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Intramuscular Injection of Osteosarcoma Cells with Limb Amputation to Study Osteosarcoma Metastasis in Mice

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TITLE:

Intramuscular Injection of Osteosarcoma Cells with Limb Amputation to Study Osteosarcoma Metastasis in Mice

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KEYWORDS:

Osteosarcoma; lung metastases; amputation; primary tumor; knee joint; mice.

SUMMARY:

To study osteosarcoma lung metastasis, we describe a modified protocol wherein intramuscular injection-induced primary tumor is followed by limb amputation.

ABSTRACT:

Osteosarcoma (OS) is a highly malignant bone tumor and one of the leading causes of cancer-related death in pediatric age groups. The 5-year survival rate of OS patients without metastases is 70%; however, the 5-year survival rate drops to 29% once metastases are diagnosed. In addition, 20% of all OS patients have been diagnosed with metastases, highlighting the importance of studying OS metastases.

A good OS metastatic mouse model is essential to investigate the mechanisms underlying OS metastases. Although there are several OS mouse models to study tumorigenesis and progression, most of these models do not reflect OS lung metastases very well. For example, tail-vein injection of OS cells to form OS pulmonary tumors is widely used but bypasses the intravasation step in the early stage of metastasis. One alternative method is to intramuscularly (IM) inject OS cells into immunodeficient athymic nude mice and observe lung metastases after the injection. However, some OS cells generate primary tumors too rapidly to allow enough time for metastases to occur. Thus, this method needs to be further improved.

Here is a refined protocol to better model OS lung metastases in mice. Briefly, primary tumors are surgically removed one week after the IM injection of OS cells, and the mice have successfully developed high penetrance of lung metastasis. OS lung metastases can be easily distinguished from lung tissue using Masson Trichrome and hematoxylin and eosin (H&E) staining. This protocol

has been applied to several OS cell lines, and OS lung metastases can be reproducibly observed. This method will be invaluable for delineating the mechanisms underlying OS metastasis.

INTRODUCTION:

OS is a highly malignant cancer and represents 4% of all new cancer cases in children in the US each year and ranks third in cancer-related death in children and adolescents[1,2]. The primary tumor of OS begins in the bone and tends to invade nearby tissues next to the tumor site, for example, muscle, tendon, or fat. OS has frequently been found in the long bones, especially the distal femur and proximal tibia; other common sites include the fibula, humerus, pelvis, or any bone in the body[3]. Surgical removal of the primary tumor followed by chemotherapy is the current standard treatment for OS. Without the follow-up chemotherapy, OS recurrence occurs in most OS patients[4,5]. Although the survival rate of OS patients without metastases after treatment is over 70%, the survival rate decreases to less than 30% for those with metastases[6,7]. All these clinical data indicate that metastases in OS are directly related to the survival rate and highlight the importance of studying the mechanisms underlying metastases in OS.

The procedures described in this paper will focus on lung metastasis because the lung is the most frequent distal organ for diagnosed OS metastases[8]. Although there are several human and murine OS mouse models for studying OS lung metastasis, these models do not model the complex processes of lung metastasis well. Tail-vein injection is a widely used model for lung metastases in the mouse model. However, because this method bypasses the process of intravasation, it does not reflect the natural events of metastatic spread. Another commonly used model for OS metastasis is the IM injection of OS cell lines followed by primary tumor formation and spontaneous lung metastasis. As most OS cell lines generate primary tumors very rapidly, this model does not allow enough time for metastasis to form. Therefore, lung metastasis formation is not consistent in the IM injection model[9,10].

One way to increase the time window for lung metastasis is to remove the primary tumor via limb amputation following the IM injection of OS cells. Specifically, when the primary tumor reaches the size predefined by an animal study protocol, it will be removed to ensure the primary tumor cells have sufficient time to metastasize to the distal organs. Using this OS metastasis model with limb amputation, high (>90%) lung metastasis incidence across several human and mouse cell lines has been consistently observed. This metastasis model can also be valuable for drug screening because the treatment window is much longer than the original IM injection model. Therefore, it allows examination of the effect of drug treatment on lung metastases instead of primary tumors. The detailed procedures of implementing this model will be described as follows.

PROTOCOL:

The protocol of intramuscular injection and amputation provided here, NCI ASP LCBG-041, has been approved by the Institutional Animal Care and Use Committee (IACUC) in the National Institutes of Health (NIH).

1. OS cell line preparation:

1.1. Maintain the cultured OS cell lines, K7M2, DLM8, Hu09-M112, and HOS-MNNG, in 10 mL of 10% fetal bovine serum (FBS)-supplemented Dulbecco's Modified Eagle Medium (DMEM) with 1% penicillin-streptomycin in 10 cm dishes at 37 °C in a humidified incubator with 5% CO_2.

1.2. To harvest the cells, remove the medium, wash the dish with 5 mL of phosphate-buffered saline (PBS), add 1 mL of trypsin for 5 min, neutralize the trypsin with 10 mL of FBS-supplemented culture medium, and spin down the cells at 193 × *g* for 5 min to collect the cells.

1.3. Apply 20 µL of cells to a cell counting chamber and count the cell number (see the **Table of Materials**). Resuspend the cells in 25 mM HEPES-buffered DMEM at a concentration of 1×10^7 cells/mL.

1.4 Store the cells in a 1.5 mL microcentrifuge tube on the ice during transportation before injection.

2. IM injection:

NOTE: Female Athymic $NCr^{Nu/Nu}$ mice, 6–8 weeks old and weighing 19–24 g, have been used as the host for this study

2.1. Before the injection, check the O_2 tank and isoflurane levels and ensure the amounts are enough for the whole procedure. Always prepare an extra O_2 tank and isoflurane to supply in case of additional need.

