**Author’s Point-by-point Response the Editorial Comments**

**Manuscript #57849\_R1**

**Editorial comments:**

**Comment A1:** *The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors. Several sentences are poorly structured.*

**Response A1:** We appreciate the Editorial comment. The manuscript was reviewed and edited by a native English speaker who is proficient in scientific writing.

**Comment A2:** *How was this compared? Do you report this herein? Please do not make claims that’s are unsupported. This model does not mimic human breast cancer progression, but attempts to mimic it.*

**Response A2:** As per the Editorial comments, we revised the short abstract as below.

**“We introduced a murine orthotopic breast cancer model and radical mastectomy model with bioluminescence technology to quantify tumor burden in which we sought to mimic human breast cancer progression.”**

**Comment A3:** *“Luciferase-expressing” may be a better fit.*

**Response A3:** As per the Editorial comments, we revised as **“Luciferase-expressing”.**

**Comment A4:** *Please expand your Introduction to include the following:*

**Comment A4-1:** *The advantages over alternative techniques with applicable references to previous studies (avoid self citations);*

**Response A4-1:** As per the Editorial comment, we added the sentenced in the Introduction section as below.

**“To mimic human breast cancer progression, we chose the #2 mammary fat pad as an inoculation site which is located in the chest. In most of the studies, breast cancer cells are inoculated subcutaneously. This technique does not require surgery and thus it is simple and straightforward. However, the subcutaneous microenvironment is quite different than the mammary grand microenvironment, which therefore results in a different pattern of cancer progression and even molecular profiles compared to mammary gland inoculated tumors. Some studies use the #4 mammary gland, which is located in the abdomen, as an inoculation site. However, because the #4 mammary glands are located on the abdomen, the most common metastatic pattern is peritoneal carcinomatosis, which is stark contrast to human breast cancer progress where peritoneal carcinomatosis occurs in less than 10% of metastatic breast cancer cases. Breast cancer generated by our technique in the #2 mammary gland metastasizes to the lung which is one of the most common human breast cancer metastatic sites.”**

**“Cancer cell inoculation via the tail vein is the most common lung metastasis mouse model, the so called “experimental metastasis”. This model is easy to generate and does not require surgery; however, it does not mimic human breast cancer progression which may result in different metastatic disease behavior. In order to mimic the human breast cancer treatment course where metastasis often occurs after mastectomy, we removed the primary tumor after orthotopic cancer cell inoculation.”**

**Comment A4-2:** *Description of the context of the technique in the wider body of literature;*

**Response A4-2:** As per the Editorial comment, we revised the sentenced in the Introduction section as below.

**“Breast cancer generated by our technique in the #2 mammary gland metastasizes to the lung which is one of the most common breast cancer metastatic sites. In this technique, we also sought to achieve a higher tumorigenesis rate with minimal tumor size variability. To do so, cancer cells suspended in a gelatinous protein mixture were inoculated under direct vision through a median anterior chest wall incision. This technique produced a high tumorigenesis rate with less variability in tumor size and shape compared to subcutaneous or non-surgical injection.”**

**“We also introduced a mouse radical mastectomy technique in which the orthotopic breast tumor is resected with surrounding tissues and axillary lymph nodes. Cancer cell inoculation via tail vein is the most common lung metastasis mice model, the so called “experimental metastasis”. This model is easy to generate and does not require surgery; however, it does not mimic human breast cancer progression which may result in different metastatic disease behavior. Thus, we removed the primary tumor after orthotopic cancer cell inoculation which mimics human breast cancer treatment course.”**

**Comment A4-3:** *Information that can help readers to determine if the method is appropriate for their application.*

**Response A4-3:** This model is applicable to most cases, thus we stated as below.

**“The techniques we described are applicable for most of breast cancer orthotopic model experiments. However, it is important to consider that the gelatinous protein mixture can affect the microenvironment and surgery can affect the stress/immune response.”**

