

Dear Editor,

On behalf of my co-authors, we appreciate the editor and reviewers very much for the instructive comments and advices on our manuscript entitled “Intratibial Osteosarcoma Cell Injection to Produce Orthotopic Osteosarcoma and Lung Metastasis Mouse Models” (ID: JoVE63072).

Following is the point-by-point responses to address the editorial and peer review comments.

Editorial comments:

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.

Response: thank you very much for your advices.

We have thoroughly proofread the manuscript to ensure no spelling or grammar issues.

2. Please clarify the corresponding author for this study as the names are different in the Editorial Manager and the main manuscript.

Response: Thanks for your question.

The corresponding author for this study is: Yanping Yang, Email: [yanpingyang@shutcm.edu.cn](mailto:yanpingyang@shutcm.edu.cn)

3. Please revise the following lines to avoid previously published work: 29-32, 144-146, 211-215.

Response: thank you very much for your advices.

The contents in lines of 29-32, 144-146, 211-215 in the original manuscript have been revised.

4. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “The present protocol describes ...”

Response: thank you very much for your advice.

Summary of “The present protocol describes intratibia osteosarcoma cell injection to produce mouse models bearing orthotopic osteosarcoma and pulmonary metastasis lesions” has been provided.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

For example: Matrigel

Response: thank you very much for your advices.

All commercial languages have been deleted.

6. Use SI units as much as possible and abbreviate all units: L, mL,  $\mu$ L, cm, kg, etc. Use h, min, s, for hour, minute, second.

Response: Thank you very much for your advice.

All these issues have been fixed.

7. Please define all abbreviations before use. For example, H&E, OS, etc.

Response: Thank you very much for your advice.

All abbreviations have been defined before. Meanwhile, U-2 OS is the official name of an osteosarcoma cell line, not our abbreviation.

8. Please avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol.

Response: Thank you very much for your advice.

These type phrases have been replaced.

9. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary.

Response: Thank you very much for your advice.

The numbering of the Protocol has been adjusted following the JoVE Instructions for Authors.

10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Response: Thank you very much for your advices.

Line 80: Please specify the age/sex/strain of the mice used in the study.

“Four-week-old male BALB/c athymic mice were provided by Shanghai SLAC Laboratory Animal Co, Ltd., and adapted for a week before the surgery for orthotopic injection of osteosarcoma cells.” has been added.

Line 84: How did you determine the logarithmic growth phase of the cells? Please mention.

“wash the cultured 143B cells at the logarithmic growth phase in 10-cm cell culture dish” has been replaced with “wash 80-90% confluent 143B cells cultured in 10cm cell culture dish”.

Please use x g for centrifugation speed.

The centrifugation speed has been changed into x g.

Line 90: How was the aspiration done? Was a pipette used? Please specify. What was the concentration of Matrigel use? Please specify.

Eppendorf pipette was used; Matrigel has been replaced by basement membrane matrix. The concentration of basement membrane matrix is 8.5mg/ml.

“Aspirate supernatant with the Eppendorf pipette and resuspend the cell pellet in 8.5mg/ml basement membrane matrix” has been added in the revised manuscript.

Line 94: How long can the cells be kept?

“Keep the cells on ice before use and the cells are used within 2h.” has been added.

Line 108: Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

“A small amount of vet ointment is applied on mouse eyes to prevent dryness while under anesthesia.” has been added.

Line 144: Was a 40% formalin solution used?

The 10% formalin solution was used.

“Fix the leg bearing osteosarcoma of each mouse in 50 ml tube filled with 20 ml formalin solution for 24 hours” has been replaced with “fix specimen of each mouse

in a 50 mL tube with 20 mL formalin solution (10%) for 24 h”:

11. Please include a single line space between each step, substep, and note in the protocol section. Please highlight 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Response: Thank you very much for your advices.

A single line space between each step, substep, and note in the protocol section has been added.

The entire Protocol is within 3 pages and highlighted with green.

12. Please revise the Discussion also to cover the significance of the protocol with respect to existing methods. Also, please include a paragraph on the limitations of the protocol.

