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Intratibial Osteosarcoma Cell Injection to Generate Orthotopic Osteosarcoma and Lung Metastasis Mouse Models

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TITLE:

Intratibial Osteosarcoma Cell Injection to Generate Orthotopic Osteosarcoma and Lung Metastasis Mouse Models

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SUMMARY:

The present protocol describes intratibia osteosarcoma cell injection to generate mouse models bearing orthotopic osteosarcoma and pulmonary metastasis lesions.

ABSTRACT:

Osteosarcoma is the most common primary bone cancer in children and adolescents, with lungs as the most common metastatic site. The five-year survival rate of osteosarcoma patients with pulmonary metastasis is less than 30%. Therefore, the utilization of mouse models mimicking the osteosarcoma development in humans is of great significance for understanding the fundamental mechanism of osteosarcoma carcinogenesis and pulmonary metastasis to develop novel therapeutics. Here, detailed procedures are reported to generate the primary osteosarcoma and pulmonary metastasis mouse models *via* intratibia injection of osteosarcoma cells. Combined with the bioluminescence or X-ray live imaging system, these living mouse models are utilized to monitor and quantify osteosarcoma growth and metastasis. To establish this model, a basement membrane matrix containing osteosarcoma cells was loaded in a micro-volume syringe and injected into one tibia of each athymic mouse after being anesthetized. The mice were sacrificed when the primary osteosarcoma reached the size limitation in the IACUC-approved protocol. The

legs bearing osteosarcoma and the lungs with metastasis lesions were separated. These models are characterized by a short incubation period, rapid growth, severe lesions, and sensitivity in monitoring the development of primary and pulmonary metastatic lesions. Therefore, these are ideal models for exploring the functions and mechanisms of specific factors in osteosarcoma carcinogenesis and pulmonary metastasis, the tumor microenvironment, and evaluating the therapeutic efficacy *in vivo*.

INTRODUCTION:

Osteosarcoma is the most common primary bone cancer in children and adolescents[1,2], which mainly infiltrates the surrounding tissue, and even metastasizes to the lungs when the patients are diagnosed. Pulmonary metastasis is the main challenge for osteosarcoma therapy, and the five-year survival rate of osteosarcoma patients with pulmonary metastasis remains as low as 20%–30%[3–5]. However, the five-year survival rate of primary osteosarcoma has been increased to about 70% since the 1970s due to the introduction of chemotherapy[6]. Therefore, it's urgently needed to understand the fundamental mechanism of osteosarcoma carcinogenesis and pulmonary metastasis to develop novel therapies. The application of mouse models that best mimic the osteosarcoma progression in humans is of great significance[7].

The osteosarcoma animal models are generated by spontaneous, induced genetic engineering, transplantation, and other techniques. The spontaneous osteosarcoma model is rarely used due to the long tumor formation time, inconsistent tumor occurrence rate, low morbidity, and poor stability[8,9]. Although the induced osteosarcoma model is more accessible to obtain than the spontaneous osteosarcoma, the application of the induced osteosarcoma model is limited because the inducing factor will affect the microenvironment, the pathogenesis, and pathological characteristics of osteosarcoma[10]. Transgenic models are helping to understand the pathogenesis of cancers since they can better simulate the human physiological and pathological environments; however, the transgenic animal models also have their limitations due to the difficulty, long-term, and high cost of transgenic modification. Moreover, even in the most widely accepted transgenic animal models generated by p53 and Rb gene modification, only 16.3% of sarcoma occurred in the four limb bones[11,12].

Transplantation is one of the most commonly used primary and distant metastatic cancer model-producing methods in recent years due to its simple maneuver, stable tumor formation rate, and better homogeneity[13]. Transplantation includes heterotopic transplantation and orthotopic transplantation according to the transplantation sites. In osteosarcoma heterotopic transplantation, the osteosarcoma cells are injected outside the primary osteosarcoma sites (bone) of the animals, commonly under the skin, subcutaneously[14]. Although the heterotopic transplantation is straightforward without the necessity to perform surgery in animals, the sites where the osteosarcoma cells are injected do not represent the actual human osteosarcoma microenvironment. Osteosarcoma orthotopic transplantation is when the osteosarcoma cells are injected into animals' bones, such as tibia[15,16]. Compared to the heterotopic grafts, orthotopic

osteosarcoma grafts are characterized by a short incubation period, rapid growth, and strong erosive nature; therefore, they are ideal animal models for osteosarcoma-related studies[17].