**Comment A5:** *References?*

**Response A5:** As per the Editorial comment, we added the citation.

**Comment A6:** *References?*

**Response A6:** As per the Editorial comment, we added the citation.

**Comment A7:** *References for the well established approaches?*

**Response A7:** As per the Editorial comment, we added the citation.

**Comment A8:** *References for the well established approaches? A description of the standard mastectomy approach in humans will help here.*

**Response A8:** As per the Editorial comment, we added the sentences in the Introduction section as below.

**“In the clinical setting, the standard of care for breast cancer patients without distant metastasis disease is mastectomy. Before mastectomy, axillary lymph node metastasis is surveyed by imaging and sentinel lymph mode biopsy. If there is no evidence of axillary lymph node metastasis, the patient is then treated by total or partial mastectomy, in which the axillary lymph node resection is omitted. Total mastectomy is a technique to resect breast cancer with the whole breast tissue en bloc, whereas partial mastectomy is to resect breast cancer with a margin of surrounding normal breast tissue only, thus conserving the remaining normal breast tissue in the patient. However, patients who preserve their remaining normal breast tissue after partial mastectomy require postoperative radiotherapy to avoid local recurrence. Patients who have axillary lymph node metastasis undertake radical mastectomy which removes the breast cancer with all normal breast tissue and axillary lymph nodes and invaded tissues en bloc. In the mouse model, surveillance for axillary lymph node metastasis and/or post-operative radiation is not reasonable or feasible. Thus, we utilized the radical mastectomy technique to avoid local recurrence.”**

**Comment A9:** *Higher success rate as compared to what?*

**Response A9:** As per the Editorial comment, we added the sentences in the Introduction section as below.

**“The aims of these methods are 1) to mimic human breast cancer progression and treatment course, 2) to conduct in vivo experiments with greater efficiency and higher success rates compared to other breast cancer inoculation or mastectomy techniques.”**

**Comment A10:** *Improve compare to what? If previous work, then references must be cited.*

**Comment A11:** *Higher than?*

**Response A10, A11:** As per the Editorial comment, we revised the sentences in the Introduction section as below.

**“In this technique, we also sought to achieve a higher tumorigenesis rate with minimal tumor size variability compared to other breast cancer inoculation techniques.”**

**Comment A12:** *Was this quantified later on? Unclear how you can claim this without a thorough analysis.*

**Response A12:** We apologize for unclear statement. It was demonstrated by our previous work.We added the sentences in the Introduction section as below.

**“This technique produced a high tumorigenesis rate with less variability in tumor size and shape compared to subcutaneous or non-surgical injection, as we have previously reported.”**

**Comment A13:** *Axillary?*

**Response A13:** We apologize for the error in the spelling. It was revised as **“axillary”.**

**Comment A14:** *Was this quantified later on? Unclear how you can claim this without a thorough analysis*

**Response A14:** We apologize for the unclear statement. It was demonstrated by our previous work.We revised the sentence in the Introduction section as below.

**“This technique produced less local recurrence compared to simple tumor resection, as we have previously reported,”**

**Comment A15:** *Needs references.*

**Response A15:** As per the Editorial comment, we added the reference.

**Comment A16:** *Please add a step to briefly describe how the cells are cultured and maintained. Please include medium used, incubation environmental conditions etc.*

**Response A16:** As per the Editorial comment, we added 2.1) as below.

**“2.1) Culture 4T1-luc2 cells, a mouse mammary adenocarcinoma cell line expressing luciferase, in Roswell Park Memorial Institute 1640 media with 10% fetal bovine serum in a humidified incubator at 37°C in 5% CO2.”**

