

Video Article

An Orthotopic Mouse Model of Spontaneous Breast Cancer Metastasis

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Abstract

Metastasis is the primary cause of mortality of breast cancer patients. The mechanism underlying cancer cell metastasis, including breast cancer metastasis, is largely unknown and is a focus in cancer research. Various breast cancer spontaneous metastasis mouse models have been established. Here, we report a simplified procedure to establish orthotopic transplanted breast cancer primary tumor and resultant spontaneous metastasis that mimic human breast cancer metastasis. Combined with the bioluminescence live tumor imaging, this mouse model allows tumor growth and progression kinetics to be monitored and quantified. In this model, a low dose (1×10^4 cells) of 4T1-Luc breast cancer cells was injected into BALB/c mouse mammary fat pad using a tuberculin syringe. Mice were injected with luciferin and imaged at various time points using a bioluminescent imaging system. When the primary tumors grew to the size limit as in the IACUC-approved protocol (approximately 30 days), mice were anesthetized under constant flow of 2% isoflurane and oxygen. The tumor area was sterilized with 70% ethanol. The mouse skin around the tumor was excised to expose the tumor which was removed with a pair of sterile scissors. Removal of the primary tumor extends the survival of the 4T-1 tumor-bearing mice for one month. The mice were then repeatedly imaged for metastatic tumor spreading to distant organs. Therapeutic agents can be administered to suppress tumor metastasis at this point. This model is simple and yet sensitive in quantifying breast cancer cell growth in the primary site and progression kinetics to distant organs, and thus is an excellent model for studying breast cancer growth and progression, and for testing anti-metastasis therapeutic and immunotherapeutic agents *in vivo*.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54040/>

Introduction

According to the American Cancer Society, breast cancer is the most frequently diagnosed form of cancer in women in the United States. Early detection in combination with recently developed targeted therapies has significantly reduced the mortality of breast cancer in the last two decades. However, breast cancer is still the second leading cause of cancer-related death in women in the United States¹. The majority of deaths of breast cancer patients are due to tumor cell metastasis. Unfortunately, most breast cancer is invasive and frequently metastasizes to the lymph node and subsequently to distant organs, including bone, lung, liver and brain.^{1,2} There is currently no effective therapy for metastatic breast cancer. Therefore, development of chemotherapeutic and immunotherapeutic agents to suppress metastatic breast cancer is of great significance.

Various breast cancer spontaneous metastasis mouse models have been developed to study the molecular mechanisms underlying breast tumor cell progression and metastasis and to be used as models for the development of therapeutic agents.^{1,3-5} However, most of these mouse models are genetic tumor models that, while excellent models for mechanistic studies, are not suitable for testing therapeutic agents since the metastasis takes months to develop in these genetic models, thereby requiring costly long-term administration of the anti-cancer agents.^{6,7} Monitoring tumor progression in live mice is also technically challenging. In contrast, a transplanted breast cancer metastasis model has the advantages of short term tumor progression and easy tracking of tumor progression in live mice. The 4T1 orthotopic breast cancer spontaneous metastasis mouse model is such a transplanted tumor model.⁸ In this model, the breast tumor cells are transplanted into the mammary fat pad to establish primary tumor nodules. The primary tumor can then be surgically removed as in human breast cancer patients. 4T1 tumor cells are highly invasive.^{8,9,3,10} Almost all tumor-bearing mice develop metastasis in 30 days after tumor transplant into the mammary fat pad. However, 4T1 tumors grow aggressively in the primary sites and the tumor sizes often exceed the limits that are allowed in most animal protocols. At this stage, the metastases are often micrometastases. Therefore, it is essential to remove primary tumors to allow metastasis progress for testing therapeutic agents. Here, we report the establishment of a simple and yet sensitive procedure of primary tumor transplant, surgical removal of primary tumor and bioluminescence imaging-based quantification of the 4T1 tumor growth and progression *in vivo*.