2.2. Anesthetize the mouse in an induction chamber with 3–5% isoflurane for 1–2 min.

2.3. When the mouse is anesthetized and stops moving, open the lid, prod the mouse, and pinch the hindlimb to ensure lack of reflex.

2.4. Take the mouse out of the chamber and transfer it to a nose cone with 2–3% isoflurane for maintenance throughout the procedure.

2.5. Apply a small amount of eye lubricant in both mouse eyes with cotton swabs to keep the eye moist.

2.6. Put the mouse on a surgery pad, gently mix the cells, and pull the leg to expose the injection area (gastrocnemius). Inject 50 µL of the cancer cells in 25 mM HEPES-buffered cultured medium into the gastrocnemius using a 1 mL syringe and 30 G needle. Ensure 50 µL (5×10^5) of the cancer cells are injected into the gastrocnemius of each mouse.

2.7. Gently put the mouse back into a new recovery cage. Put a heating pad under the cage for better recovery.

2.8. Wait and make sure the mouse is awake, alert, and with normal movement. Then, put the cage back into the rack and monitor the animal at least once a day after the injection until amputation surgery.

3. Tumor size monitoring:

NOTE: Different OS cell lines have different tumorigenesis rates. A small-scale pilot study is highly recommended to understand the suitable date for amputation after IM injection.

3.1. Measure the tumor size daily, starting from 5 days post implantation until 2 weeks, to estimate the tumor growth rate.

NOTE: Although the tumor grows under the muscle, the approximate tumor size can still be measured through the shape of the tumor using a caliper.

3.2. Measure the largest and smallest diameter of the primary tumor for calculating the estimated tumor size using Eq (**1**).

$$(Tumor\ volume\ (mm^3) = \frac{largest\ diameter\ as\ length\ (mm) \times smallest\ diameter\ as\ width\ and\ depth\ (mm^2)\ )}{2} \quad (\mathbf{1})$$

NOTE: The result of this calculation serves as the approximate tumor size and can help decide the amputation date. It is recommended to wait until the tumor reaches at least 200 mm^3 before the amputation.

4. Amputation:

4.1. Check O_2 tank and isoflurane levels and ensure the amounts are enough for the whole procedure. Always prepare extra O_2 tank and isoflurane to supply in case of additional need.

4.2. Anesthetize the mouse in an induction chamber with 3–5% isoflurane for 1–2 min

4.3. When the mouse stops moving, open the lid, gently prod the mouse, and pinch the hindlimb to ensure a lack of response.

4.4. Transfer the mouse to a nose cone for anesthesia maintenance on isoflurane throughout the procedure. Turn down the isoflurane flow to 2–3% to maintain the anesthesia and put a preheated heating pad under the surgery pad to maintain the mouse body temperature during the surgery.

4.5. Apply a small amount of eye lubricant in both eyes with a cotton swab to keep the eye moist.

4.6. Change gloves and apply 70% ethanol to clean gloved hands. Open the autoclaved surgical pack, including scissors, forceps, and a 9 mm wound clip applier with clips.

4.7. Prepare the sterile surgical site by applying chlorhexidine acetate three times using cotton swabs. Afterward, scrub the surgery area (knee joint) 3 times with 70% ethanol to reduce the risk of infection. Use a sterile gauze sponge to gently remove the remaining ethanol on the skin before the surgery.

4.8. Pull the leg straight and then bend the leg to expose the surgical location (knee joint). Pinch the toe to look for the paw withdrawal reflex to ensure the mouse is still under anesthesia.

4.9. Hold and bend the leg slightly to reveal the knee joint. Then, cut the knee joint with scissors and directly cut through the bone and muscle. Use a gauze sponge to hold the surgical area and apply some pressure to prevent bleeding.

4.10. Use forceps to hold the edges of the skin and apply 3–5 wound clips to close the skin around the surgical area.

4.11. Administer 2 mg/kg bupivacaine via subcutaneous injection to the mouse.

4.12. After the amputation, put the mouse in a clean recovery cage with a heating pad under the cage to maintain the body temperature. Wait until the mouse is awake and alert before operating on the next mouse.

4.13. After the mouse is awake, put the mouse into another clean cage to separate the awake mouse from the mouse under anesthesia to prevent the awake mouse from attacking the mouse under anesthesia. Ensure that all mice are fully awake without bleeding before transferring the cage back to the rack.

4.14. After amputation, monitor the mice twice daily for the following 2 weeks. Apply bupivacaine once daily at 2 mg/kg through subcutaneous injection until there are no signs of pain.

NOTE: Signs of pain after the surgery include hunched posture, rough hair, unkemptness, and piloerection.

4.15. Remove the wound clips in the first week, based on the wound healing process. As some clips might fall during the first week, be prepared to apply new wound clips.

NOTE: The open wound should heal in 1–2 weeks.

4.16. From the third week onwards, monitor the mice once daily throughout the study.

NOTE: As lung metastases show minor signs before becoming severe, closely monitor each

mouse's breath and movement, which is crucial to detect at the correct time to observe lung metastases. Mice with severe lung metastases can show the following signs: shortness of breath with increasing breath rate, movement decrease, and poor appetite. With the cell lines being tested in this protocol, lung metastases can be found ~0.5–3 months post implantation, depending on the cell line (**Table 1**).