**Comment A17:** *Do they adhere to the dish?*

**Response A17:** As per the Editorial comment, we revised the sentence of 2.2) as below.

**“2.2) Wash adherent 4T1-luc2 cells in a 10 cm dish with phosphate-buffered saline (PBS)”**

**Comment A18:** *Please change “degrees” to “ °C” throughout.*

**Response A18:** As per the Editorial comment, we revised “degree” to **“°C”** throughout.

**Comment A19:** *What is the composition? Please add it to the table of materials.*

**Response A19:** As per the Editorial comment, we revised 2.2) as below and added materials in the table of materials.

**“Then, add 4ml growth media (RPMI-1640 with 10% FBS)”**

**Comment A20:** *Transfer the cell suspension? unclear*

**Response A20:** As per the Editorial comment, we revised 2.2) as below.

**“transfer the cell suspension into a 15 mL conical tube”**

**Comment A21:** *Please convert to g.*

**Response A21:** As per the Editorial comment, we revised “1500 rpm” to **“180 x g”.**

**Comment A22:** *Please revise for grammar.*

**Response A22:** As per the Editorial comment, we revised the sentence as below.

**“we use 1x104/20 μL of final concentration, to avoid reaching euthanasia criteria (tumor size > 2cm) within 2 weeks.”**

**Comment A23:** *Mention oxygen flow rate.*

**Response A23:** As per the Editorial comment, we added about oxygen flow as below.

**“3.1) Put the mice in the anesthesia induction chamber with 2.5% isoflurane and 0.2 L/min oxygen flow until the mice breathe calmly (2-3 min).”**

**Comment A24:** *Please mention how you ensure depth of anesthesia (e.g. toe pinch).*

**Response A24:** As per the Editorial comment, we added about oxygen flow as below.

**“3.3) Confirm adequate anesthesia by the lack of reaction to toe pinch.”**

**Comment A25:** *Limbs?*

**Response A25:** As per the Editorial comment, we revised “legs and arms” to **“limbs”.**

**Comment A26:** *Are analgesics provided? Mention dosage.*

**Response A26:** Analgesics was not required, but we anesthetized the mice by isoflurane. We revised the sentence as below.

**“3.9) Remove the sutures under anesthesia same as 3.1) 7 days after surgery.”**

**Comment A27:** *In how many animals did you observe this? I’m also not sure about what purpose this statement serves here. Is this typical for this model? Is this reported by others for this particular cell line? Please provide references. This seems incredibly early for the number of cells injected. How did you confirm the presence of lung metastasis without lung resection and imaging? How do you know that the injected cells were not localized in the lungs rather than a true metastasis?*

**Response A27:** The purpose of this statement is to illustrate that timing of lung metastasis is varied by cell line or injected cell number. We confirmed that 100% of mice in over 50 mice experiments developed lung metastasis at 8 days after inoculation. Thus, we described it here to show the timing of lung metastasis in this particular setting. To clarify these points, we revised the sentence as below.

**“Note: Timing of mastectomy is very important. If it is too early, lung metastasis does not occur. If it is too late, the primary tumor has invaded major blood vessels which make complete oncologic resection challenging. Thus, we tested multiple time points for mastectomy to determine which time point produced the appropriate balance in waiting for metastasis before resection became too challenging. After doing so in over 50 mouse experiments it was demonstrated that mastectomy at 8 days after cancer cell inoculation (or when tumor size reaches 5 mm) was the ideal time point to achieve that balance13.”**

**Comment A28:** *Unclear, needs grammar revision*

**Response A28:** As per the Editorial comment, we revised the sentence as below.

**“Make a 5 mm skin incision in 2 mm left from the surgical scar which was made at the initial cancer cell inoculation, using the micro dissection scissors.”**

**Comment A29:** *Forelimb?*

**Response A29:** As per the Editorial comment, we revised “arm” to **“forelimb”.**

**Comment A30:** *How many? How do you identify them? Can you provide a photograph to aid identification?*

**Response A30:** We apologize for the unclear statement. Most of the time, the axillary lymph nodes are not visible at the time of mastectomy; however, en bloc resection with axillary lymph nodes is important to avoid visible recurrence by bioluminescence in order to monitor lung metastasis. To clarify these points, we revised the sentence as below and added the citation of our previous publication which contains the picture of the mastectomy.