Response: Thank you very much for your advices.

The significance of the protocol with respect to existing methods has been added in the revised Discussion as follows: “Although the primary bone tumors and bone metastasis have been recently reported to be achieved by implantation of solid tumor graft into bone, and the animals developed reproducible growth, as well as lung metastasis eventually[23], however, the authors directly implanted fresh or cryopreserved tumor fragments into the proximal tibia, which showed the disadvantage of open surgery caused potential infection and failure of developing tumor engraftment; moreover, the volume of implanted tumor fragments without strict controlling will result in significant difference in produced tumor volume, which is difficult in following application, such as evaluating the therapeutic efficacy *in vivo*. Here, we reported a simple and reproducible technique to establish the intratibia primary osteosarcoma with later pulmonary metastasis mouse models via intratibia injection of osteosarcoma cells, which showed the advantages of best mimicking the clinical development characteristics of osteosarcoma in human; accurate numbers of osteosarcoma cells being directly injected into tibia using micro-volume syringe allowing identical tumor formation rate (100%) and tumor volume; avoiding the possibilities of infection or even death using open surgery techniques; allowing lively

monitor and quantifying osteosarcoma growth and metastasis using the bioluminescence live imaging system after the injected osteosarcoma cells are labeled with bioluminescence; preventing injected osteosarcoma cells from directly reaching blood stream and colonizing in the lung to form pulmonary embolism and/or false positive pulmonary metastasis by resuspending the injected osteosarcoma cells in appropriate concentration of basement membrane matrix since the basement membrane matrix has the property of coagulation above room temperature and immediately coagulates to support and restrict osteosarcoma cells within the basement membrane matrix after being injected into mouse tibia.”

A paragraph on the limitations of the protocol has been added as follows “There are several limitations of our protocol. Mice used in this protocol are genetic immune system defect nude mice without thymus that prevents them from immunologically rejecting human cells and widely used in preclinical trials, which are not applicable for immune functional research. Furthermore, not all osteosarcoma cell lines are identically applicable in these models, the tumorigenesis abilities of 143B, MNNG, MG-63 and U-2 OS cells are higher than the Saos-2 cells.”

13. Please submit each figure individually in a high-resolution format in your Editorial Manager account.

Response: Thank you very much for your advices.

Each figure has been submitted individually in a high-resolution format in our Editorial Manager account.

14. Figure 2: Please reference Figure 2 in the manuscript text. Please ensure that all the Figures are referenced sequentially in the manuscript text.

Response: Thank you very much for your advices.

Figure 2 has been referenced in manuscript text and all the Figures have been referenced sequentially in the manuscript text.

15. Figure 3C: What do the error bars stand for (standard error or standard deviation)? Please specify. Also, please provide the number of samples used (n number).

Response: Thank you very much for your advices.

“Error bars stand for standard deviation (n=8)” has been specified in the FIGURE

## LEGEND of Figure 3C.

16. Figure 3E, 4A: Please provide a color bar for the images for better understanding.

Response: Thank you very much for your advices.

Color bars have been provided for Figure 3E, 4A.

17. Please do not abbreviate journal Titles in References.

Response: Thank you very much for your advices.

Journal Titles have been provided in References.

18. Please ensure that all the materials/equipment used in the Protocol are included in the Table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Response: Thank you very much for your advices.

Table of the essential supplies, reagents, and equipment has been provided as required.

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### Reviewers' comments:

Reviewer #1:

Review for JoVE63072

General comments: The manuscript is nicely written and presented. Figures are very nice. A few major comments:

1) Please discuss in the paper the similarities/differences and advantages/disadvantages of your technique to the following two papers, one of which was recently published in JoVE: Modeling Primary Bone Tumors and Bone Metastasis with Solid Tumor Graft Implantation into Bone. Hildreth BE 3rd, Palmer C, Allen MJ. J Vis Exp. 2020 Sep 9;(163):10.3791/61313

Models of bone metastasis. Campbell JP, Merkel AR, Masood-Campbell SK, Eleftheriou F, Sterling JA. J Vis Exp. 2012 Sep 4;(67):e4260. doi: 10.3791/4260.