5. Humane euthanasia criteria endpoints:

5.1. Euthanize the mice postoperatively if any of the following signs have been found: bleeding, wound dehiscence, infection, or if the animals cannot ambulate. To euthanize the mice, look for the following symptoms of metastasis described in step 6.1.

6. Monitoring signs of metastasis and necropsy:

NOTE: A necropsy of at least one mouse is recommended to confirm the status of lung metastases when observing any of the following signs: shortness of breath, poor body condition, reducing movement, and pale ears/muzzle.

6.1. To euthanize the mouse, put it in a chamber full of CO_2. Wait for at least 2–3 min until there are no signs of breathing, and then perform mouse spinal dislocation to ensure death.

6.2. Use forceps and scissors to open the abdomen for abnormalities or metastases. Open the chest to observe the condition of the lung metastases.

6.3. Use forceps to hold the trachea and vena cava and cut the distal site of the trachea and vena. Lift the remaining trachea and vena with the heart and lung and isolate the lung by removing the heart from the lung.

6.4. Collect the lung metastases, fix them in 5 mL of 10% neutral-buffered formalin at room temperature overnight, wash with 1x PBS, and store in 10 mL of 70% ethanol in a 4 ˚C refrigerator until used for histology stains, such as H&E staining (see the **Table of Materials**).

6.5. If protein or RNA is needed, quickly collect samples (in 15 min), freeze them in liquid nitrogen immediately, and store them in a –80 ˚C freezer.

REPRESENTATIVE RESULTS:

To model OS lung metastases, nude mice were initially used as the host with IM injection of the following OS cell lines, K7M2 and DLM8. However, these IM-injected OS cells generated large primary tumors in 2–3 weeks, and the mice had to be euthanized. Importantly, no metastasis was found in the lung (**Figure 1**).

Upon injection of the OS cells through the tail vein, most cell lines successfully formed lung metastases. However, tail-vein injection can only determine whether these cells can colonize in the lung environment without indicating whether these cells can complete different steps of

metastasis. To overcome this limitation, a tibia amputation procedure was performed after the primary tumor became palpable (**Figure 2**). After the amputation, the surgical wound healed in 1–2 weeks.

A small proportion of the mice (4/20) died immediately after the amputation, which may be because the original cutting site was not always at the knee joint. Some of the cutting sites were close to the distal femur, which possibly led to extra bleeding and caused the death of these mice. Indeed, no mouse died after the surgery in a follow-up experiment when the cutting site was precisely at the knee joint. The timing of lung metastasis formation varies depending on the OS cell lines (**Table 1**). For example, HOS-MNNG and K7M2 formed lung metastases 1–2 months post amputation (**Figure 3** and **Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Histology of primary tumors and lungs from IM injection in the quadriceps. (**A**) H&E staining of a large K7M2 primary tumor. (**B**) H&E staining of a nonmetastatic lung from the mouse with the K7M2 primary tumor. (**C**) H&E staining of a large Hu09-M112 primary tumor close to the femur. (**D**) H&E staining of a nonmetastatic lung from the mouse with the Hu09-M112 primary tumor. Scale bars = 1 mm. Abbreviations: IM = intramuscular; H&E = hematoxylin and eosin.

Figure 2: Images showing each step of amputation of the tibia with primary OS tumor. (**A**) A primary OS tumor can be easily seen grown from the tibia/gastrocnemius. The arrow indicates the primary tumor. (**B**) The surgical cutting site, the knee joint, is easier to be seen by bending the knee. The arrow indicates the knee joint. (**C**) A partial knee joint can be seen after the tibia amputation, which indicates the cutting site was right on the knee joint. (**D**) Applying clips to the skin to close the surgical wound. (**E**) Relative position of bone, muscle, tumor, and amputation point (blue line). The figure was created with BioRender.com. Abbreviation: OS = osteosarcoma.

Figure 3: H&E staining of the primary tumor and lung metastases from a mouse with HOS-MNNG IM injection followed by a tibia amputation. (**A**) H&E staining of the surgical removal of the tibia with the primary tumor. The primary tumor is attached to the bone and the muscle. (**B**) A low-magnification image of lung metastases. The square indicates the area magnified and shown in panel **C**. (**C**) A high-magnification image of lung metastases. Scale bars = 1 mm (**A, B**), 500 µm (**C**). Abbreviations: IM = intramuscular; H&E = hematoxylin and eosin.

Figure 4: H&E staining of the primary tumor and lung metastases from a mouse with K7M2 IM injection followed by a tibia amputation. (**A**) The primary tumor is attached to the knee joint. H&E staining from the surgical removal of the tibia together with the primary tumor. (**B**) A low-magnification image of multiple lung metastases. The square indicates the area magnified and shown in panel **C**. (**C**) A high magnification image of lung metastases. Scale bars = 1 mm (**A, B**), 500 µm (**C**). Abbreviations: IM = intramuscular; H&E = hematoxylin and eosin.

Table 1: Time of lung metastases with different cell lines in the amputation model.

DISCUSSION:

The protocol introduced here is to model the complex processes of OS lung metastases by IM injection of either human or murine OS cell lines into the nude mice, followed by amputation of the primary tumor. This procedure is a modification of the original OS metastasis model of IM injection of OS cells[11]. Removing the primary tumor can help extend the development time for OS lung metastasis. In the current procedure, the injection site is changed to the gastrocnemius from the quadriceps in the original model. If the injection site is the quadriceps, a whole leg needs to be amputated to remove the primary tumor. However, if the injection site is the gastrocnemius, surgical removal of the tibia is sufficient to remove the primary tumor. Thus, this change can lower the risk of excessive bleeding and increase the survival of mice after the surgery. In addition, the invasion of tumor cells into the trunk region, such as the pelvis, should be avoided because the invaded tumor cells will continue to generate tumors even after the primary tumor in the quadriceps is removed. By changing the injection site to the gastrocnemius, the chance of the primary tumor invading the trunk is considerably reduced.

