT:
5' LTR
EF1α
Renilla
RFP
TTK
3' LTR
4T1-RRT

Figure 3

Click here to access/download;Figure;Fig 3.tif

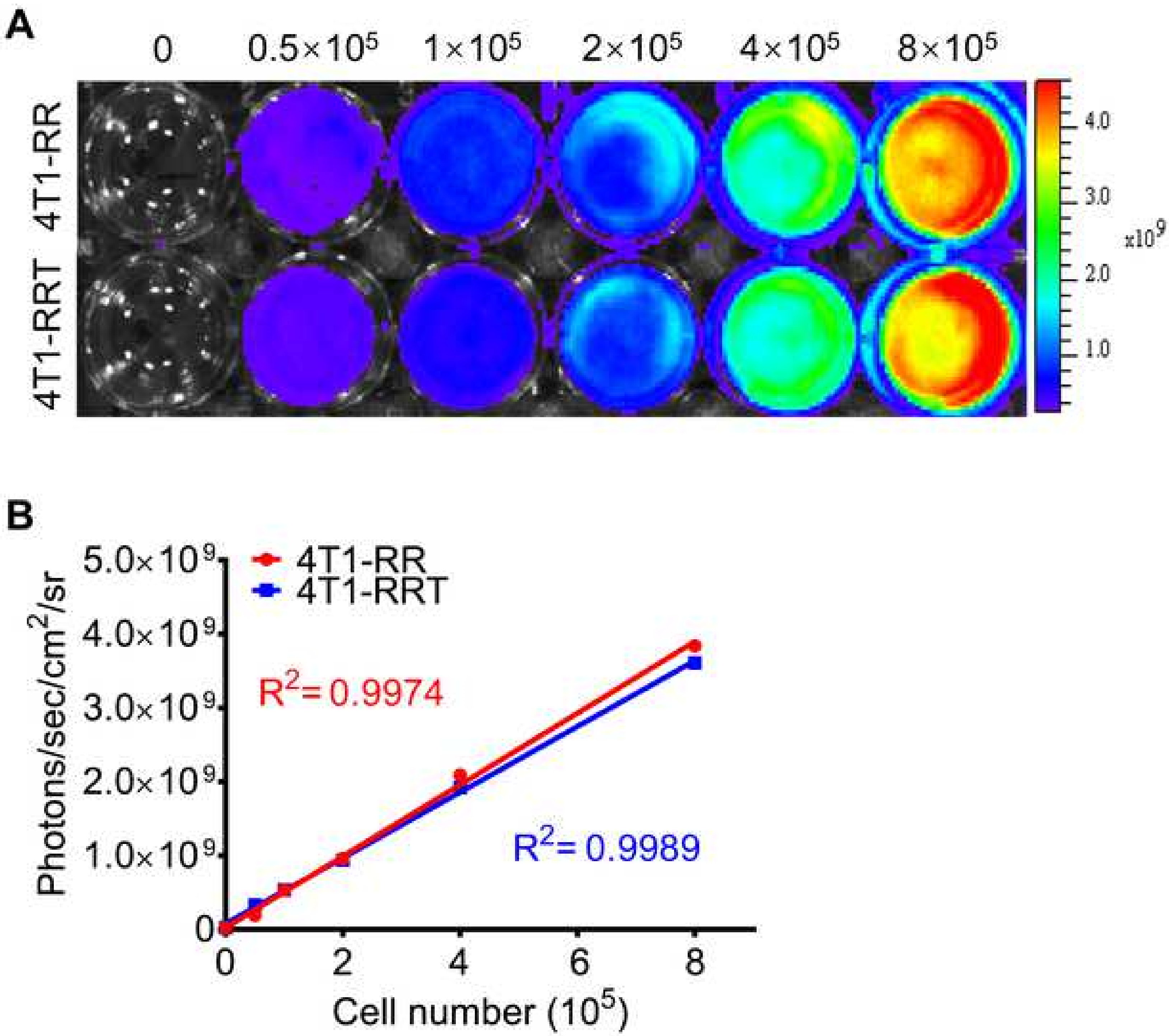

