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Measurement of Bone Metastatic Tumor Growth by a Tibial Tumorigenesis Assay

Baotong Zhang[1, 2, 3, *], Xin Li[4, 5], Wei-Ping Qian[3, 6], Daqing Wu[4, 5, *] and Jin-Tang Dong[1, 2, 3, *]

[1]Department of Human Cell Biology and Genetics, Southern University of Science and Technology School of Medicine, Shenzhen, China

[2]Department of Hematology and Medical Oncology, Emory University School of Medicine, Atlanta, GA, 30322, USA

[3]Winship Cancer Institute, Emory University, Atlanta, GA, 30322, USA

[4]Center for Cancer Research and Therapeutic Development and Department of Biological Sciences, Clark Atlanta University, Atlanta, GA, USA

[5]Department of Biochemistry and Molecular Biology, Georgia Cancer Center and Medical College of Georgia, Augusta University, Augusta, GA, 30912 USA

[6]Department of Surgery, Emory University School of Medicine, Atlanta, GA, 30322, USA

*For correspondence: zhangbt@sustech.edu.cn; dwu@cau.edu; dongjt@sustech.edu.cn

[Abstract] Bone metastasis is a frequent and lethal complication of many cancer types (*i.e.*, prostate cancer, breast cancer, and multiple myeloma), and a cure for bone metastasis remains elusive. To recapitulate the process of bone metastasis and understand how cancer cells metastasize to bone, intracardiac injection and intracaudal arterial animal models were developed. The intratibial injection animal model was established to investigate the communication between cancer cells and the bone microenvironment and to mimic the setting of prostate cancer patients with bone metastasis. Given that detailed protocols of intratibial injection and its quantitative analysis are still insufficient, in this protocol, we provide hands-on procedures for how to prepare cells, perform the tibial injection, monitor tibial tumor growth, and quantitatively evaluate the tibial tumors in pathological samples. This manuscript provides a ready-to-use experimental protocol for investigating cancer cell behaviors in bone and developing novel therapeutic strategies for bone metastatic cancer patients.

Keywords: Bone metastasis, Mouse model, Tibial injection, Prostate cancer, Tumorigenesis, Osteolysis

[Background] Bone metastasis is the most frequent metastasis for many cancer types, especially for those arising from prostate (90%), breast (70%), multiple myeloma (90%), lung (40%), and kidney (40%) (Coleman, 2006). The median survival of patients with bone metastasis ranges from approximately one year for lung cancer to 3-5 years for prostate cancer, breast cancer, and multiple myeloma (Campbell *et al.*, 2012). Bone metastases often require radiotherapy and lead to bone pain, hypercalcemia, pathologic fracture, and spinal cord or nerve root compression (Coleman, 2006). Bone metastases also develop resistance to chemotherapy, as docetaxel-treated prostate cancer patients with bone metastasis still have poor prognoses (Coleman *et al.*, 2020). Therefore, effective therapeutic strategies are still urgently needed for bone metastatic cancer patients.

Bone metastases can be classified as osteolytic or osteoclastic types according to their predominant

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characteristics in radiographic images. Although lysis or sclerosis may appear to predominate in some types of bone metastases, osteoclastic and osteoblastic activities are usually simultaneously involved in the formation of bone metastatic lesions. The outgrowth of cancer cells in the bone microenvironment is a process in which cancer cells communicate with osteoclasts and osteoblasts frequently via paracrine factors and receptors on their cell surfaces. Therefore, the behaviors of cancer cells in bone metastasis are distinct from those in primary tumors, thus potentially requiring different therapeutic methods.