Response: Thank you very much for your advices.

The significance of the protocol with respect to existing methods has been added in the revised Discussion as follows:

“Although the primary bone tumors and bone metastasis have been recently reported to be achieved by implantation of solid tumor graft into bone, and the animals developed reproducible growth, as well as lung metastasis eventually[23], however, the authors directly implanted fresh or cryopreserved tumor fragments into the proximal tibia, which showed the disadvantage of open surgery caused potential infection and failure of developing tumor engraftment; moreover, the volume of implanted tumor fragments without strict controlling will result in significant difference in produced tumor volume, which is difficult in following application, such as evaluating the therapeutic efficacy *in vivo*. Here, we reported a simple and reproducible technique to establish the intratibia primary osteosarcoma with later pulmonary metastasis mouse models via intratibia injection of osteosarcoma cells, which showed the advantages of best mimicking the clinical development characteristics of osteosarcoma in human; accurate numbers of osteosarcoma cells being directly injected into tibia using micro-volume syringe allowing identical tumor formation rate (100%) and tumor volume; avoiding the possibilities of infection or even death using open surgery techniques; allowing lively monitor and quantifying osteosarcoma growth and metastasis using the bioluminescence live imaging system after the injected osteosarcoma cells are labeled with bioluminescence; preventing injected osteosarcoma cells from directly reaching blood stream and colonizing in the lung to form pulmonary embolism and/or false positive pulmonary metastasis by resuspending the injected osteosarcoma cells in appropriate concentration of basement membrane matrix since the basement membrane matrix has the property of coagulation above room temperature and immediately coagulates to support and restrict osteosarcoma cells within the basement membrane matrix after being injected into mouse tibia.”

Another literature has reported the bone metastasis model establishment by intracardiac inoculation or intratibial inoculation of breast cancer cells[24]; however, cells used in this literature are breast cancer cells, which have different biological and clinical characteristics with osteosarcoma cells; moreover, both the intracardiac and the intratibial inoculation established cancer models in bone are formed by cancer cell colonizing directly or reaching through blood stream rather than metastasis lesions formed by cancer cell dissemination from the primary cancer lesions.

2) That being said, intratibial injections are associated with a high rate of artifactual

pulmonary metastasis following injection, if the technique is performed with a high pressure injection. Expand on this with regard to your technique. Above references address this.

Response: Thank you very much for your advices.

The injection technique is not performed with a high pressure.

“Slowly inject about 10  $\mu$ L (ignore pre-existing solution in the needle) of 143B cell suspension into the tibia (about  $2 \times 10^5$  cells) of each athymic mouse without application of high pressure.” after being drilled a hole through the tibia platform towards the distal end of the tibia (medullary cavity) with a micro-volume syringe (Figure 2B, 2C)” has been provided in the “2. Surgery for Orthotopic Injection of Osteosarcoma Cells” section.

3) More detail is needed about mice to use - age, strain, gender, etc. There is some concern on using mice older than 6 weeks, for there could be mineralization of the proximal tibial growth plate, making needle insertion challenging.

Response: Thank you very much for your advices.

“Four-week-old male BALB/c athymic mice were provided by Shanghai SLAC Laboratory Animal Co, Ltd., and adapted for a week before the surgery for orthotopic injection of osteosarcoma cells. ” has been added.

Specific comments by line number:

Response: Thank you very much for your advices.

1) Line 57 - Change "Transgenic model is" to "Transgenic models are"

This has been changed.

2) Line 67 - Change "under the subcutaneous" to "under the skin, subcutaneously"

This has been changed.

3) Line 94 - Emphasize the importance of keeping Matrigel on ice. This has been changed.

4) Line 101 - While 2% isoflurane is mentioned, add the flow rate of oxygen in L/min. This has been changed.

5) Line 111 - Make sure all figures are accurately referenced. Figure 1A here is one example. This has been changed.

6) Line 112 - For 6., is the needle placed through or adjacent to the patellar ligament as it goes through the skin/joint capsule. This has been changed.