The most commonly used animals are mice, dogs, and zebrafish[18,19]. The spontaneous model of osteosarcoma is usually used in canines because osteosarcoma is one of the most common tumors in canines. However, the application of this model is limited because of the long tumor formation time, the low tumorigenesis rate, poor homogeneity, and stability. Zebrafishes are often used to construct transgenic or knockout tumor models because of their rapid reproduction[20]. But zebrafish genes are different from human genes, so their applications are limited.

This work describes the detailed procedures, precautions, and representative images for producing the primary osteosarcoma in the tibia with pulmonary metastasis *via* intratibia injection of osteosarcoma cells in athymic mice. This method was applied to create the primary osteosarcoma in mouse tibia for therapeutic efficacy evaluation, which showed a high reproducibility[21,22].

PROTOCOL:

All animal experiments were approved by the animal welfare committee of Shanghai University of Traditional Chinese Medicine. Four-week-old male BALB/c athymic mice were adapted for a week before the surgery for orthotopic injection of osteosarcoma cells.

1. Preparation of cells

1.1. On the day of osteosarcoma cell (143B-Luciferase) injection, wash 80%–90% confluent cells cultured in a 10 cm cell culture dish twice with PBS (pH 7.4) and trypsinize with 1.5 mL of 0.25% trypsin for 3 min. Then, add 6 mL of 10% serum-containing MEM media to quench the trypsin, and collect the cells in a 15 mL centrifuge tube.

NOTE: 143B-Luciferase cell line is obtained from 143B cell line transfect with pLV-luciferase vector[23].

1.2. Aspirate 20 µL of cell suspension into the chamber of cell counting plate and calculate cell concentration using an automatic cell counter (see **Table of Materials**).

1.3. Centrifuge the cells at 800 x *g* for 5 min at room temperature.

1.4. Aspirate the supernatant with a pipette and resuspend the cell pellet in an 8.5 mg/mL basement membrane matrix (see **Table of Materials**) to a final concentration of 2 x 10^7 cells/mL.

1.5. Keeping the cells on ice, bring them to the surgery room. The cells are to be used within 2 h.

NOTE: To avoid inaccurate injection doses (for example, due to the dead space in syringes), an extra cell suspension is prepared (usually two times the required volume of cell suspension). The basement membrane matrix is kept on ice all the time since it has coagulation property above room temperature[24].

2. Surgery for orthotopic injection of the osteosarcoma cells

NOTE: The surgery tools are shown in **Figure 1.**

2.1. Mice were raised in specific pathogen-free conditions. All procedures were done in an aseptic cabinet with sterile tools.

2.2. Anesthetize the mice by exposing them to 2% isoflurane and 98% oxygen (oxygen flow rate, 2 L/min).

2.3. Apply a small amount of vet ointment on the eyes to prevent dryness while under anesthesia.

NOTE: Perform the entire procedure in a well-ventilated area. Before osteosarcoma cell injection, ensure that each mouse is under deep anesthesia by gently touching the foot; if the mouse still has responses, such as twitch or jerk, wait for a long time until the above responses disappear.

2.4. Keep each mouse in a supine position. Hold the ankle of the mouse using the thumb and index finger and disinfect the injection site of the tibia with a 70% ethanol swab.

NOTE: To tightly hold the mouse ankle, both the thumb and index fingertips are of great importance for the subsequent procedures.

2.5. Rotate the ankle joint of each mouse outward to move the tibia and fibula, and bend the knee joint to a suitable position until the proximal tibia plateau (the top of the tibia) is clearly visible through the skin (**Figure 2A**).