Diverse *in vivo* animal models have been established to study bone metastasis. Intracardiac injection has been utilized as the gold standard to develop bone metastasis by injecting cancer cells into the left ventricle in mice, with a goal of recapitulating the bone metastasis process, including cancer cell survival in the bloodstream, extravasation, cancer cell arrest in bone, and bone metastatic tumor growth (James *et al.*, 2015). Similarly, intracaudal arterial injection was established to provide an easy-to-use model with higher efficiency of bone metastasis (Kuchimaru *et al.*, 2018). However, the intratibial injection model is used to mimic the scenario when cancer cells have metastasized to the bone, thus providing a better focus on the crosstalk between cancer cells and the bone microenvironment. Herein, we will focus on tibial tumorigenesis by using the intratibial injection model to mimic the setting of cancer patients who have developed bone metastasis.

In this protocol, we take the prostate cancer cell line PC-3 as an example, describe the hands-on procedures of cell preparation, intratibial injection, monitoring tibial tumor growth, and post tissue collection analyses, highlight the critical steps in cell preparation and intratibial injection, and detail how to quantitatively analyze the tibial tumors in histological samples.

Materials and Reagents

1. Nude mice (The Jackson Laboratory, catalog number: 002019)
2. NOD SCID mice (The Jackson Laboratory, catalog number: 001303)
3. Povidone Iodine Prep Pads (DYNAREX, catalog number: 1108)
4. Sterile Alcohol Prep Pads (DYNAREX, catalog number: 1116)
5. 27G ½" syringe (BD, catalog number: 305620)
6. 40 μm Cell Strainer (Fisher Scientific, catalog number: 087711)
7. PC-3 cells (ATCC, catalog number: CRL-1435)
8. Ham's F-12K (Kaighn's) Medium (ThermoFisher, catalog number: 21127022)
9. FBS (Sigma, catalog number: F2442)
10. 0.05% trypsin-EDTA (ThermoFisher, catalog number: 25300054)
11. 0.4% trypan blue solution (Sigma, catalog number: T8145)
12. PBS (Sigma, catalog number: 806552-1L)
13. Isoflurane Inhalation Anesthetic (Southern Anesthesia & Surgical, catalog number: PIR001325)
14. Human Kallikrein 3/PSA Quantikine ELISA Kit (R&D system, catalog number: DKK300)
15. 14% EDTA (pH 7.4) (American Research Product, catalog number: BM-150A)
16. 10% neutral-buffered formalin (Sigma, catalog number: HT501128)

17. 100% Ethanol (KOPTEC, catalog number: 89426-252)
18. Hematoxylin (Electron Microscopy Sciences, catalog number: 26043-05)
19. Eosin Y-solution (Millipore Sigma, catalog number: 102439)
20. Permount (Fisher Scientific, catalog number: SP15100)
21. Xylene (Fisher Chemical, catalog number: X54)
22. Immu-Mount (Epredia, catalog number: 9990402)
23. Sodium acetate anhydrous (Sigma, catalog number: S-2889)
24. L-(+) tartaric acid (Sigma, catalog number: T-6521)
25. Fast Red Violet LB salt (Sigma, catalog number: F-3381)
26. Napthol AS-MX phosphate (Sigma, catalog number: N-4875)
27. Basic Staining Buffer for TRAP staining (see Recipes)
28. TRAP Staining Solution (see Recipes)
29. 10% EDTA (see Recipes)

Equipment

1. Refrigerated centrifuge (Eppendorf, model: 5810R)
2. Gas Anesthesia System (PerkinElmer, model: XGI-8)
3. Heating pad (Sunbeam Products, catalog number: 2139885)
4. IVIS® Spectrum Imaging System (PerkinElmer)
5. MX-20 X-ray system (Faxitron [Tucson, Arizona])
6. -80°C freezer (Fisher, catalog number: TSU600ARAK)
7. Embedding Center (Leica, catalog number: EG1160)
8. Rotary Microtome (Leica, catalog number: RM2135)
9. NanoZoomer Whole Slide Scanner (Olympus, Hamamatsu)

Software

1. NDP.view2 (HAMAMATSU, https://www.hamamatsu.com/us/en/product/type/U12388-01/index.html)
2. Living Image software (PerkinElmer, https://www.perkinelmer.com.cn/product/li-software-for-lumina-1-seat-add-on-128110)

Procedure

A. Collection of fresh cultured cancer cells
   1. Seed prostate cancer PC-3 cells in F-12K medium with 10% FBS. For PC-3 cells, 0.5 million cells per 10 cm dish in 10 ml of media is ideal for cell culture. Culture cells at 37°C and 5% CO_2 in a cell culture incubator.