Figure 4

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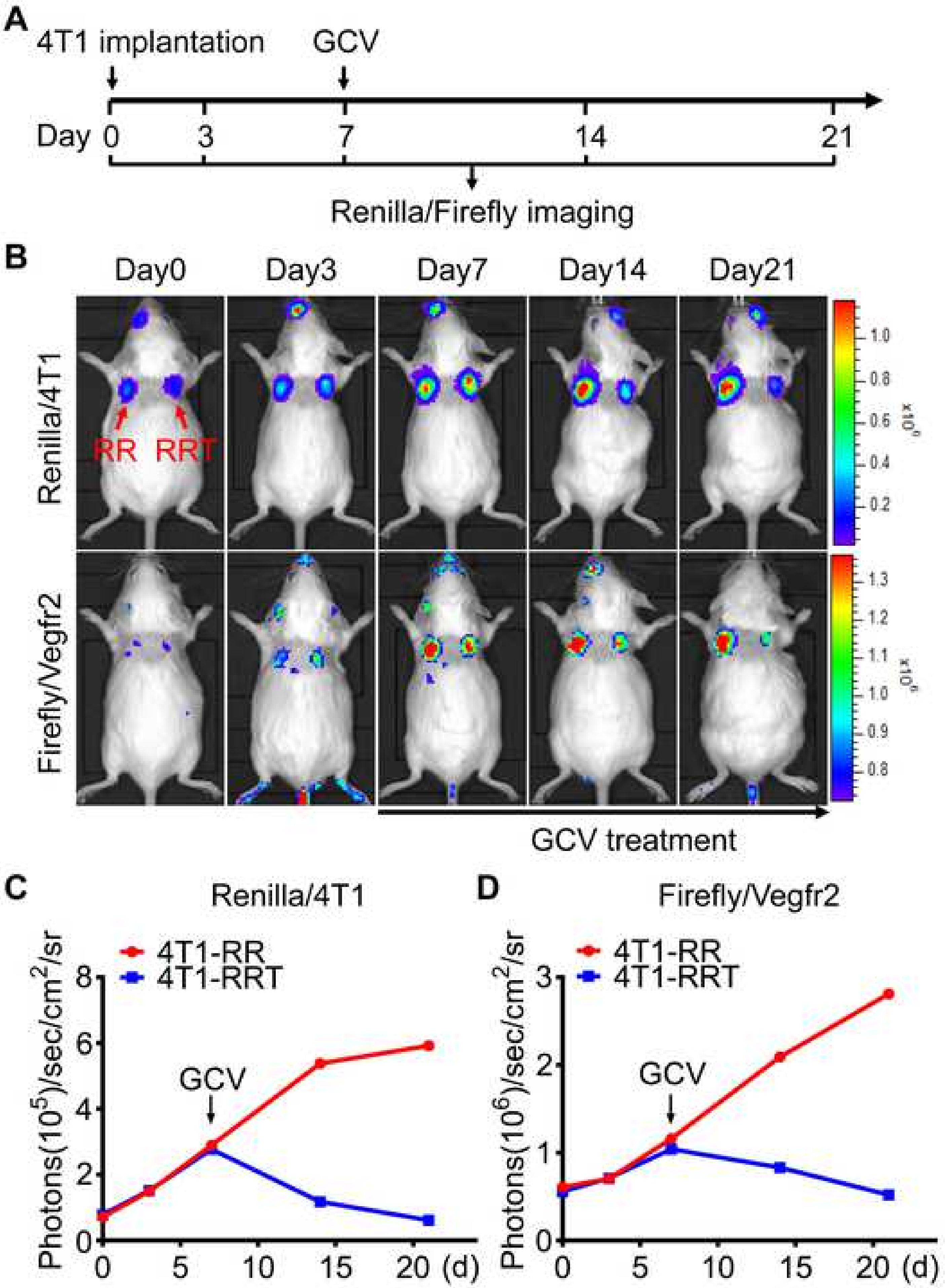