Protocol

All procedures follow guidelines and approved protocols by Georgia Regents University Animal Use and Care Committee.

1. Establishment of Orthotopic Breast Cancer Tumor

1. One day before the experiment, culture approximately 2×10^6 4T1-Luc tumor cells in a 10-cm culture dish in 10 ml RPMI medium containing 10% FBS. Incubate the culture dish in a CO₂ incubator at 37 °C and 5% CO₂.
2. On the day of tumor cell injection, remove culture medium from the culture dish with vacuum, add 5 ml sterile phosphate buffered saline (PBS), pH 7.4 to the culture dish, and gently shake the dish to wash the culture surface, remove the PBS by vacuum.
3. Add 2 ml trypsin-EDTA (0.05% Trypsin and 0.53 mM EDTA) to the culture dish, incubate it in the CO₂ incubator at 37 °C for about 5 min. Check cells on an inverted microscope to make sure that all cells are detached from the bottom of the culture dish.
4. Quench the trypsin by adding 8 ml serum-containing medium, transfer cells to a 15-ml conical tube and centrifuge the cells at approximately 450 x g for 3 min at RT.
5. Remove the supernatant by vacuum and resuspend cells in 10 ml PBS. Centrifuge cells at approximately 450 x g for 3 min. Remove the supernatant by vacuum, and resuspend cells in 10 ml PBS.
6. Pipette 10 µl of the cell suspension onto a hemocytometer under the cover glass. Count the cells to determine the cell concentration. Resuspend cells in PBS at a density of 2×10^5 cells/ml HBSS. Transfer cells to a 1.5 ml sterile microfuge tube and keep cells on ice until use.
7. Use female BALB/c mice of 6 - 8 week for tumor cell injection.
 1. Shave hair near surrounding nipple #3 using an electronic hair trimmer. Injection of 4T1 cell into the mammary gland 3 fat pad reproducibly generates lung metastasis. Clean the shaved area by using the cotton swab dipped into 70% ethanol. Resuspend cells by inverting the microcentrifuge tube 2 - 3 times. Gently aspirate 50 µl of cell suspension into a ½ CC 27 G 1/2 tuberculin syringe.
 2. Inject the tumor cells into the mammary fat pad under #3 mammary gland of a BALB/c mouse. Place the mouse in a clean cage and return the cage to animal housing facility. Wait approximately 21 - 30 days for tumor development. Tumor growth should be monitored on a regular basis as per institution's policies for tumor studies.

2. Live Mouse Bioluminescence Imaging of Tumor Growth

1. Image mice every 3 days after tumor injection Transfer mice to the imaging facility. Inject 100 µl luciferin (30 mg/ml in PBS) intraperitoneally (i.p.) to mice prior to imaging. After approximately 10 min, anesthetize mice in an induction chamber with 2% isoflurane/O₂ flow. Use the forceps to touch the leg of the mouse. No response to the touch confirms that the mouse is fully unconscious.
 1. Place the mice in the imaging chamber with continuous 2% isoflurane and O₂ at a flow rate of 2 L/min. Place the mouse such that the area of interest (i.e., the mammary pad of the initial tumor injection) faces the camera of the imaging system. Ensure the nose and mouth of the mouse are firmly set within the anesthetic tubing. Animals should be anesthetized per veterinary standards at the institution.
2. Acquire bioluminescence imaging with an array of various exposure time. For instance, set exposure to 30, field of view (FOV) to 25, object height to 1.5 cm, and obtain luminescent photographic images with low power X-ray. Set the units of measurement to "radiance."
 1. Use an automatic linear color range with a maximum of approximately 2.4×10^7 radiance units. After acquiring initial images, adjust settings and obtain an optimized-image (**Figures 3 & 4**).
3. Return mice to clean cages and ensure mice regain consciousness with ambulation before returning the animals to the housing facility.
4. Analyze data with the living imaging software associated with the imaging instrument. Using an imaging program, draw an enclosed line around the region of interest (ROI). Use the imaging software to determine the radiance intensity of the ROI. Record the relative intensities of each ROI for each mouse.