**“Extend the incision toward the root of the forelimb to remove the tumor, the skin including the surgical scar and the lesion in contact with the tumor as well as the axillary lymph node basin in which most of the time no visible lymph node exist at the time of mastectomy13.”**

**Comment A31:** *Are analgesics provided? Mention dosage.*

**Response A31:** Analgesics was not required, but we did anesthetize the mice by isoflurane. We revised the sentence as below.

**“4.6) Remove the sutures under anesthesia same as 3.1) 7 days after surgery.”**

**Comment A32:** *Shouldn’t this section be placed before section 4? Or do you image the animal after mastectomy?*

**Comment A33***At what time points after inoculation?*

**Response A32, 33:** We apologize for the confusing title and lack of adequate explanation. Bioluminescence can be used for both primary tumor (without mastectomy) and lung metastasis (mastectomy model) quantification regularly. To clarify these points, we revised the title and added the note as below.

**“5. Bioluminescent quantification of the primary tumor (orthotopic inoculation without mastectomy) or lung metastasis (mastectomy model)”**

**“Note: For primary tumor burden quantification, the bioluminescence is measured twice a week from the day after orthotopic inoculation. For lung metastasis quantification, the bioluminescence is measured twice a week from the day after mastectomy.”**

**Comment A34:** *Degree Celsius?*

**Response A34:** As per the Editorial comment, we revised “degrees” to **“°C”.**

**Comment A35:** *In prone position?*

**Response A35:** The mouse is placed in the supine position. We revised the sentence as below.

**“5.6) Fit each mouse with a nose cone inside the imaging system in the supine position”**

**Comment A36:** *This seems pretty long? Wouldn’t Luciferin clear out by this*

**Response A36:** Peak times varied by individuals and by the day of image acquisition; however, sometimes it takes around 50 minutes to reach peak bioluminescence. We have no experience of problems due to luciferin clear out. In our technique, cells were suspended in 90% Matrigel. We think due to a high concentration of Matrigel, it takes a long time for luciferin delivery to cancer cells.

**Comment A37:** *What is the acquisition duration per image?*

**Response A37:** It depends on exposure time. As we described in 5.7.1), we usually use “auto” exposure time which the software automatically chooses as the suitable exposure time. It is decided by the signal intensity of the original cell and the size of tumor burden, in another words, total luciferase-luciferin shining amount.

**Comment A38:** *By ex vivo what? Sound incomplete.*

**Response A38:** As per the Editorial comment, we revised the title as below.

**“6. Lung metastasis tumor burden quantification by ex vivo imaging”**

**Comment A39:** *Why was this time point chosen? Also, is this done on the animals that have undergone mastectomy?*

**Response A39:** As per the Editorial comment, we added “Note” as below.

**“Note: lung metastasis quantification is applicable for both orthotopic inoculation with and without mastectomy models. In the mastectomy model, ex vivo imaging or survival observation is chosen depending on the purpose. In the orthotopic inoculation (without mastectomy) model, most cases produce primary tumor size euthanasia criteria (2.0 cm) approximately 21 days after inoculation.”**

**Comment A40:** *By ex vivo what? This is incomplete. Do you mean “Ex vivo imaging”?*

**Response A40:** We apologize for the error. As per the Editorial comment, we revised as **“ex vivo imaging”.**

**Comment A41:** *Luciferase image? Mention acquisition settings?*

**Response A41:** As per the Editorial comment, we revised the sentence as below.

**“6.12) Capture the bioluminescence image (same as 5.7.1) and 5.7.2)) 5 min after euthanasia (20 min after luciferin injection).”**

**Comment A42:** *This progression is not always follows. Lung metastasis can happen by the lymphatic and/or blood route, and there is likely no sequence as such where the lung metastasis follows the nodal metastasis. Please cite a reference to back this up.*