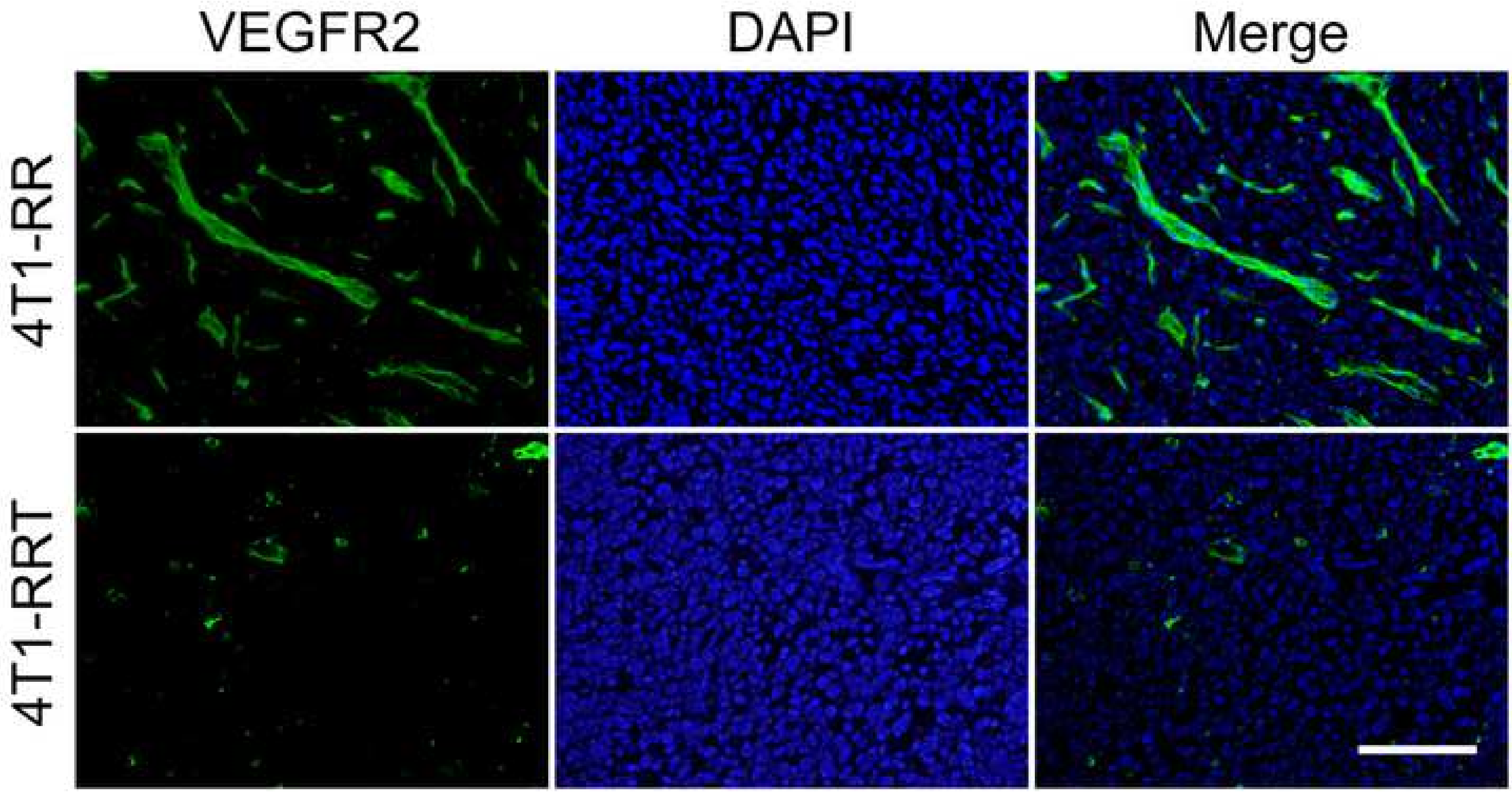
VEGFR2
DAPI
Merge
4T1-RR
4T1-RRT

LV-RR and LV-RRT Packaging Conditions

Components	MEM medium	3-plasmid system	4-plasmid system
pLV-RR/pLV-RRT vector[A]	0.25 mL	1.5 µg	1.5 µg
Gag-Pol + Rev expression vector[B]		1.0 µg	
Gag-Pol expression vector[C]			0.75 µg
Rev expression vector[D]			0.3 µg
VSV-G expression vector[E]		0.5 µg	0.45 µg
Liposome	0.25 mL	7.5 µL	7.5 µL

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.25% Trypsin-0.53 mM EDTA	Gibco	25200072	
1.5 mL Tubes	Axygen Scientific	MCT-105-C-S	
15 mL Tubes	Corning Glass Works	601052-50	
293T	ATCC	CRL-3216	
4T1	ATCC	CRL-2539	
60 mm Dish	Corning Glass Works	430166	
6-well Plate	Corning Glass Works	3516	
Biosafety Cabinet	Shanghai Lishen Scientific	Hfsafe-900LC	
Blasticidine S Hydrochloride (BSD)	Sigma-Aldrich	15205	
Cell Counting Kit-8	MedChem Express	HY-K0301	
CO_2 Tegulated Incubator	Thermo Fisher Scientific	4111	
Coelenterazine (CTZ)	NanoLight Technology	479474	
D-luciferin Potassium Salt	Caliper Life Sciences	119222	
DMEM Medium	Gibco	C11995500BT	
Fetal Bovine Serum (FBS)	BIOIND	04-001-1A	
Fluorescence Microscope	Nikon	Ti-E/U/S	
Ganciclovir (GCV)	Sigma-Aldrich	Y0001129	
Graphics Software	GraphPad Software	Graphpad Prism 6	
Insulin Syringe Needles	Becton Dickinson	328421	
Isoflurane	Baxter	691477H	
Lentiviral Packaging System	Biosettia	cDNA-pLV03	
Liposome	Invitrogen	11668019	
Living Imaging Software	Caliper Life Sciences	Living Imaging Software 4.2	
Living Imaging System	Caliper Life Sciences	IVIS Lumina II	
MEM Medium	Invitrogen	31985-070	
Penicillin-Streptomycin	Invitrogen	15140122	
Phosphate Buffered Saline (PBS)	Corning Glass Works	R21031399	
Polybrene	Sigma-Aldrich	H9268-1G	
RPMI1640 Medium	Gibco	C11875500BT	
SORVALL ST 16R Centrifuge	Thermo Fisher Scientific	Thermo Sorvall ST 16 ST16R	
Ultra-low Temperature Refrigerator	Haier	DW-86L338	