NOTE: Higher radiance intensities indicate greater levels of luminescent cells, and thus greater tumor growth.
5. Visually examine the mouse every 3 days for adverse effects.

NOTE: Mice will be weighed once a week. A weight loss of 15% body weight will be considered adverse effect and the tumor-bearing mice will be euthanized as described in step 5. Mice with ulcerated tumors will also be euthanized.

3. Surgical Removal of Primary Tumors

NOTE: Autoclave scissors, forceps and the wound clips and wound clip applier (**Figure 1**).

1. Perform the surgery in a sterile hood to maintain a sterile condition to reduce risk of infection. One day before surgery, shave the area surrounding the tumors of the tumor-bearing mice with an electric hair trimmer.
2. Approximately 2 - 4 hr before surgery, feed the mice chewable carprofen tablets formulated for mice at a dose of 5 mg/kg body weight.
3. Put the mice in a chamber containing 2% isoflurane in oxygen for approximately 30 sec. Continuously anesthetize the mouse using an anesthesia apparatus during the entire surgical period.
4. Use a sterile cotton stick to apply vet ointment to the eyes of the mouse to prevent dryness while under anesthesia. Use the forceps to touch the leg of the mouse. No response to the touch confirms that the mouse is fully unconscious.
5. Disinfect the skin area around the tumor site with three alternating wipes of a soap disinfectant scrub. Wipe the surgical area with a 70% alcohol wipe.
6. Use a sterilized scalpel (**Figure 1**) to cut a small incision near the tumor.

7. Insert the tip of a pair of sterilized scissors to the incision to cut the skin around the tumor to expose the tumor nodule (**Figure 2A**). Hold the tumor with sterile forceps and use the scissors to separate the tumor from the skin (**Figure 2B**). Save the tumor sample by freezing in a -80 °C freezer or by fixing in 10% formalin solution for future use.
8. Close the surgical site with 9 mm wound clips using the auto-clip applicator (**Figure 1**). Return the mouse to a clean cage with autoclaved bedding. The animals should be monitored per institutional guidelines.
NOTE: The mouse usually regains sufficient consciousness and starts to move in the cage approximately 10 - 30 min after the surgery.
9. Administer carprofen to mice again 24 hr after surgery. Carprofen is for post-surgery analgesia and prescribing of post-surgery analgesia should be verified by direct consultation with the institution's veterinary staff. Remove wound clips after the wound heals (usually within 5 - 7 days after surgery). Remove wound clips using the auto-clip remover.

4. Live Mouse Bioluminescence Imaging of Tumor Metastasis

1. Image mice every 3 days after surgery as described in step 2.

5. Validation of Lung Metastases Using India Ink Inflation of Tumor-bearing Lungs

NOTE: To validate the luciferase live tumor imaging results, perform India ink inflation of tumor-bearing mouse lungs to quantify tumor nodules. 4T1 cells also metastasize to other organs and tissues (**Figure 5**) and therefore, histological examination of these organs to validate tumor metastasis should be performed if needed. This protocol use lung metastasis as a example.