7) Line 116 - Remove the word "late" unless a better word is used. This has been changed.

8) Line 121 - Change "falling sensation" to "movement into the medullary canal" This has been changed.

9) Line 122-123 - This is a very nice validation - x-ray confirmation. This has been changed.

10) Line 126 - If you inject 10  $\mu$ L, how are you accounting for the amount of pre-existing solution in the needle/hub?

“slowly inject about 10  $\mu$ L (ignore pre-existing solution in the needle) of 143B cell suspension into the tibia (about  $2 \times 10^5$  cells) of each athymic mouse.” has been added. Therefore, 10 $\mu$ L volume includes the amount of pre-existing solution in the needle/hub, which is consistent for each mouse.

11) Line 133 - I would not recommend GFP as a fluorescent protein for in vivo use. Some of the GFPs will be obscured by the fluorescence coming from the ring structure of hemoglobin in red blood cells. This has been changed.

12) Line 214 - Change "with a light sensitive camera" to "by optical imaging" This has been changed.

13) Line 214 - Remove the word "to" This has been changed.

Reviewer #2:

Manuscript Summary:

This manuscript documents a procedure for intratibial injection of human osteosarcoma cells in nude (immunocompromised) mice as a model for primary and metastatic osteosarcoma cell growth. The manuscript describes a procedure that is firmly established in the field. The only possible innovation is the use of Matrigel in the implant (vs. cells in media or PBS). However, the authors do not discuss whether Matrigel ameliorates the concern of immediate hematogenous dissemination of cells and embolization in the lungs as has been described by Maloney et al (see below). The description of the procedure and the images are clear, but the manuscript is repetitive of resources that have been previously published, including in the JoVE (and on YouTube! Valérie Raymond, Injection intratibiale 2019, <https://www.youtube.com/watch?v=f4b2fw4OIzQ>).

### Major Concerns:

The paper lacks thorough citations and acknowledgment of previous work in the field. The protocol is not original to the authors and the information described is repetitive from previous publications. The authors fail to address concerns about the model and its representation of the metastatic process, as well as innovations that have been recently published that have addressed this concern.

1. The authors cite the paper by Maloney et al (Clin Orthop Relat Res. 2018 Jul; 476(7): 1514-1522), but they fail to note Maloney's major conclusion that intratibial injection of osteosarcoma cells results in embolization of cells to the lung, so the dissemination of cells to the lung may not be, in effect, metastasis, but rather just a different route to inject cells that reach the blood stream and colonize the lung - in essence, no differently than intracardiac or intravenous injection models that result in lung colonization.

Response: Thank you very much for your question.

As we described in the discussion “Here, we reported a simple and reproducible technique to establish an intratibia primary osteosarcoma with later pulmonary metastasis mouse models via intratibia injection of osteosarcoma cells, which showed the advantages of .... preventing injected osteosarcoma cells directly reaching blood stream and colonizing in the lung to form pulmonary embolism and/or false positive pulmonary metastasis by resuspending the injected osteosarcoma cells in appropriate concentration of basement membrane matrix since the basement membrane matrix has the property of coagulation above room temperature and immediately coagulates to support and restrict osteosarcoma cells within the basement membrane matrix after being injected into mouse tibia”.

Moreover, by lively monitor and quantifying osteosarcoma growth and metastasis with a bioluminescence live imaging system after intratibia injection of luciferase labeled 143B osteosarcoma cells, we found that the intratibia osteosarcoma lesions formed by direct colonization of injected osteosarcoma cells are detectable 1 week after intratibia injection of osteosarcoma cells; however, the pulmonary osteosarcoma lesions are detectable at least 4 weeks after intratibia injection of osteosarcoma cells. If the pulmonary osteosarcoma lesions are directly formed by injected osteosarcoma cells that reach the blood stream and colonize the lung, the pulmonary osteosarcoma lesions should be detectable at the same time point as the intratibia osteosarcoma

lesions (1 week after intratibia injection of osteosarcoma cells) rather than at least 4 weeks after intratibia injection of osteosarcoma cells.