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Author(s):	Kaiyue Zhang, Chen Wang, Ran Wang, Shang Chen and Zongjin Li

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Institution:	School of Medicine Nankai University
Title:	PI

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Nankai University School of Medicine

Zongjin Li, MD, PhD
Professor
Department of Pathophysiology
Nankai University School of Medicine

94 Weijin Road
Tianjin, China
Phone: +(86) 22-2350 9332
Email: zongjinli@nankai.edu.cn

Apr 7, 2019

Dear Editor,

We would like to re-submit a manuscript, manuscript ID: JoVE59763, entitled "Dual Bioluminescence Imaging of Tumor Progression and Angiogenesis" for consideration in *JoVE*.

We appreciate the helpful comments and suggestions by the editors. Our manuscript has been revised according to the editorial comments. Detailed replies to editorial comments are listed below. **Changes to the manuscript are highlighted in *blue* font.**

Editorial Comments:

1. Please copy-edit the manuscript. There are many scattered typos throughout.

We are so sorry for our mistakes. We have checked and corrected the spelling mistakes in our manuscript. Besides, we re-wrote some sentence to make our protocol easier to read. All modifications are highlighted in *blue* font.

2. Please reference Figure 5 in the manuscript text.

As you required, we have referenced **Figure 5 in** our manuscript (line 328).

3. Additional comments are in the attached manuscript.

A copy of the additional comments (highlighted in *grey*) followed by a detailed reply can be found below.

4. Please provide the legends for the supplemental figures in the manuscript text and upload the supplemental figures individually as image files.

According to your suggestions, we have separated the supplemental figures and their legends. The legends were provided after the legend for **Figure 5** in the manuscript. The supplemental figures were uploaded individually.

Sincerely,

Zongjin Li

Additional Comments:

3.1 Please reference Figure 1 before Figure 2. Figure 1 could be referenced in the Introduction.
According to your suggestions, we have referenced **Figure 1** in the Introduction. Thank you for your comment.

3.2 Liposome or Lipofectamine 2000? I changed it to liposome suspension (step 1.2?).
Thank you for your revision. The liposome suspension is what we want to describe in step 1.4.

3.3 Please be more specific here.
Sorry for our unclear description. The polybrene should be blend in medium by pipetting in step 2.2.

3.4 What happens after centrifugation? Aspiration?
Thank you for your question. As previously reported, co-centrifugation of cells and lentivirus particles could increase the transduction efficiency {Rouas, 2002 #24}. Centrifugation may increases the probability of contact between the lentivirus particles and the cells.

3.5 Please revise for clarity. The grammar is confusing. At the proportion?
We are so sorry for our mistake. The steps of sub-culturing 4T1 cells were re-organized in our manuscript.

3.6 What temperature? 5% CO_2 at 37 °C?
Sorry for our unclear statement. The cells were cultured in a humidified incubator with 5% CO2 at 37 °C. We have clarified this cultured condition of 4T1 cells in our manuscript.

3.7 How is sufficient depth of anesthesia confirmed? Is eye ointment used?
We are so sorry for our innocent omission. We have added the above procedures you mentioned in our manuscript.

3.8 How is this done? What is used?
Sorry for our unclear statement. The hair of mice was removed by using electric shaver and hair removal cream. We have re-written this step in our manuscript.

3.9 How much is injected? 1 x 10^6 cells per 100 µL? Or is this just the concentration? For how many mice? Step 4.6 mentions more than one mouse.
We appreciate your kind suggestions. There were 1 x 10^6 4T1-RR cells at a 100 µL total volume as well as 1 x 10^6 4T1-RRT cells at a 100 µL total volume which injected to each mouse in step 4.6.