1. Keep mice in their existing home cage for euthanasia. When housed with other animals, relocate those mice not being euthanized to a different cage.
2. Remove the cage filter top and replace with the CO₂ filter top. Turn on the compressed CO₂ gas cylinder (100%) that is connected to the CO₂ filter top. Set the flow rate at 2 L/min and keep CO₂ on for at least 10 min. If the mice are still breathing at the end of 10 min, leave the CO₂ on until at least 1 min after mice stop breathing. Turn off CO₂ cylinder control and the flow meter. Wait for 3 more minutes and remove the filter top.
3. Place the sacrificed-mouse on its back on a polystyrene board. Pin the legs to ensure unobstructed access to the trachea. Spray the mice with 70% ethanol.
4. Using a pair of scissors, cut along the midline of the mid-abdomen of the mouse through the ribcage and up toward the salivary glands. Observe the trachea. Use forceps to remove tissues surrounding the trachea to expose the trachea.
5. Thread a pipette tip underneath the trachea. Holding the tip with one hand, gently lift the trachea up and away from the body.
6. Rotate the platform holding the mouse 180°. Use a ½ CC 27 G 1/2 tuberculin syringe to inject India Ink (10% India ink and 0.1% Ammonium Hydroxide) into the lungs via the trachea. Completely inflate the lungs with ink until a strong resistance is felt.
7. Use a pair of scissors to cut the trachea. Use forceps to hold the mouse and insert another set of forceps under the lungs and pull the lung lobes out of the mouse. Rinse the lungs briefly in a beaker containing water.
8. In a chemical fume hood, transfer the lungs to a glass scintillation vial containing 3 ml of Fekete's solution (50% Ethanol, 6% Formaldehyde, and 3% glacial acetic acid). Cap the vial to prevent the solution from evaporating.
NOTE: The tissues can be stored in Fekete's solution indefinitely.
9. After a few minutes, observe tumor nodules as white dots on the black lungs (**Figure 5**). The white tumor nodule is visible by eyes. Transfer the lungs to a dish in a fume hood and count the number of white spots. Each white spot represents a single metastatic tumor nodule.

Representative Results

Establishment of Orthotopic Breast Cancer Mouse Model

4T1 is an aggressive mammary carcinoma cell line. Injection of as little as 1×10^4 cells into the mammary fat pad can lead to establishment of a single tumor nodule in the site of injection (**Figure 2A**). Therefore, the tumor mimics human primary mammary carcinoma. Almost 100% mice develop the orthotopic tumor. The tumor size can be quantified using a digital caliper or by live tumor imaging (**Figure 3**). Tumors are detectable approximately 3 days after tumor injection using a bioluminescence imaging system, and the luminescence intensity increases as tumor size increases (**Figure 3**). Therefore, this model is an ideal orthotopic breast cancer model for testing efficacy of chemotherapeutic and immunotherapeutic agents against all stages of mammary carcinoma.

Spontaneous Metastasis

In human breast cancer patients, the primary tumors are surgically removed after diagnosis. However, a large number of patients already have LN or distant metastasis at the time of the diagnosis. In the procedure, most of the mice have developed LN and distant metastasis after 30 days of tumor transplant. The described surgical procedure completely removes the primary tumor (**Figure 2B**). No residual primary tumors are detected at the primary tumor sites 10 - 30 days after surgery (**Figure 2B**). Surgical removal of the primary tumor extends the life span of the tumor-bearing mice but does not prevent tumor metastasis to various parts of the mice. These metastases can be detected by the live imaging method (**Figure 4**). This model thus is a spontaneous metastasis breast cancer model that mimics human breast cancer patients. This model is useful for: 1) studying spontaneous breast cancer metastasis (for example, 4T1 cells can be transfected with a gene of interest to test the functions of these genes in breast cancer spontaneous metastasis); and 2) testing the efficacy of chemotherapeutic and immunotherapeutic agents against spontaneous breast metastasis in an immune-competent host. The sites of and the degrees of metastasis can be detected by live imaging (**Figure 4**) and validate with a second approach (**Figure 5**). Luminescence imaging can quantify tumor burden of the entire lung. Ink inflation of tumor-bearing lungs allows accurate counting of the actual tumor nodules of each lung (**Figure 5**), thereby, representing a complimentary quantification method of tumor metastasis.