2.6. Attach the needle to a 1 mL syringe and point the needle tip toward the injection site. Ensure that the syringe needle is parallel to the long axis of the tibia.

2.6.1. Percutaneously insert the needle through or adjacent to the patellar ligament as it goes through the skin/joint capsule; then, rotate the syringe (1/2 to 3/4-circle) to drill a hole through the tibia platform toward the distal end of the tibia (medullary cavity) for osteosarcoma cell injection with a micro-volume syringe (**Figure 2B,C**).

NOTE: Simultaneous rotation of the tibia can be felt while drilling if the needle tip is accurate.

Ensure the needling moves forward with the syringe rotation rather than being directly pushed forward until about half of the needle is in the tibia.

2.7. Check whether the syringe needle made a prominent movement into the medullary canal to ensure successful drilling.

NOTE: Perform an X-ray examination (see **Table of Materials**) to confirm the proper position of the needle and collect the images.

2.8. Load 143B osteosarcoma cell suspension (from step 1.5) into a micro-volume syringe and replace the 1 mL syringe in the tibia with the 143B cell-loaded micro-volume syringe (**Figure 2D**). Slowly inject ~10 µL (ignore pre-existing solution in the needle) of 143B cell suspension into each athymic mouse's tibia (about 2 x 10^5 cells) without applying high pressure.

2.9. Press the injection site with a cotton swab for 20–30 s when the micro-volume syringe is removed.

2.10. Put each mouse back into a clean cage and closely monitor until the mouse is completely recovered from anesthesia (about 10 min).

2.11. Monitor the tumor growth *in vivo* using an X-ray imaging system. Measure the longer diameter (a) and the short diameter (b) of the cancer mass every week with a caliper for tumor volume (V) calculation: $V = 1/2 \times a \times b^2$.

NOTE: Intratibia injection of luciferase or fluorescent protein labeled osteosarcoma cells enables tracking of primary and metastatic osteosarcoma lesions.

3. Pathologic examination (collecting primary and pulmonary metastatic osteosarcoma specimen for analysis)

3.1. Six weeks after osteosarcoma cell injection, sacrifice the mice by cervical dislocation after exposing them for CO_2 inhalation.

3.2. Keep the mouse in a supine position and stretch both the hind limbs.

3.3. Separate the whole legs bearing osteosarcoma from the inguinal area.

NOTE: Ensure that all legs are separated from the same anatomical site.

3.4. Prepare the histological specimen of legs bearing osteosarcoma by removing the skin, muscles, and feet, and then fix the specimen of each mouse in a 50 mL tube with 20 mL formalin

solution (10%) for 24 h, followed by decalcification in 10% EDTA solution for 14 days with occasional buffer change.

3.5. Embed the specimen in paraffin and prepare sections for histological examination following previously published work[25].

3.6. Gently separate the lungs and put them into a 50 mL tube filled with 20 mL formalin solution (10%). After 24 h, transfer the lungs of each mouse into a 15 mL tube with 70% ethanol. Embed the lungs in paraffin for Hematoxylin and Eosin (H&E) staining and immunohistochemistry assay[25].

REPRESENTATIVE RESULTS:

Successful orthotopic (primary) osteosarcoma and metastatic pulmonary models depend on the accurate orthotopic injection of osteosarcoma cells. Here, an orthotopic (primary) osteosarcoma model *via* intratibial osteosarcoma cell injection was successfully developed. **Figure 3A** shows a representative mouse bearing orthotopic (primary) osteosarcoma, and **Figure 3B** shows a representative isolated orthotopic (primary) osteosarcoma. The tumor volume was measured once a week with a caliper and calculated as described in step 2.11 (**Figure 3C**). The orthotopic (primary) osteosarcoma growth *in vivo* was tracked by both the X-ray and the bioluminescence (when the injected cells were labeled with luciferase) live imaging system**.** The X-ray images were obtained from the first week to the sixth week after 143B osteosarcoma cell injection (**Figure 3D**). Furthermore, the image of orthotopic (primary) osteosarcoma growth *in vivo* was obtained after luciferase labeled 143B cells were injected into the mouse tibia (**Figure 3E**).