**Response A42:** As per the Editorial comment, we added the citation.

**Comment A43:** *Provide the equation used for this or a reference for tumor volume measurements using calipers.*

**Response A43:** As per the Editorial comment, we added the sentence as below.

**“The tumor volume was estimated using the following equation: volume = (length) x (width)2/2.”**

**Comment A44:** *This is vague, unclear what is meant by this. Do you mean that they show similar trends?*

**Response A44:** We apologize for unclear statement. We revised the sentence as below.

**“Quantification of the tumor burden by bioluminescence and caliper measurement showed similar trends in the representative results”.**

**Comment A45:** *Imaging?*

**Response A45:** We apologize for the error of the grammar. We revised as **“ex vivo imaging”.**

**Comment A46:** *Please provide references for standard mastectomy.*

**Response A46:** As per the Editorial comment, we added citation for standard mastectomy.

**Comment A47:** *How can you be confident that all the luminescence signal you measure is coming from mets? Could it not be residual tumor that was not resected?*

**Response A47:** As we described in the Representative results section as below, if local recurrence occurs, it can be easily distinguished from the lung metastasis.

**“if the post-operative day one bioluminescence exceeds 1.00E + 06 photons (10 times larger than other individuals), there is a high possibility of local residual tumor13. If there are remnant cancer cells present, a palpable tumor appears within 2 weeks.”**

**Comment A48/A49:** *Examples?*

**Response A48/A49:** We apologize for the unclear description for that. We revised the sentence as below.

**“This model is particularly useful in the preclinical study of the development of novel therapeutics13. As we have previously published, a Src inhibitor, AZD0530, which showed efficacy in preclinical mice model using but failed in clinical trial for breast cancer treatment, showed efficacy in the primary lesion utilizing orthotopic inoculation without mastectomy but not in lung metastasis utilizing our mastectomy model. Although anti-cancer drugs, e.g. anthracyclines, are sometimes used in humans as neoadjuvant therapy to treat primary breast tumors, the vast majority of drugs are used as adjuvant therapy where drugs are given after surgery to reduce the risk of recurrence by treating clinically undetectable cancer or as palliative treatment for metastatic cancer1,7,18.”**

**Comment A50:** *Reference?.*

**Response A50:** As per the Editorial comment, we added the citation.

**Comment A51:** *Which drug? What is the dosage. This appear out of the blue and distracts the reader.*

**Response A51:** We apologize for the confusing description. We revised the sentence as below.

**“To test any of drug efficacies for metastatic lesions, it should be administrated after mastectomy.”**

**Comment A52:** *Ex vivo?*

**Comment A53:** *How was this done? In vivo or ex vivo? If in vivo, do you study the minimum met size for detection, and if ex vivo, do you extract all organs and perform imaging?*

**Response A52/53:** We apologize for the unclear description. We revised the sentences as below.

**“This model allows for lung metastatic lesion quantification as well as whole body metastatic lesion quantification as long as metastatic tumor burden has a detectable bioluminescence utilizing in vivo imaging.”**

**Comment A54:** *Please report the percentage recurrence*

**Response A54:** As per the Editorial comment, we added the sentence as below.

**“In our hands, local recurrence rate was less than 5% in over 50 mice experiments.”**

**Comment A55:** *References?*

**Response A55:** As per the Editorial comment, we added citation.

**Comment A56:** *What was the percentage of such cases in your study?*

**Comment A57:** *Is this for all the animals? Did you exclude animals with local recurrence?*

**Comment A58:** *What about the local recurrence?*

**Comment A60:** *What about the local recurrence?*

**Response A56/A57/A58/A60:** The percentage of the local recurrence cases is less than 5% and these animals were excluded from any of further analysis. To clarify this point, we revised the sentence as below.