3.10 How do the mice recover from anesthesia?

Sorry for our incomplete statement. The anesthetic animals should be returned to the cage in warm environment, and monitor frequently until the mouse wake up. We have added this step in our manuscript.

3.11 How is sufficient depth of anesthesia confirmed? Eye ointment?
We are so sorry for our innocent omission. We have added the above procedures you mentioned in our manuscript.

3.12 Please make into a sentence. How much of what is injected? Please provide all volumes and concentrations throughout.
We appreciate your comments. We have re-written this step to illustrate the volumes and concentrations of substrates we used in the manuscript. Thanks for your suggestions.

3.13 How much is injected?
Sorry for our unclear statement. We have added the volume and the concentration of D-luciferin in step 5.7.

3.14 What is being incubated? Substrate or subject?
Sorry for our unclear statements. We have re-written the sentence to describe that the mouse were kept at room temperature for 10 min.

3.15 Please specify the step numbers.
Thank you for your comment. We have added the step numbers that we mentioned in step 5.11.

Supporting Information

Dual Bioluminescence Imaging of Tumor Progression and Angiogenesis

Kaiyue Zhang[1], Chen Wang[1], Ran Wang[2], Shang Chen[1] and Zongjin Li[1*].

[1]Nankai University School of Medicine, Tianjin, China;
[2]State Key Laboratory of Medicinal Chemical Biology and College of Pharmacy, Nankai University, Tianjin, China.

*Corresponding Author:
Zongjin Li, MD, PhD
Email: zongjinli@nankai.edu.cn
Tel: +86-22-23509332

Figure S1

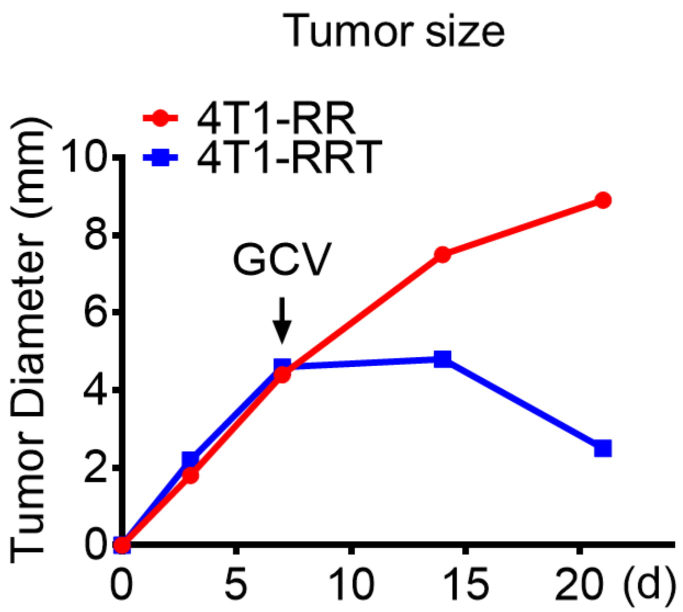


Figure S1. The curve of tumor size during tumor progression in vivo. The tumor size of 4T1-RR and 4T1-RRT cells increased after implantation, but the tumor size of 4T1-RRT cells sharply decreased post GCV treatment.

Figure S2

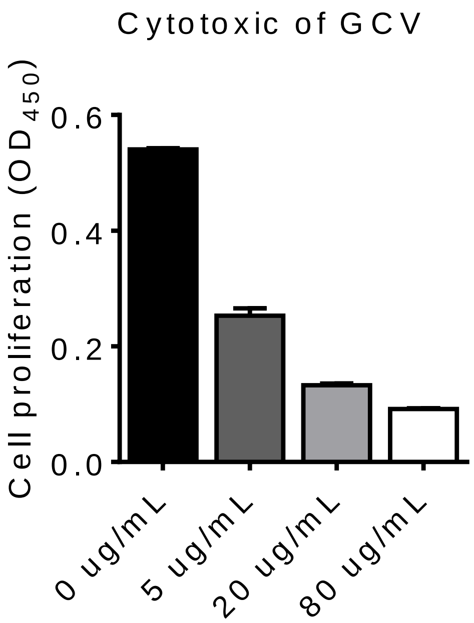


Figure S2. The cytotoxic effect of GCV on 4T1-RRT cells. With increased concentrations of GCV, the viability of 4T1-RRT cells was significantly inhibited.