One trade-off of the change in the injection site from the gastrocnemius is that the volume of injected tumor cells decreases from 100 µL to 50 µL to avoid inducing excess stress to the surrounding muscle by the injection. The reduction in injection volume might be problematic for some less malignant tumor cells. Thus, this protocol can better model OS lung metastases by changing several steps in the original IM injection protocol and adding a simple surgical removal of the primary tumor. This protocol has been used to test the metastatic ability of several OS cell lines, and lung metastases were consistently observed.

IM injection has been used instead of orthotopic intrabone injection in this protocol. Although intrabone injection is one of the most commonly used injection routes to model primary tumor formation and metastasis of OS, primary tumors and metastases from IM injection generally grow faster than those from the intrabone injection. Steinber et al.[12] reported that intratibial injection of K7M2 OS cells could directly seed OS cells into the lung, due to which an intratibial injection-induced OS model is not recommended as a spontaneous model of metastasis. Although intratibial injection has been considered a standard method to model OS[13], its contradictory results make it unsuitable for modeling spontaneous OS metastasis. In the IM OS injection model, cancer cells have not been found to enter the venous system to seed the lung directly. Further experiments need to be done to completely rule out the risk that the lung metastases have not arisen from the primary tumor in this amputation model. For example, amputation could be performed at different time points after IM injection of the OS cells to check if there are lung metastases. However, IM injection for OS is a reliable model to generate primary tumors instead of lung metastases.

It is worth noting that the timing of amputation affects the result: too early or too late limb removal can generate completely different results and complicate interpretation. If the primary tumor is removed when it is extremely small, fewer circulating tumor cells will enter the bloodstream to form lung metastasis. If the surgical removal of the primary tumor is extremely late, the primary tumor may have already invaded surrounding tissues, making it much more difficult to completely remove the primary tumor. The remaining invading tumor cells could generate a second primary tumor, leading to the early euthanization of the mouse and a large

variation in the cohorts. Therefore, it is crucial to carefully choose the amputation time for different OS cell lines. The approach of estimating the amputation time is described in protocol section 4.

Two critical steps need to be considered to perform a successful OS amputation study. The first important step to pay extra attention to is during the removal of the tibia. After surgical removal of the tibia, the survival rate of the mice drastically decreases if the cutting site is not the knee joint. Choosing either the distal femur or proximal tibia as the cutting site can potentially increase the risk of bleeding and reduce the survival rate. There should be only slightly bleeding if the cutting site is precisely on the knee joint. In addition, observe the cutting area for any signs of tumor invading the knee joint. If tumors consistently invade the knee joint, the amputation date needs to be adjusted to an earlier date.

The second critical step is how to decide the correct timing to terminate the study for OS-induced lung metastases. The termination date of the cohort can be adjusted based on the status of the lung metastases. For example, if an endpoint with more severe lung metastasis is desired, the necropsy of the cohort can be delayed. However, if the mouse examined has already had severe metastasis and the rest of the cohort exhibit signs of shortness of breath and poor body condition, it is recommended to perform the necropsy of the rest of the cohort.

This protocol provides a practical method of screening for molecules affecting lung metastasis. Compared to the tail-vein injection protocol, this protocol is a better model of the complex processes of lung metastasis of OS. This protocol may be better at assessing the effect of a drug on lung metastasis because the primary tumor has been removed. This protocol uses immunocompromised mice, such as nude mice or NSG mice. In the future, by combining with syngeneic OS cell line models, this protocol can be used to test the effects of immunotherapeutic agents on OS lung metastasis to help generate novel therapeutic strategies to combat this devastating cancer.

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DISCLOSURES:
The authors declare no conflicts of interest.

REFERENCES:
1. Campbell, J. P., Merkel, A. R., Masood-Campbell, S. K., Elefteriou, F., Sterling, J. A. Models of bone metastasis. *Journal of Visualized Experiments: JoVE*. (67), 4260 (2012).
2. Durfee, R. A., Mohammed, M., Luu, H. H. Review of osteosarcoma and current management. *Rheumatology and Therapy.* **3** (2), 221–243 (2016).
3. Jacques, C. et al. Small animal models for the study of bone sarcoma pathogenesis: characteristics, therapeutic interests and limitations. *Journal of Bone Oncology.* **12**, 7–13 (2018).