The pulmonary metastasis caused by the intratibial injection of luciferase labeled osteosarcoma cells was successfully tracked *in vivo* by a bioluminescence live imaging system (**Figure 4A**). The metastatic colonies in the isolated lung tissues were also visualized under the stereomicroscope (**Figure 4B**). The metastatic lesions were further confirmed by H&E staining on paraffin-embedded lung tissues (**Figure 4C**).

FIGURE LEGENDS:

Figure 1: Surgery Tools. (**A**) 1 mL scale syringe. (**B**) Micro-volume syringe.

Figure 2: Representation of the intratibial injection surgery. (**A**) The intratibial injection site of an athymic mouse. (**B**) A sterile 1 mL syringe with an accompanied needle was percutaneously inserted into the tibia toward the distal end *via* the proximal tibia plateau (the top of the tibia). (**C**) A lateral view of the drilling process. The syringe needle was parallel to the long tibia axis (solid line). (**D**) Intratibial injection with osteosarcoma cell loaded micro-volume syringe.

Figure 3: Visualization of the osteosarcoma growth in mice. (**A**) Successful mouse orthotopic osteosarcoma model. (**B**) Isolated orthotopic osteosarcoma. (**C**) Tumor volume was measured

with a caliper and calculated using the following formula: tumor volume = 0.5 x longer diameter x short diameter x short diameter. Error bars stand for standard deviation (n = 8). (**D**) X-ray images were obtained from the same mouse at a different time (from 1–6 weeks). (**E**) Image obtained on the 28th day after luciferase labeled 143B cells were injected into the mouse tibia. The red arrows indicated the luminescence intensity of the orthotopic (primary) osteosarcoma.

Figure 4: Pulmonary metastasis of osteosarcoma. (**A**) Image obtained on the 28th day after luciferase labeled 143B cells were injected into the mouse tibia. The red arrows indicated the luminescence intensity of the pulmonary metastasis. (**B**) The isolated lungs with osteosarcoma metastases. The red arrows indicated the metastatic colonies (x20). (**C**) H&E staining showed metastatic lesions in lung tissues (scale bar = 200 μm).

DISCUSSION:

Orthotopic injection of osteosarcoma cells is an ideal model to study the function and mechanism of specific factors in osteosarcoma carcinogenesis and development to evaluate the therapeutic efficacy. To avoid differences in tumor growth, most active osteosarcoma cells at 80%–90% confluent with the same number are carefully injected into the tibia of each mouse, and the cell trypsinization time is strictly controlled without affecting the cell viability. As cell clumps affect cell counting leading to inaccurate cell numbers being injected into the tibia of each mouse, the cell suspension needs to be appropriately mixed up and down with a pipette to avoid the formation of cell clumps.

Another critical aspect that must be taken into consideration is the resuspended solution for osteosarcoma cells. The injected cells are resuspended in a basement membrane matrix instead of in PBS or culture medium. Moreover, a high concentration of basement membrane matrix is challenging to be pipetted and affects the accurate volume; thus, an appropriate concentration of basement membrane matrix is required[26]. To drill a hole through the tibia platform for osteosarcoma cell injection, the needling moves forward with the syringe rotation rather than being directly pushed forward until about half of the needle is in the tibia. More particularly, immunodeficient mice are applied to establish an orthotopic osteosarcoma model using human osteosarcoma cells[27]. Meanwhile, the injection procedure is performed in biological safety cabinet using sterile surgical tools. Since mice may experience uneasiness after anesthesia and surgery, the mice must be closely monitored on the first week postsurgery.