**“thus those animals with local resurrence (<5% in our experience) should be excluded from any of further analysis.”**

**Comment A59:** *Please report the criteria*

**Response A59:** As per the Editorial comment, we revised the sentence as below.

**“Since there is no primary lesion, mice cannot meet euthanasia tumor criteria which are generally defined as tumor size > 2.0 cm or ulceration of the primary tumor.”**

**Comment A61/A62:** *Please report the sample sizes, how many animals in total?*

**Response A61/A62:** As per the Editorial comment, we added mice number.

**Comment A63:** *Is the number of days report, days after inoculation or days after mastectomy?*

**Response A63:** As per the Editorial comment, we revised x axis title as **“Time after inoculation (Days)”.**

**Comment A64:** *Is the number of days report, days after inoculation or days after mastectomy? This image strongly suggests that there was local recurrence and that the signal you see is not only metastasis. How do you claim that the signal in B is from mainly metastatic lesions? I do not believe this was decoupled here.*

**Response A64:** As per the Editorial comment, we revised the x axis title as **“Time after inoculation (Days)”.** And as we described, once local recurrence occurred, it grows very fast and signal intensity is much higher and reaches palpable size within 2 weeks. We confirmed that the signal was from the lung by lung ex vivo imaging and whole body imaging after lung removal. To clarify this point, we added the sentence as below.

**“It was confirmed that signals were from lung metastasis utilizing ex vivo imaging, and no local recurrence was confirmed by whole body imaging after lung removal.”**

**Comment A65:** *You mean no neoadjuvant or adjuvant treatment correct? Mastectomy is a form of treatment.*

**Response A65:** As per the Editorial comment, we revised the sentence as below.

**“Kaplan-Meier survival curve of mastectomy model without any of drug treatment.”**

**Comment A66:** *References?*

**Comment A67:** *This needs references.*

**Comment A68:** *Needs references.*

**Comment A69:** *References?*

**Comment A70:** *References?*

**Comment A71:** *References?*

**Response A66/A67/A68/A69/A70/A71:** As per the Editorial comment, we added references.

**Comment A72:** *How do you know that the lung mets are metastasis and not cancer spread from contact (as you mention here)?*

**Response A72:** We confirmed lung metastasis by histology and lung ex vivo imaging, and also confirmed no shining lesion present by whole body imaging after lung removal. To clarify it, we added the sentence as below.

**“We confirmed lung metastasis by histology and lung ex vivo imaging, and we also confirmed no shining lesion present by whole body imaging after lung removal.”**

**Comment A73:** *In how many days?*

**Response A73:** As per the Editorial comment, we revised the sentence as below.

**“In our 4T1 orthotopic model, cancer cells eventually metastasize to the lungs in all cases within 21 days.”**

**Comment A74:** *Please reference other works where this is shown. My understanding is this is very common, but it is important to cite references to published literature.*

**Comment A75:** *References?*

**Response A74/A75:** As per the Editorial comment, we added references.

**Comment A76:** *Even if they are shaved?*

**Response A76:** Yes, even after they are shaved, there is a pigmentation issue. To clarify this point, we added the sentence as below.

**“Even after the fur is removed, there is pigmentation on the skin27.”**

**Comment A77:** *What is your success rate so far? Please report this.*

**Response A77:** Once an investigator is getting used to this technique, it is easy to achieve 100% success rate. To clarify this point, we added the sentence as below.

**“In our model which we introduced here, once an investigator has learned this technique, it is easy to achieve a 100% tumorigenesis rate.”**

**Comment A78:** *How do you quantify and estimate this?*

**Response A78:** It is largely dependent on the model. In our case, we compared tumorigenesis rate tumor size variability in multiple settings (subcutaneous inoculation, orthotopic inoculation without surgical incision and our model) in previously published work, as described in the Discussion section.

**Comment A79:** *How is this relevant here? Seems like you are just trying to cite more of your work in this paper*

**Response A79:** As per the Editorial comment, we removed the sentence.

**Comment A80:** *All of the references are self citations. Please include references to work from other groups and minimize the self citations.*

**Response A80:** Since we added more citations, now less than half the bibliography is composed of publications from our group.