4. Klein, M. J., Siegal, G. P. Osteosarcoma: anatomic and histologic variants. *American Journal of Clinical Pathology.* **125** (4), 555–581 (2006).
5. Overholtzer, M. et al. The presence of p53 mutations in human osteosarcomas correlates with high levels of genomic instability. *Proceedings of the National Academy of Sciences of the United States of America.* **100** (20), 11547–11552 (2003).
6. Picci, P. Osteosarcoma (osteogenic sarcoma). *Orphanet Journal of Rare Diseases.* **2**, 6 (2007).
7. Pratt, C. B., Meyer, W. H., Rao, B. N., Parham, D. M., Fleming, I. D. Osteosarcoma studies at St. Jude Children's Research Hospital from 1968 through 1990. *Cancer Treatment and Research.* **62**, 323–326 (1993).
8. Ren, L. et al. Characterization of the metastatic phenotype of a panel of established osteosarcoma cells. *Oncotarget.* **6** (30), 29469–29481 (2015).
9. Tan, J. Z. et al. Multidisciplinary approach to diagnosis and management of osteosarcoma - a review of the St Vincent's Hospital experience. *International Seminars in Surgical Oncology: ISSO.* **3**, 38 (2006).
10. Vasquez, L. et al. Analysis of prognostic factors in high-grade osteosarcoma of the extremities in children: a 15-year single-institution experience. *Frontiers in Oncology.* **6**, 22 (2016).
11. He Y. et al. cFOS-SOX9 axis reprograms bone marrow-derived mesenchymal stem cells into chondroblastic osteosarcoma. *Stem Cell Reports.* **8** (6), 1630–1644 (2017).
12. Maloney C. et al. Intratibial injection causes direct pulmonary seeding of osteosarcoma cells and is not a spontaneous model of metastasis: a mouse osteosarcoma model. *Clinical Orthopaedics and Related Research.* **476** (7), 1514–1522 (2018).
13. Sottnik J. L., Duval D. L., Ehrhart E. J., Thamm D. H. An orthotopic, postsurgical model of luciferase transfected murine osteosarcoma with spontaneous metastasis. *Clinical & Experimental Metastasis.* **27** (3), 151–160 (2010).

Figure 1

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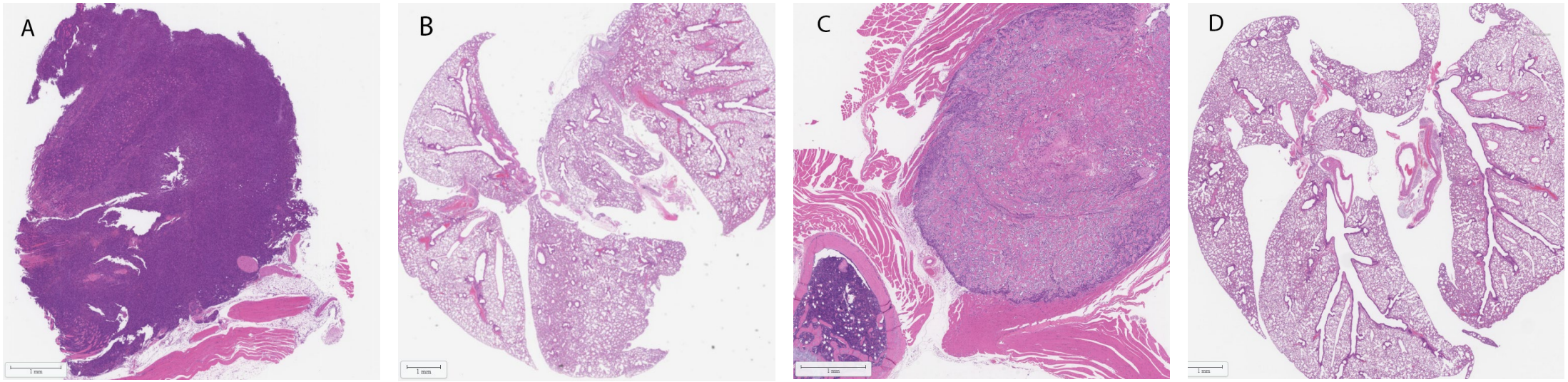

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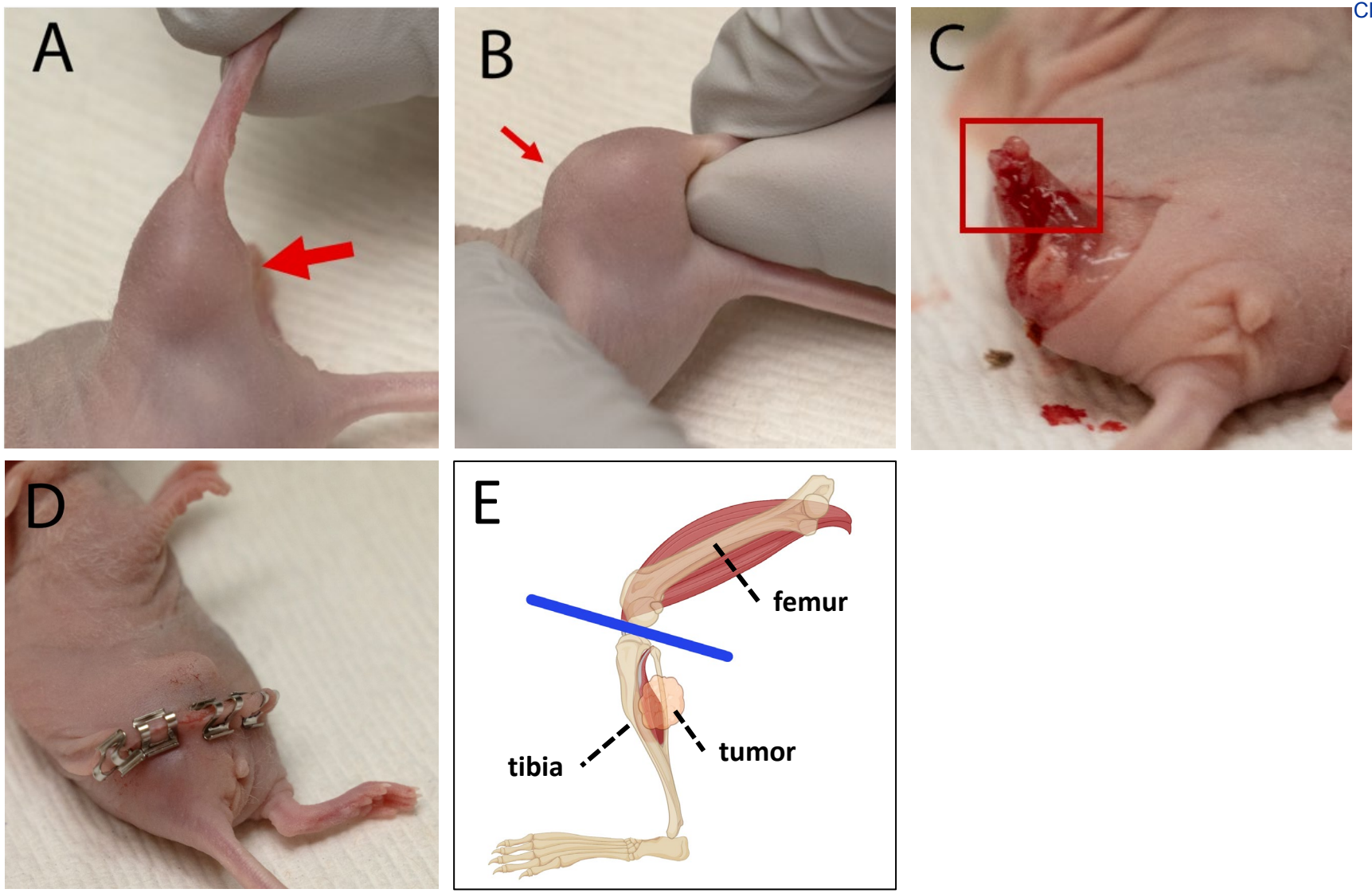