Intratibia injection of osteosarcoma cells labeled with fluorescent protein or luciferase enables the tracking of primary and metastatic lesions using optical imaging[28]. Osteosarcoma is never allowed beyond the size limit as in the IACUC-approved protocol; meanwhile, ulcerations may occur in enormous size tumor mass, which may lead to failed immunohistochemical analyses. 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[29]; 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 control will result in a significant difference in produced tumor volume, which is difficult in following application, such as evaluating the therapeutic efficacy *in vivo*. Here, a simple and reproducible technique is reported to establish the intratibia primary osteosarcoma with later pulmonary metastasis mouse models *via* intratibia injection of osteosarcoma cells. This showed the advantages of best mimicking the clinical development characteristics of osteosarcoma in humans; accurate numbers of osteosarcoma cells being directly injected into tibia using micro-volume syringe allowing identical tumor formation rate (100%) and tumor volume. The method ensures avoiding the possibilities of infection or even death using open surgery techniques and allowing lively monitor and quantifying osteosarcoma growth and metastasis using the bioluminescence live imaging system after the injected osteosarcoma cells are labeled with bioluminescence. This prevents the injected osteosarcoma cells from directly reaching the bloodstream and colonizing in the lungs 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. The immediate coagulation support and restrict osteosarcoma cells within the basement membrane matrix after being injected into the mouse tibia.

Another literature has reported the bone metastasis model establishment by intracardiac inoculation or intratibial inoculation of breast cancer cells[30]; 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 bloodstream rather than metastasis lesions formed by cancer cell dissemination from the primary cancer lesions.

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

In conclusion, the present primary and pulmonary metastatic osteosarcoma models generated by orthotopic osteosarcoma cell injection are handy tools to study the tumor microenvironment, efficacy of therapeutics on osteosarcoma growth and/or metastasis. In addition, by intratibia injection of the genetically modified osteosarcoma cells specifically targeting a gene, the models are helpful to explore the key oncogenes and tumor suppressors in osteosarcoma growth and pulmonary metastasis.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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Figure 1