Figure S3

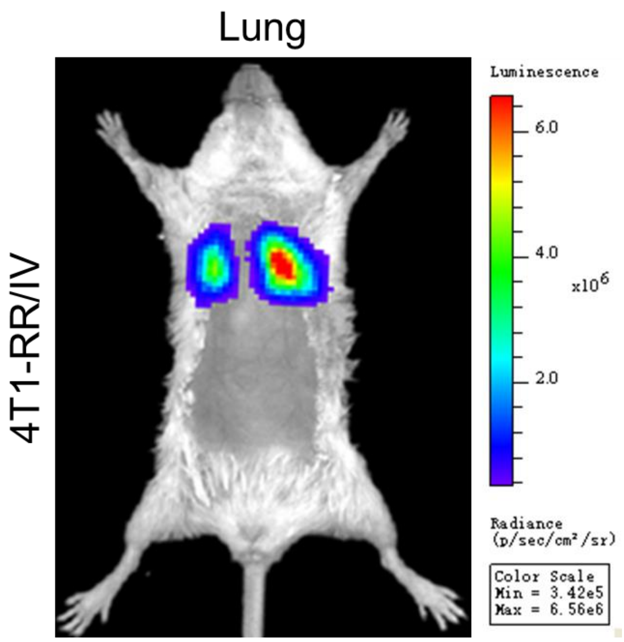
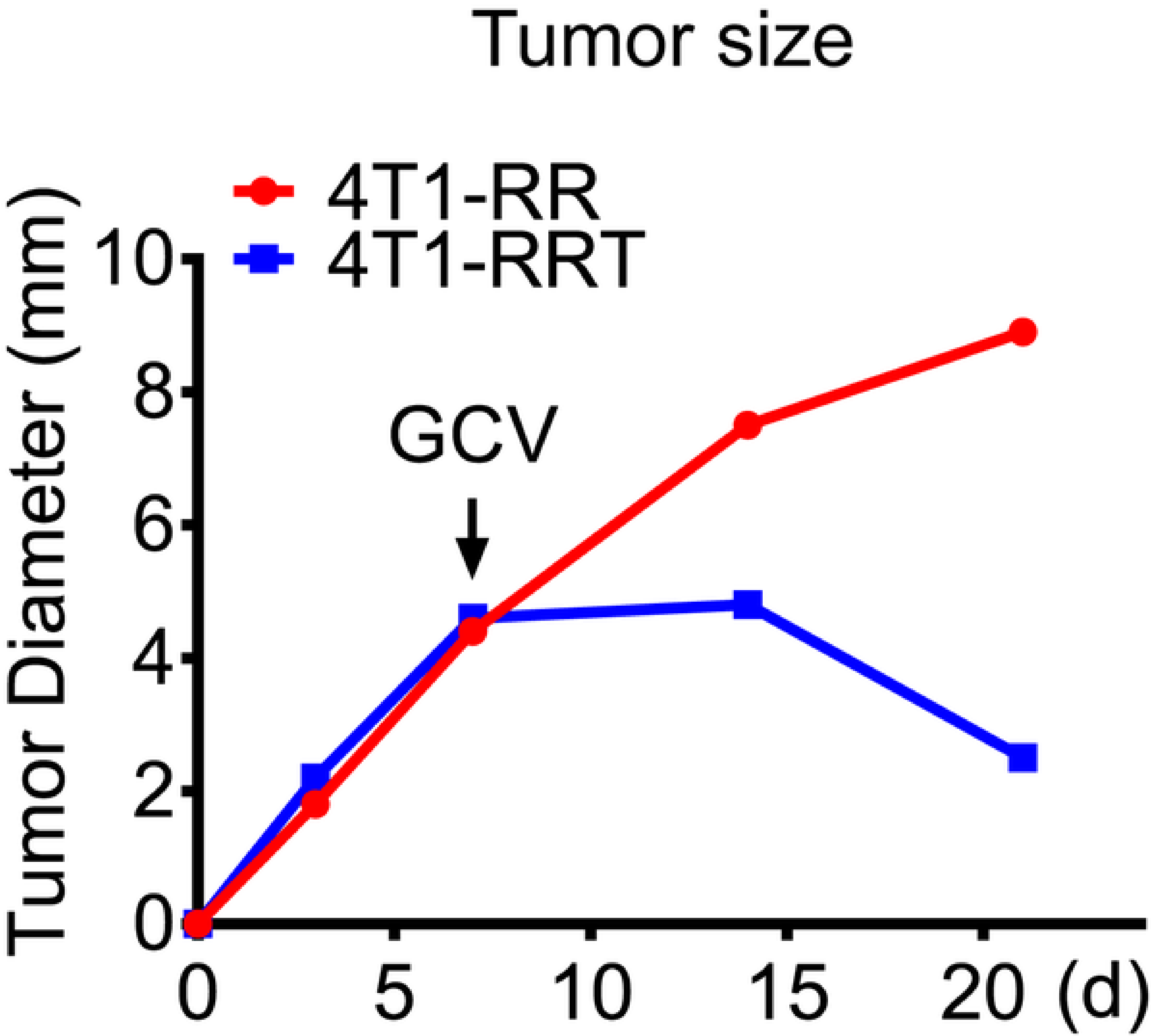
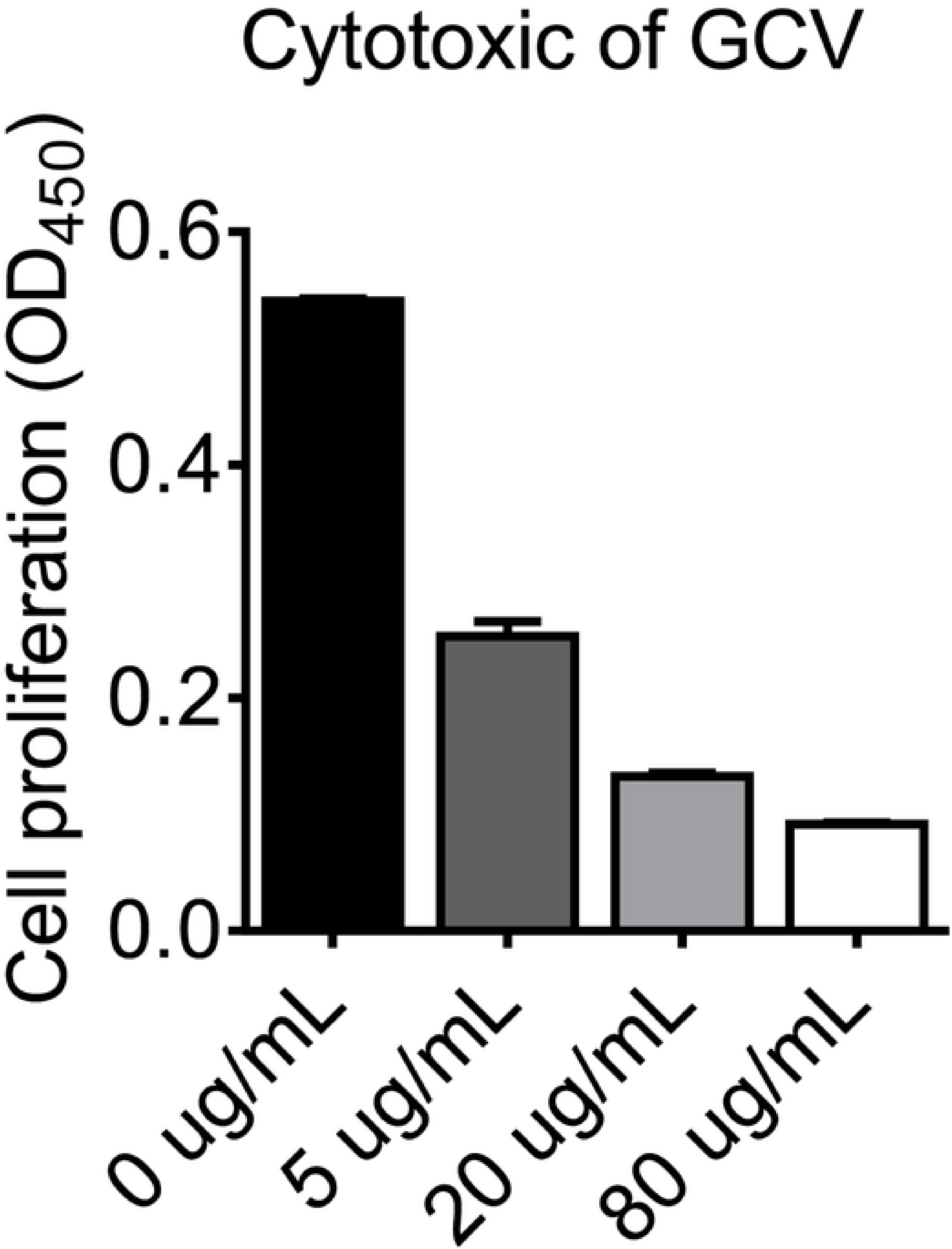
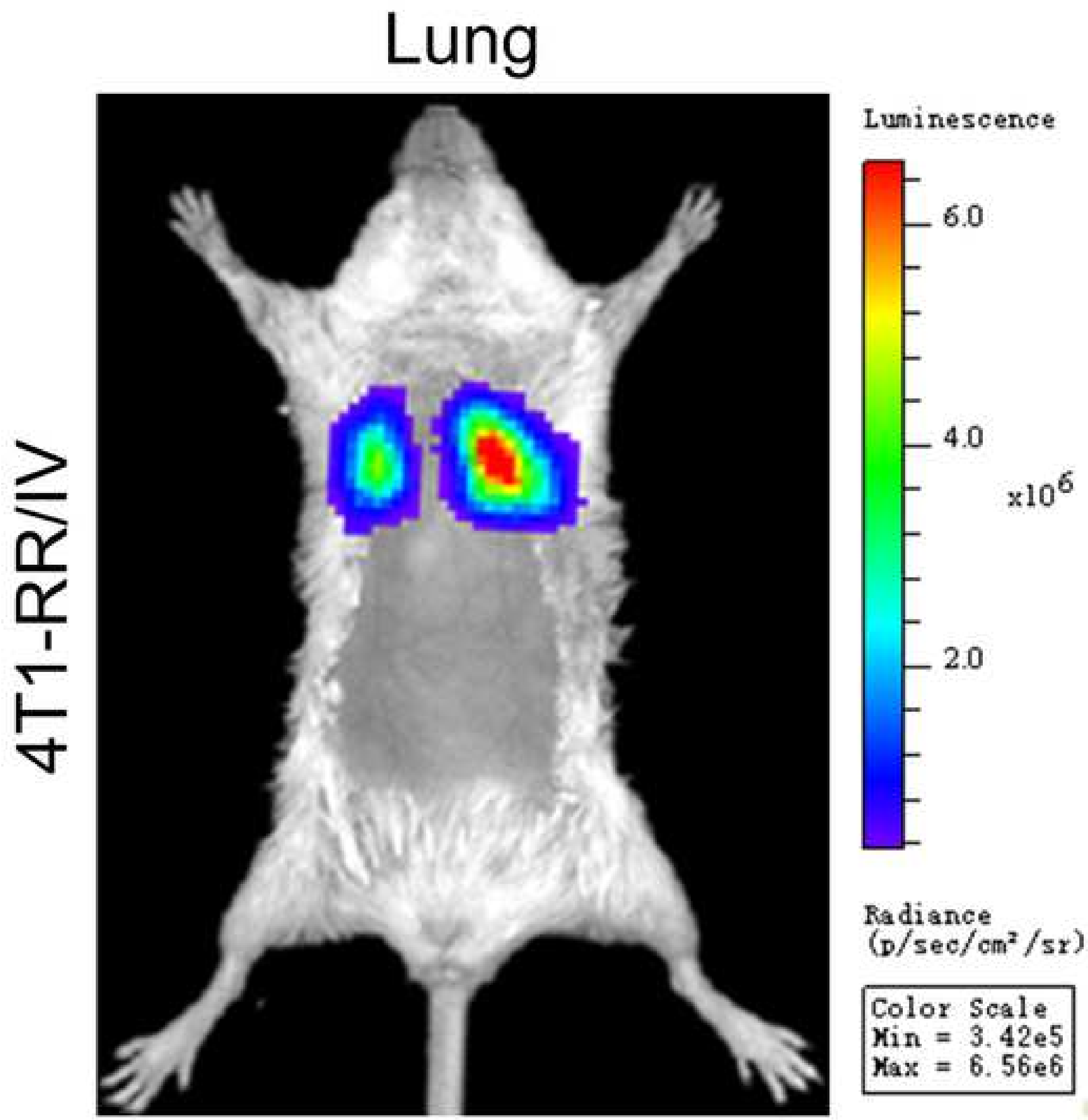


Figure S3. The BLI image of 4T1-RR cells in the lungs. After tail vein injection of 4T1-RR cells, the Rluc signal of cells was detected by BLI.


Tumor size
4T1-RR
4T1-RRT
Tumor Diameter (mm)
10
8
6
4
2
0
GCV
0
5
10
15
20
(d)



Cytotoxic of GCV
Cell proliferation (OD450)
0.6
0.4
0.2
0.0
0 ug/mL
5 ug/mL
20 ug/mL
80 ug/mL


Lung
4T1-RR/IV
Luminescence
6.0
4.0
2.0
x10^6
Radiance
(p/sec/cm²/sr)
Color Scale
Min = 3.42e5
Max = 6.56e6