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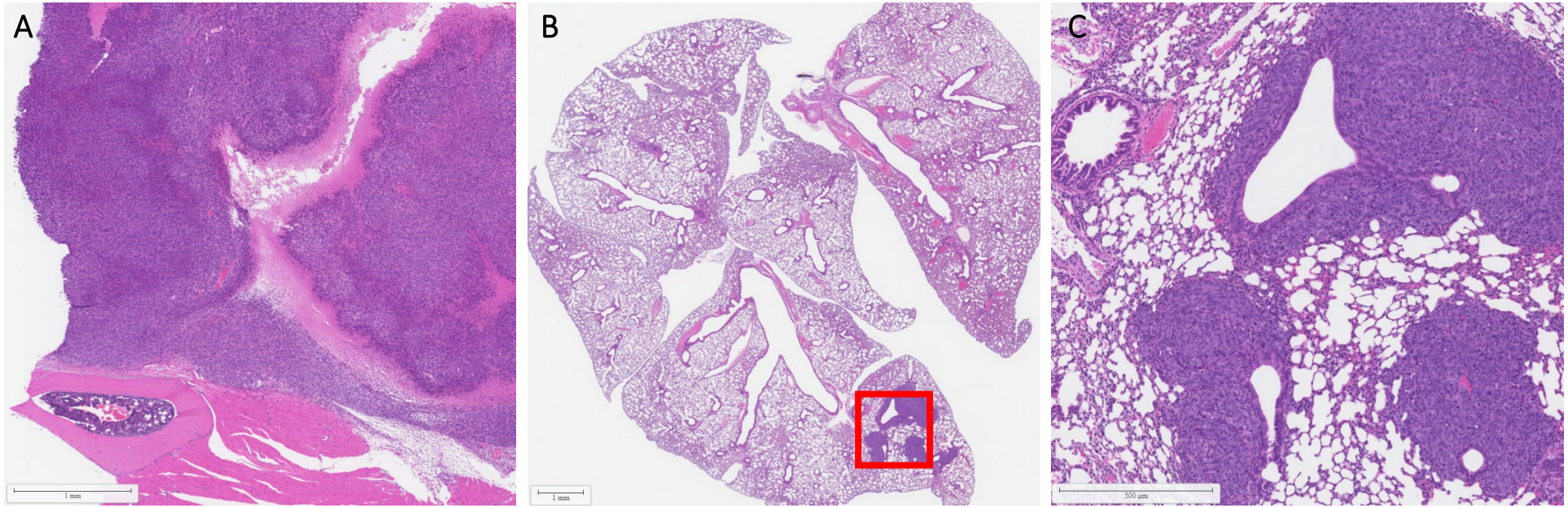