2. Passage the PC-3 cells every three days when cells reach 80-90% confluence. See below procedures for subculturing PC-3 cells:
    a. Remove medium and wash the cells with 2 ml of sterile PBS.
    b. Add detaching reagent 0.5% trypsin-EDTA and incubate the dishes at 37°C until the cells are fully detached from the dishes (approximately 2 min for PC-3 cells).
    c. Add 2 ml of fresh media containing 10% FBS to inactivate the trypsin and collect the cells into 15 ml (or 50 ml) sterile centrifuge tubes.
    d. Centrifuge at 1,200 × *g* for 3 min to pellet the cells. Remove the supernatant and resuspend the cells in 3 ml of fresh medium. Count the cells and perform the cell culture procedure described in Step A1.
3. Collect cultured PC-3 cells for tibial injection. For PC-3 cells, approximately 2-2.5 million cells will be collected per dish when the cells are 80%-90% confluent in a 10 cm dish. Perform steps described in Steps A2a-A2c to collect the cells in sterile centrifuge tubes. Follow the procedures below to obtain a single cell suspension for tibial injection.
    a. Centrifuge at 1,200 × *g* for 3 min to pellet the cells. Remove the supernatant and resuspend cells in 20 ml of sterile PBS. Centrifuge at 1,200 × *g* for 3 min to pellet the cells again. Wash the cells with PBS by following this procedure twice.
    b. Resuspend the cells in 20 ml of sterile PBS, pipette thoroughly to obtain a single cell suspension. Filter the cells with a 40 µm Cell Strainer to guarantee a single cell suspension.
    c. Count the cells in 0.4% trypan blue solution to assess cell viability and calculate the total number of live cells.
    d. Centrifuge at 1,200 × *g* for 3 min to pellet the cells and remove the supernatant. Approximately 300-500 µl of PBS will remain in the centrifuge tube. Use the pipette to measure the exact volume and then add the appropriate volume of PBS to obtain a cell suspension with a density of 5×10^7 cells/ml.
    e. **Critical step**: Prepare sufficient cells. Approximately 1.5 to 2-fold the required number of cells is ideal. For instance, if 10 million cells are needed for tibial injection, then prepare 15-20 million cells. Use fresh cells for tibial injection. Suspend the cells in PBS until tibial injection; for instance, proceed to Steps A3a-A3d within an hour before tibial injection.

B. Tibial injection
1. Mouse preparation for tibial injection. General requirements for the tibial injection procedure follow the standards of rodent survival surgery.
    a. Sanitize the work surface and anesthetize nude mice by using 3% isoflurane.
    b. Maintain the anesthesia with 2.5% isoflurane and check the toe reflex of muscle tone by pinching the toes. The animal is well anesthetized when no toe reflex is present.
    c. If NOD SCID mice are used, remove the hairs from the ankle joint to expose the injection area before anesthesia.
    d. Administer buprenorphine at 0.1 mg/kg by intraperitoneal injection.

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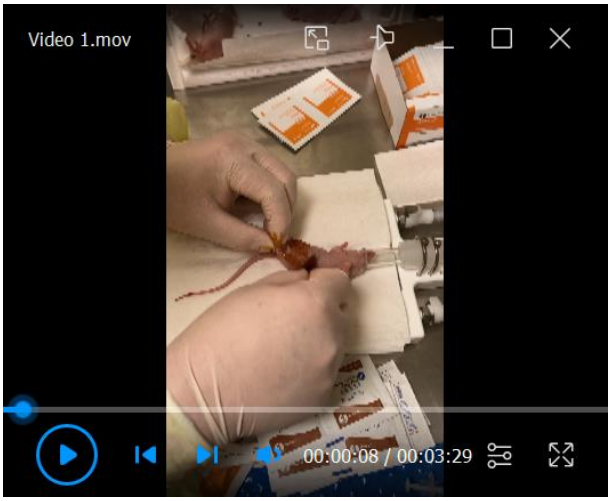
e. Clean both legs of the mouse with 10% povidone/iodine swab/solution followed by 70% ethanol. Repeat three times.