Figure 1. Surgery Tools. 1. Stainless scalpel, 2. Forceps, 3. Stainless scissors, 4. Autoclip wound clip applicator, 5. Autoclip wound clip remover. [Please click here to view a larger version of this figure.](#)

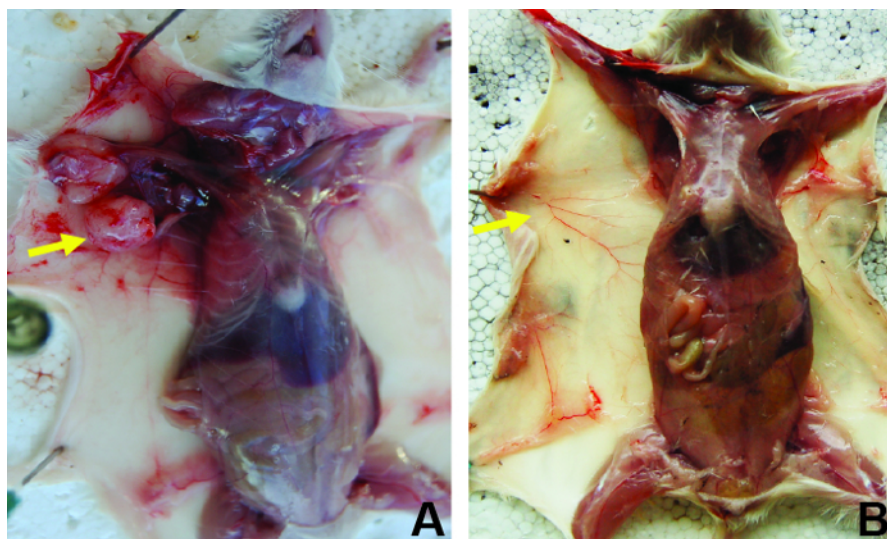


Figure 2. The Orthotopic 4T1 Tumor Model. **A.** 4T1 tumor cells (1×10^4 cells in 100 μ l PBS) cells were injected into the mammary fat pad. Thirty days after tumor injection, the mouse was sacrificed and examined for tumor growth. Shown is the tumor-bearing mice with a single tumor nodule at the site of injection (yellow arrow). **B.** The tumor as shown in **A** was surgically removed. Shown is the 4T1 tumor-bearing mice 10 days after surgical removal of the primary tumor. [Please click here to view a larger version of this figure.](#)

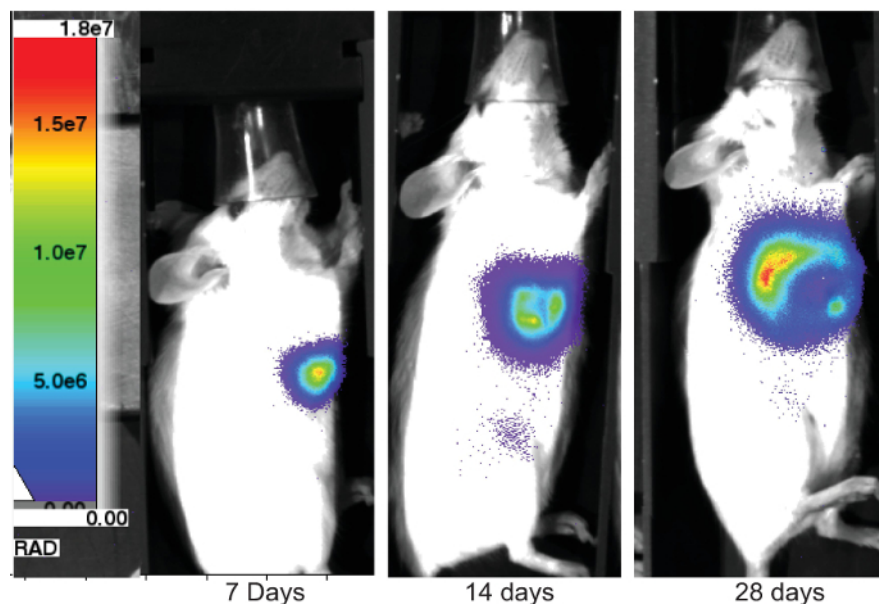


Figure 3. Live Tumor Imaging by Luciferase-based Live Tumor Imaging. 4T1 tumor cells (1×10^4 cells in 100 μ l PBS) cells were injected into the mammary fat pad. The mouse was imaged at days 7, 14 and 28. Shown is the luminescence intensity of the primary tumor. [Please click here to view a larger version of this figure.](#)