Figure 4

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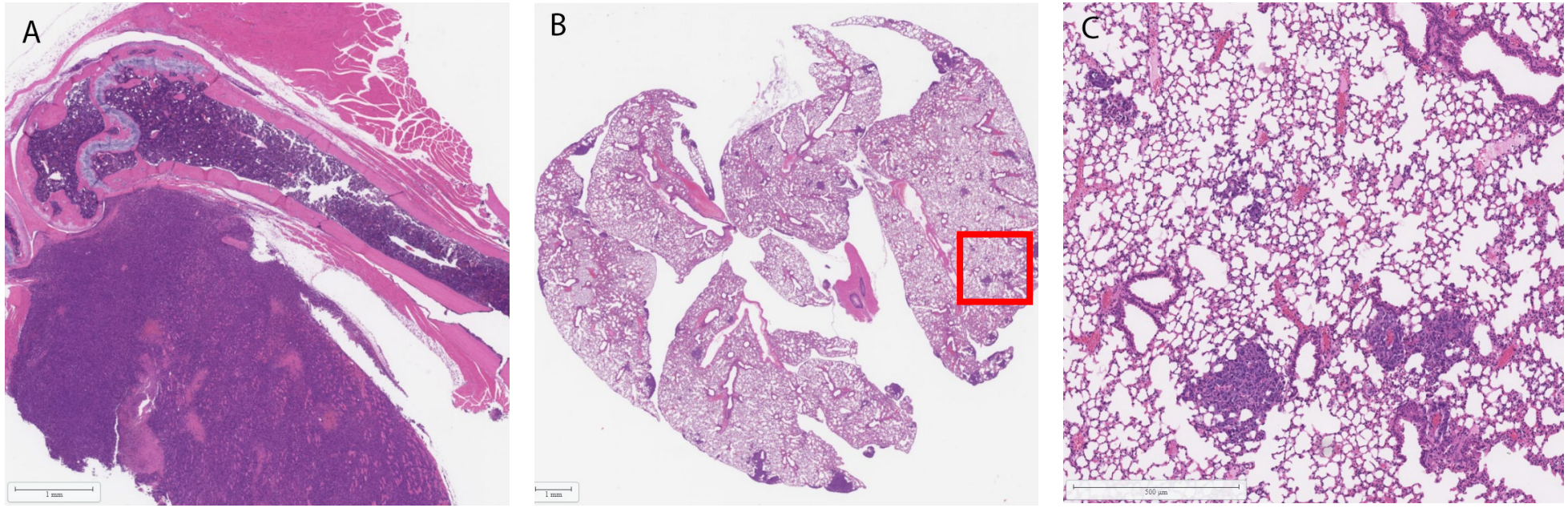

Table 1

Click here to access/download;Table;YCT table for lung metastases.xlsx

	Weeks for primary tumor to reach 200 mm^3	Average period for spontaneous lung metastases	Percentage of experimental metastases
K7M2	1	4-5 weeks	100%
DLM8	1	5-6 weeks	100%
HOS-MNNG	2	5-7 weeks	100%
Hu09-M112	3	7-9 weeks	90%

Editorial comments:

Editorial Changes

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The spelling and grammar issues have been corrected.

2. Please provide the approval number for the ethical approval obtained for the protocol. Also, please ensure that all the steps highlighted for filming are approved by the animal welfare committee of your institute.

NCI ASP LCBG-041 is our animal study protocol. Animal procedures were approved by the NCI Animal Care and Use Committee. The filming for the procedure been described here is approved by the NCI animal care and use committee.

3. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) and before the punctuation.

The corrections have been made.

4. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

The discussion part in protocol has been moved to discussion section and only action items are remained.

5. Please provide the volume added for the various reagents, media, chemicals used in the study.

The volume for reagents, media, chemicals have been included.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure

that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

The corrections have been made to the manuscripts for the protocol section.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

The corrections have been made to the protocol section.

8. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The personal pronouns in the manuscript have been modified.

9. Step 1.1: Please provide the source of the cell lines used here.

The OS cell-lines have been purchased from ATCC. For cell line DLM8 and Hu09-M112 the resource need to confirm with **Jing**.

10. Step 1.2: How was trypsinization done? What was used for neutralizing? How were the cells counted? Please describe all associated steps.

The missing information has been included.

11. Step 2.2: Please specify the mouse age, sex, strain, weight used in the study?

The missing information has been included.

12. Step 6.4: How were lung samples collected? Please provide all the specific details for doing this. How was the staining done? Please provide details.

Alternatively, please provide a reference demonstrating how this can be done.

The description of how lung been collected has been include to the protocol. The H&E staining was done by Histoserv, Inc.

13. Please include a single line space between all the steps and ensure that the highlight is up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Modification has been made.

14. As we are a methods journal, please also include in the Discussion the following in detail along with citations:
a) Critical steps within the protocol
b) Any limitations of the technique

An extra discussion showed the critical steps and limitation of this technique were included.

15. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

16. Please ensure that the table of materials contains all the essential supplies, reagents, and equipment used in the study.

The table of materials si checked and more supplies are added to the table.

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Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The authors provide an easy step-by-step procedure for an alternative model of limb tumour amputation in mice. This amputation model differs from previous established studies (PMID: 26278104, 23988893, 11315100 and 26320182) in that this model describes amputation at the level of the knee rather than at the coxofemoral joint, as described in the previous studies. The authors describe the advantages of this model which include: increased survival of mice after surgery and invasion of the primary tumour into the trunk region. A disadvantage includes the reduction of injection volume into the gastrocnemius muscle (more sheer stress on tumour cells).

Major Concerns:
1) Can the authors include the animal protocol number to lines 82-84?

The animal protocol number has been included to line2 82.

Can the authors comment on how the limb remnant (femur and attached muscle) heals over time?

The surgical wound healed in 1 week, but the femur with attached muscle were under the skin which can not be seen. According to those mice healing period, mice can move around smoothly in 1-2 weeks, so I expects the limb remnant heals around 1-2 weeks after the surgery.

Are there complications such as tissue trauma from the movement of the limb remnant?

There was no complication issue related to the surgery as we observed. But after the amputation, these mice did show slightly imbalance during the movement.

Have there been issues with the limb remnant interfering with the surgical wound?

As long as the clips applied to the wound after the surgery were tight enough, there was no issue been reported. But less movement during the healing period has the found in the amputated mice. The movement of the mice went back to normal once the wounds were healed.

Have the authors previously published with this model? If so, can you provide the appropriate references?

No. This is the first time we report this model.