2. Cell preparation for tibial injection. Mix the cells again to obtain a homogeneous cell suspension. Remove the plunger and pipette 20 µl of cells (1 million cells) into a 27G ½" syringe from the bottom. Install the plunger again and remove the air by slowly pushing the plunger inside. Hold the syringe vertically with the needle pointing up and gently tap the syringe to remove any bubbles. A drop of cell suspension will come out of the needle, ensuring no air remains in the syringe.
3. Tibial injection (see Video 1).
   a. Using forefinger and thumb, gently grasp the lateral malleolus, medial malleolus, and lower half of the tibia, and bend the leg in a movement combining flexion and lateral rotation. The knee will be visible and accessible at this time.
   b. While firmly grasping the ankle/leg of the mouse, insert a needle without cell suspension under the patella, through the middle of the patellar ligament, and into the anterior intercondylar area in the top of the tibia. We use a blank syringe first to avoid the blockage of the needle by bone tissues.
   c. When inserting the needle into the tibia, guide the syringe through the growth plate using steady and firm pressure with a slight drilling action. Upon penetration of the tibial growth plate, the needle will encounter markedly less resistance.
   d. Use a gentle and lateral movement of the needle to ensure the needle is in the tibia and through the growth plate. The movement will be limited because the needle is in the proper place within the tibia.
   e. Remove the blank syringe and insert the syringe with the cell suspension following the same route. Gently move the needle to ensure the needle is in the tibia and through the growth plate. Inject a volume of 20 µl of cell solution very slowly. At this point, no resistance should be felt.



Video 1. Procedures of tibial injection in a nude mouse.

This video was made at Emory University according to guidelines from the Emory University on Animal Care and approved by the Animal Research Ethics Board of Emory University under protocol #PROTO201700737.

4. Place the mice on a heating pad during the recovery period. Monitor the mice every 12 h during the first 72 h.

C. Monitoring tumorigenesis in the tibia
   1. Monitor bone lesions via X-ray/micro-computed tomography (CT).
      a. Turn on the machine and wait until the “READY” green light is on.
      b. Warm up the machine for 300 s under 20 KV.
      c. Anesthetize the mice via intraperitoneal injection of ketamine (80 mg/kg)/xylazine (10 mg/kg) prior to surgery. Monitor anesthesia depth by pinching toes.
      d. Open the door and place a film in the cassette at the bottom with the “+” laser guide in the center.
      e. Place the mice on top of the tray with the proper enlargement, center it with the “+” laser guide, and close the door tightly.
      f. Perform X-ray imaging under 25 KV for 60 s.
      g. Following scanning, turn the machine off, develop and fix the films, and recover the mice in their cages.
   2. Monitor tumorigenesis in tibia by IVIS once a week if the tumor cells are prelabeled with luciferase.
      a. For the cancer cells prelabelled with firefly luciferase, administer D-luciferin at 150 mg/kg via intraperitoneal injection. Wait 15-20 min before imaging for maximum luciferase signal.
      b. Anesthetize the mice with 3% isoflurane and maintain at 2% isoflurane. Monitor anesthesia depth by pinching toes.
      c. Acquire bioluminescence images with a Xenogen IVIS Spectrum Imaging System (PerkinElmer) and measure the photon flux in the tibial area of mice by using Living Image software (PerkinElmer). Please follow the equipment instructions to complete this step.
      d. Following scanning, recover the mice in their cages.
   3. Monitor Prostate-Specific Antigen (PSA) levels in serum.
      a. Some prostate cancer cell lines (*i.e.*, LNCaP, C4-2, C4-2B) and patient derived xenografts (*i.e.*, LuCaP 23.1) secrete PSA during tumorigenesis in bone (Li *et al.*, 2021). Determine the PSA levels in cell lines and patient derived xenografts by literature search or experiments.
      b. Diverse methods can be used for blood collection from mice, and 150 µl of blood per mouse is sufficient for the measurement of PSA levels in serum. Herein, collect blood from the lateral tail vein as below:
         i. Warm the animals on a heating pad at least 30 min before blood collection.