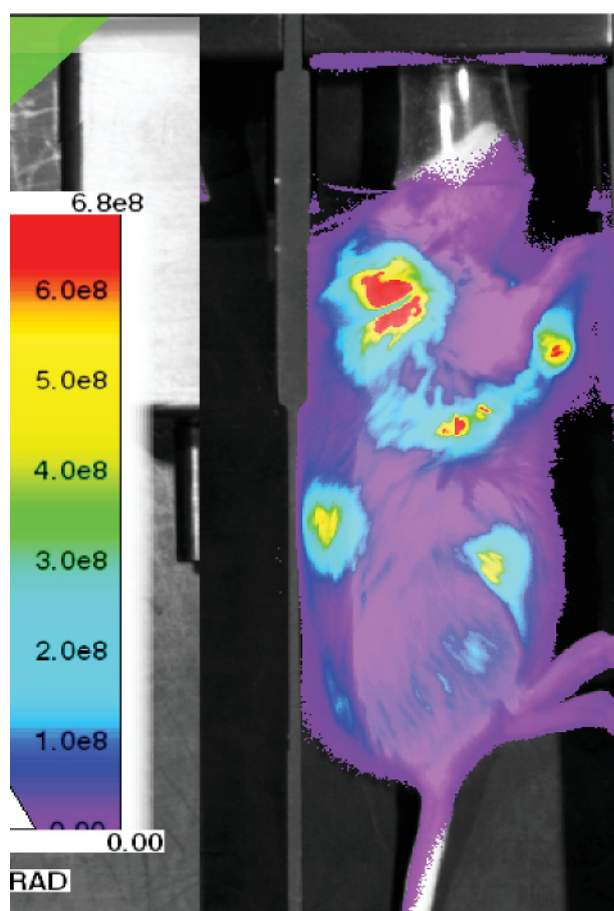


Figure 4. Visualization of Tumor Progression by Luciferase-based Live Tumor Imaging. 4T1 tumor cells (1×10^4 cells in 100 μ l PBS) cells were injected into the mammary fat pad. The primary tumor was surgically removed as shown in **Figure 3**. The mouse was then imaged 17 days after surgical removal of the primary tumor. Shown is image of metastasis. [Please click here to view a larger version of this figure.](#)

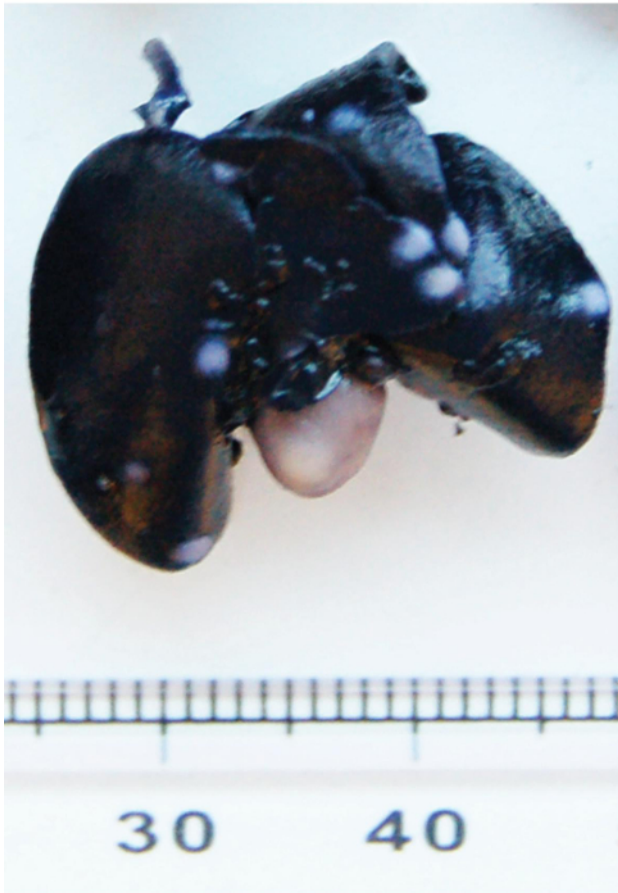


Figure 5. Visualization of Lung Tumor Nodules by India Ink Inflation. The lung of the tumor-bearing mouse was inflated with India ink and fixed in Fekete's solution. The white dots are lung metastases. [Please click here to view a larger version of this figure.](#)