2) Figure 1 is largely negative data with respect to large, fast growing tumours with no spontaneous lung metastases. The amputation model can substantiated by providing positive data showing successful spontaneous lung metastases from the limb amputations using the DML8, Hu09-M112, or HOS-MNNG cells.

We removed the tail-vein model in figure 2, and replace the HOS-MNNG spontaneous lung metastases after amputation as the positive data to support our model in Figure 3.

3) The DLM8 experimental metastasis (tail-vein tumour injection) data in Figure 2 has no relevance to the current study unless being compared to the DLM8 spontaneous lung metastasis from the proposed alternative amputation data. If no comparison of this data is made, remove the DLM8 experimental metastasis data or replace with DLM8 spontaneous lung metastasis data from the amputation model.

As you suggested, we removed the original tail-vein in Figure 2 and replaced with HOS-MNNG amputation model in Figure 3. (The order of Figures have been changed)

4) In Figure 3, can you list the cell line model being used? Can you provide corresponding whole-mount image or histology of the resulting spontaneous lung metastases from this model?

K7M2 was the cell line we used to generate the set of photos. The cell line was used for the amputation model in Figure 4 was also K7M2 which was corresponding to present the lung metastases in Figure 2. (The order of Figures have been changed)

5) Figure 4 only shows K7M2 data, whereas the text (lines 236-237) says Figure 4 shows both K7M2 and DML8 data.

We already removed DLM8 for the text.

6) As this will be a resource for osteosarcoma researchers, can the authors provide a table detailing the length of time for primary tumour growth and the time when lung metastases form for each cell line model used (K7M2, DLM8, Hu09-M112)? Do the authors have such data for the SAOS2 and HOS-MNNG cells? Can a table include the surgical success rate for each cell line tested?

A table of specific information about different cell lines induced amputation model has been included. We did not test the SAOS2 in this model. We also removed the SAOS2 in the original cell lines list.

Minor Concerns:
1) In addition to the accompanying video, can the authors provide a rough diagram/drawing with tumour, bones & muscles of the leg with a precise amputation point in Figure 3? A diagram will help orient researchers when repeating this procedure.

A simple diagram has been included in Figure 2 to show the relative position of bones, muscles and tumor for the amputation.

2) With respect to lines 268-273, can you provide the reference you are comparing your model to?

A reference using intramuscular injection tumorigenesis model in our lab has been included.

Reviewer #2:

Manuscript Summary:

The authors present a method for improved investigation of lung metastasis in osteosarcoma (OS) mouse models via amputation of an intra-muscular injected primary tumor. As an introduction, the need for additional treatments of OS, especially lung metastases, is discussed, as well as shortcomings of the current research models. A detailed protocol provides description of cell preparation, injection, and amputation, as well as monitoring of both tumor and mice. Images of the amputation procedure are provided. Results are shown from mice injected with K7M2 and DLM8 cells, with H&E images of primary tumor and lung metastases. Further discussion addresses the benefits of this procedure compared with previous methods such as quadriceps IM injection and intra-bone injection, and mentions the potential use of this model system for screening of metastasis-specific drugs.

Major Concerns:

*None

Minor Concerns:

*** Please provide a citation for the "original model" using a quadriceps injection that is mentioned in the discussion.**

A reference using intramuscular injection tumorigenesis model in our lab has been included.

*** Please specify the gender and origin of the mice used in the study.**

The information has been included to line 97.

*** Based on the images, it appears like it might be difficult to cut directly at the knee joint without allowing any tumor cells to remain behind. What is the rate of local recurrence after amputation? If a cut further up the leg is required, can cautery solve any of the excessive bleeding issues that led to reduced survival?**

The recurrence rate after amputation was about 5%. The images of the tumor in Figure 2, we intended to let the tumor grew bigger than 200 mm^3, which was just for the reader to better see the location of tumor. In the actual amputation, once

the tumor size is around 200 mm^3, the amputation will be performed, which currently with very low chance of local recurrence. The cautery to solve the bleeding issue is a very good suggestion, we will consider use this method combining cutting more of femur to eliminate the chance of recurrence.

*** Can this procedure be applied to other cell types, including other bone cancers but also cancer types which metastasize to the bone and lung i.e. breast or prostate?**

We think it is very likely to apply to other kind of cancer. In our hand, breast cancer, mda-mb-231, orthotopic fat pad injection mouse model also represented similar results. The primary tumor got too big and the study need to terminate. But there were only few small spots of lung metastases been found. If the primary tumor can be removed, then theoretically the process of lung metastasis can be prolonged. If the primary cancer removal does not affect the survival, then we think the amputation should be able to apply to other types of metastatic cancer.

*** As intra-bone models have largely been used in the past, some support of their statements in the discussion regarding invasion of the IM tumor into the bone would help establish this model as clinically relevant. Please provide evidence that the cells invade bone consistently after IM injection.**

With a further look at our study, for the amputation purpose, there were no bone invasion been identified. It only happens in the human OS IM injection model for tumorigenesis, which usually took 2-3 months. So the bone invasion in the discussion has been removed to avoid misunderstanding.

*** The osteosarcoma mouse model is not novel - intra-tibial or intra-bone injections have been used in OS models for decades and amputations have been performed as well. However, it has been shown that intra-tibial injections provide direct seeding of lung metastases, and are thus no different from IV injection for metastasis study (Maloney. et al, Clin Orthop Relat Res. 2018 Jul;476(7):1514-1522). The authors mention that no lung metastasis is seen with the IM injection after short periods of time, so this method may be beneficial over intra-bone methods in that regard. Discussion of this aspect of the model would be valuable.**

The reference you suggested has been included in the discussion.