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A B

Figure 2

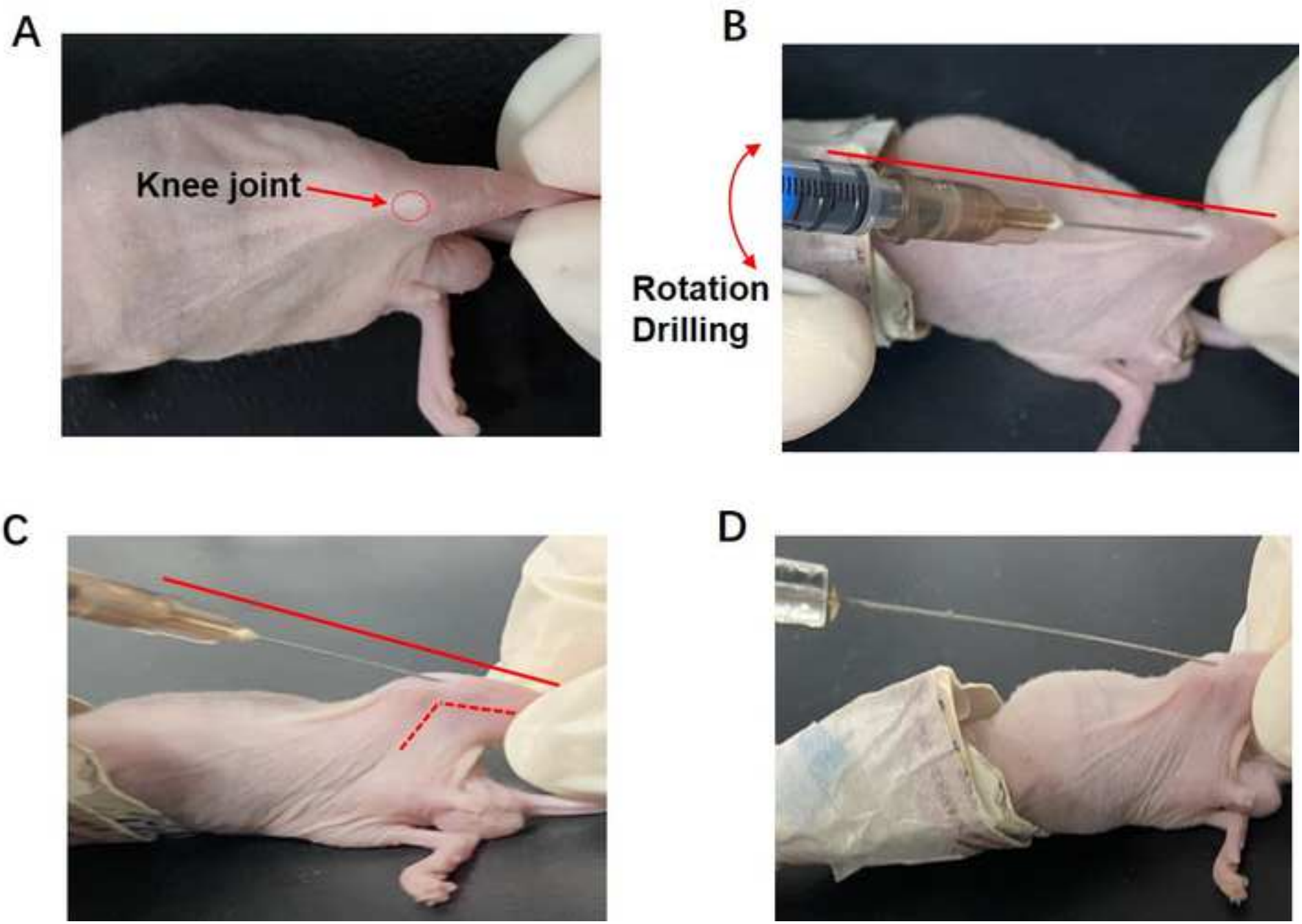

Figure 3

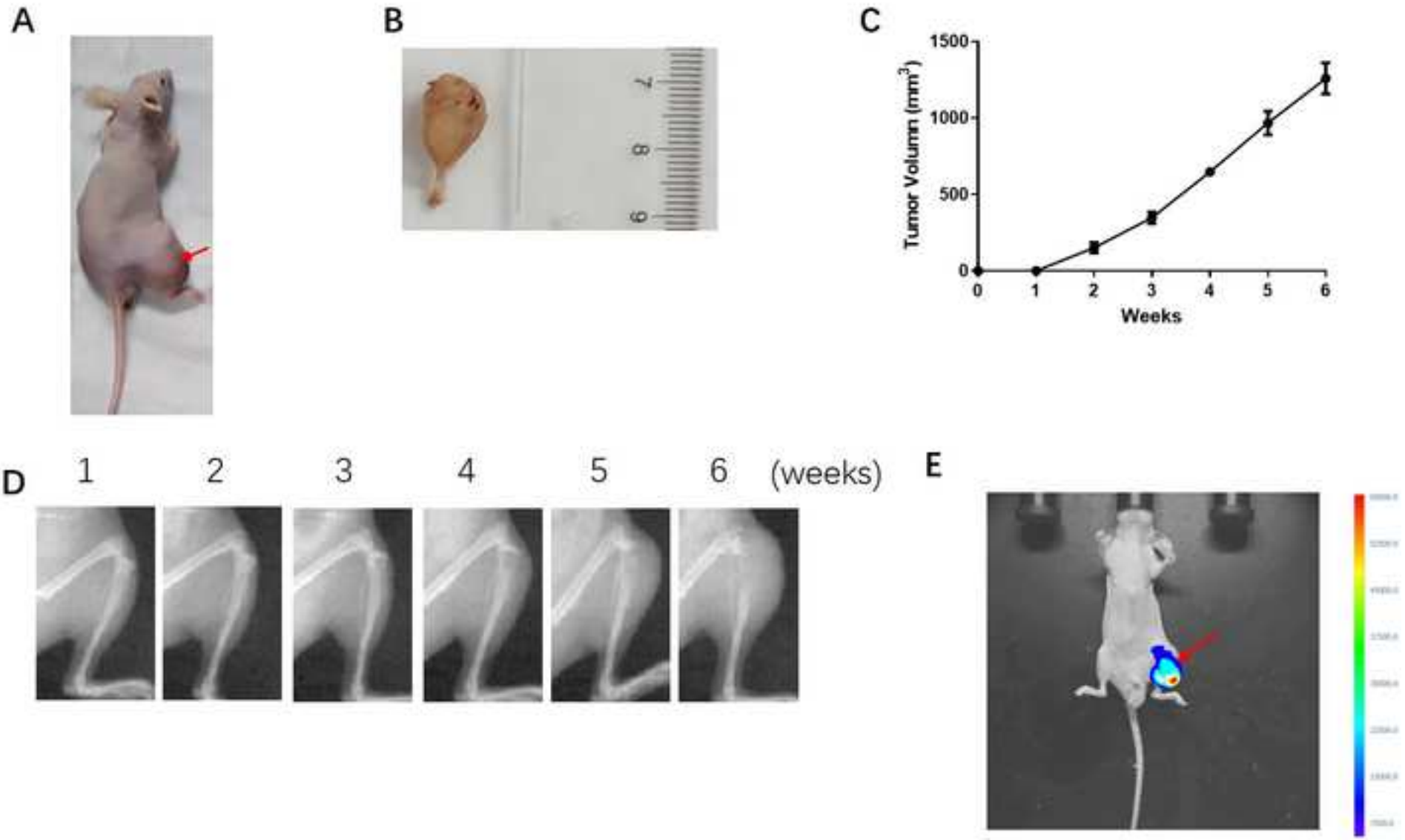

Figure 4

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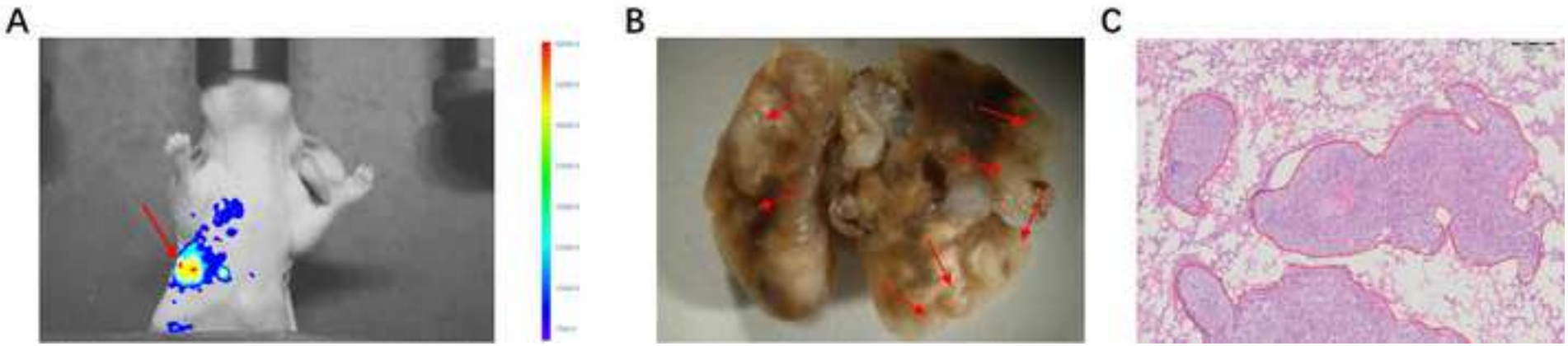

Dear Editor,

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

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, µL, 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.

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, Elefteriou 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 μL (ignore pre-existing solution in the needle) of 143B cell suspension into the tibia (about 2×10^5 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 ul, how are you accounting for the amount of pre-existing solution in the needle/hub?

"slowly inject about 10 μL (ignore pre-existing solution in the needle) of 143B cell suspension into the tibia (about 2×10^5 cells) of each athymic mouse." has been added. Therefore, 10μL 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 at 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 os 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.