Discussion

Many types of transgenic mouse models of breast cancer metastasis have been developed.¹ These transgenic mice have high tumor incidence ranging from 60 to 100%. However, the metastasis incidence of these transgenic mice is much lower than the tumor incidence (14 - 100%). Tumor cells metastasize to LN and lungs in the majority of these transgenic mouse models of breast cancer metastasis. The metastasis latency varies from model to model and ranges from 2 to 8 months.^{6,11-16} These transgenic mouse models of breast cancer metastasis are excellent systems for studying the genetic and molecular mechanisms underlying breast cancer metastasis. However, the low metastasis incidence and long metastasis latency limit the usefulness of these models in the test of anti-cancer agents.

The orthotopic implantation of 4T1 cells in the mammary fat pad, with the formation of primary tumors and subsequent metastatic growth, resembles multiple stages from malignant breast cancer, including primary tumor formation, lymph node metastasis and distant organ metastasis. Moreover, syngenic transplantation avoids immunologic host-versus-graft reaction allowing the study of the contribution of tumor microenvironment, including immune system, to malignant tumor progression. This model is also useful for studying host anti-tumor immune response. Therefore, injection of 4T1 cells in the mammary fat pad represents a fast and quantitative method to study breast cancer metastasis.

The orthotopic 4T1 transplant tumor model has a 100% tumor incidence in less than 30 days and 100% metastasis incidence in less than 60 days (**Figure 2-4**). Furthermore, unlike most of the transgenic mouse models of breast cancer metastasis, 4T1 tumor cells also metastasize to the bone (**Figure 4**), thereby, resembling human breast cancer metastasis.

Stable expression of luciferase-coding cDNA in 4T1 tumor cells allows monitoring of tumor growth and progression in live mice over time (**Figure 3**). The tumor burden and metastasis kinetics can be quantified based on the luminescence intensity. Furthermore, the metastasis sites can also be identified by the luciferase imaging (**Figure 4**) and validated with a complementary approach (**Figure 5**). Therefore, this breast cancer spontaneous metastasis model is an excellent model for determining the efficacy of anti-metastasis agents *in vivo*.

Most of the surgery involves closure of cuts with sutures. Here, we observed that stainless wound clips work well (**Figure 1**). The wound clip can be easily sterilized and easily applied with the autoclip applicator (**Figure 1**). The wound clips can be easily removed with the clip remover (**Figure 1**) after the wound has been healed. We have observed no infections with this surgical technique in over 20 surgeries.

One limitation of this model is the fast tumor growth rate. Majority of the metastases-bearing mice dies in 1 - 2 months after surgery due to extensive metastasis burden. Another limitation is that 4T1 is a mouse cell line and its response to experimental drugs could differ to the response from human cells. This should be considered in study design for experimental drug testing.

In summary, we have optimized a simple and yet sensitive procedure of orthotopic breast cancer spontaneous metastasis model. This model mimics human breast cancer metastasis. The tumor progression kinetics can be monitored and quantified over time. This model is an ideal one not only for studying breast cancer growth and metastasis, but also for testing the efficacy of chemotherapeutic and immunotherapeutic agents¹⁷ in suppression of spontaneous breast cancer metastasis.

Disclosures

Mice were purchased from the Jackson Laboratory and housed in the Georgia Regents University animal facility. Experiments and care/welfare were in agreement with federal regulations and an approved protocol by the Georgia Regents University IACUC committee. The authors have nothing to disclose.

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