Therefore, the pulmonary osteosarcoma lesions formed in our models are caused by cancer cell dissemination from the primary intratibia osteosarcoma lesions and takes much longer time to be detectable than the primary intratibia osteosarcoma lesions.

2. The procedure for intratibial injection has been described by numerous investigators, including previous publications in JoVE (Campbell et al, J Vis Exp. 2012; (67): 4260). The authors should expand the depth of citation to acknowledge this is a well-established procedure and should focus the manuscript to document any new information that is not already available through other media (peer reviewed manuscripts and even YouTube videos).

Response: Thank you very much for your question.

This article has been cited in our revised manuscript, as we described in the discussion “Another literature has reported the bone metastasis models establishment by intracardiac inoculation or intratibial inoculation of breast cancer cells[24]; however, cells used in this literature are breast cancer cells, which have different biological and clinical characteristics with osteosarcoma cells; moreover, both the intracardiac and the intratibial inoculation established cancer models in bone are formed by cancer cell colonizing directly or reaching through blood stream rather than metastasis lesions formed by cancer cell dissemination from the primary cancer lesions.”

3. A recent paper from Hildreth et al (Modeling Primary Bone Tumors and Bone Metastasis with Solid Tumor Graft Implantation into Bone, J Vis Exp. 2020 Sep 9;(163):10.3791/61313. doi: 10.3791/61313) shows a major improvement in the procedure by engrafting fresh or cryopreserved tumor fragments into the tibia using minimally invasive surgery. This innovation (essentially creating a patient-derived xenograft as opposed to cell line xenografts) should be acknowledged, and differences in methodology should be described in the Discussion.

Response: Thank you very much for your advices.

The following information have been described in the revised Discussion: “Although

the primary bone tumors and bone metastasis have been recently reported to be achieved by implantation of solid tumor graft into bone, and the animals developed reproducible growth, as well as lung metastasis eventually[23], however, the authors directly implanted fresh or cryopreserved tumor fragments into the proximal tibia, which showed the disadvantage of open surgery caused potential infection and failure of developing tumor engraftment; moreover, the volume of implanted tumor fragments without strict controlling will result in significant difference in produced tumor volume, which is difficult in following application, such as evaluating the therapeutic efficacy *in vivo*. Here, we reported a simple and reproducible technique to establish the intratibia primary osteosarcoma with later pulmonary metastasis mouse models via intratibia injection of osteosarcoma cells, which showed the advantages of best mimicking the clinical development characteristics of osteosarcoma in human; accurate numbers of osteosarcoma cells being directly injected into tibia using micro-volume syringe allowing identical tumor formation rate (100%) and tumor volume; avoiding the possibilities of infection or even death using open surgery techniques; allowing lively monitor and quantifying osteosarcoma growth and metastasis using the bioluminescence live imaging system after the injected osteosarcoma cells are labeled with bioluminescence; preventing injected osteosarcoma cells from directly reaching blood stream and colonizing in the lung to form pulmonary embolism and/or false positive pulmonary metastasis by resuspending the injected osteosarcoma cells in appropriate concentration of basement membrane matrix since the basement membrane matrix has the property of coagulation above room temperature and immediately coagulates to support and restrict osteosarcoma cells within the basement membrane matrix after being injected into mouse tibia.”

#### Minor Concerns:

The authors indicate that other species (dogs, zebrafish) have been used to establish orthotopic (intratibial) osteosarcoma xenografts in mice, but they fail to include the relevant references describing those experiments. This work and any potential differences (methodological or regarding the biology of osteosarcoma in these species) should be included in the Introduction and/or Discussion.

Response: Thank you very much for your advices.

“The most commonly used animals are mice, dogs and zebrafish [15, 16]. The spontaneous model of osteosarcoma is usually used in canine, because osteosarcoma is the most common tumors in canine. However, the application of this model is limited because of long tumor formation time, low tumorigenesis rate, poor homogeneity and stability. Zebrafish are often used to construct transgenic or knockout tumor models because of their rapid reproduction [17]. But zebrafish genes are different from human genes, so applications are limited.” has been provided in the Introduction section.