ii. Restrain the animal using the mechanical restraint device of choice with the tail protruding.
iii. Clean the collection site with 70% ethanol.
iv. Align the needle parallel to the lateral tail vein with the beveled edge of the needle facing up.
v. Insert the needle into the vein starting at the tip of the tail and gently aspirate to collect blood via the syringe. This step is easier if a butterfly needle and vacuum blood collection tube are used.
vi. Apply gentle pressure with gauze until bleeding has stopped.

c. After blood collection, allow the blood to clot by leaving it undisturbed at room temperature (RT) for 30 min. Remove the clot by centrifuging tubes at 2,000 × *g* for 10 min. Carefully collect the supernatant in yellow, which is designated serum. Approximately 50 µl can be collected from 150 µl whole blood. Store the serum in a -80°C freezer until use.
d. Measure the PSA levels in serum using PSA ELISA kits (*i.e.*, R&D System, #DKK300) following the manufacturer's instructions.

D. Collection of bone tissue and histological analysis

1. Bone tissue processing
   a. Euthanize the animals. Dissect the tibia, the femur, and the joint together from both lateral limbs. At least some of the femur should remain to account for the possible migration of tumor cells from the tibia to the femur. Remove as much muscle tissue as possible. Store the bone tissues in 10% neutral-buffered formalin (10 ml/bone sample) at RT up to two weeks for optimal results.
   b. Decalcify bone tissues with 10% EDTA (pH 7.4) at 4°C for 10 days. The fluid volume to tissue ratio is critical for the decalcification process. Use at least 40 volumes of EDTA to decalcify bone tissues. For instance, if the bone tissues occupy 0.5 ml, then use 20 ml EDTA. Shake intermittently to make sure the decalcification solution is flowing around the bone. Change the decalcification solution on day 5.
   c. After decalcification, rinse the bone tissues with distilled water once and store them in 70% alcohol (10 ml/bone sample) for further standard tissue processing.
   d. Section the tissues embedded in paraffin at 5 µm-thick to include the tibia, knee joint, and the distal femur.
2. Hematoxylin & eosin (H&E) staining
   a. Deparaffinize the unstained slides in xylene and rehydrate them in an ethanol gradient as below:
      - 3 × 3 min Xylene
      - 2 × 3 min 100% ethanol
      - 1 × 3 min 95% ethanol
      - 1 × 3 min 80% ethanol

- 1 × 3’ min 70% ethanol
- 1 × 3 min deionized water

b. Stain slides with hematoxylin for 10 min, rinse with deionized water twice, and incubate with PBS for 3 min. Rinse again with deionized water twice.

c. Stain slides with eosin for 30 s and then proceed to dehydration procedures as below：

- 2 × 3 min 95% ethanol
- 2 × 3 min 100% ethanol
- 3 × 10 min xylene

d. Coverslip slides using Permount. Place a drop of Permount on the slide using a glass rod, taking care to leave no bubbles. Dry overnight in the hood.

3. Tartrate-resistant acidic phosphatase (TRAP) staining

a. Prepare Basic Staining Buffer and TRAP Staining Solution (see Recipes) and prewarm to 37°C.

b. Deparaffinize the unstained slides in xylene and rehydrate them in an ethanol gradient as below:

- 3 × 3 min Xylene
- 2 × 3 min 100% ethanol
- 1 × 3 min 95% ethanol
- 1 × 3 min 80% ethanol
- 1 × 3’ min 70% ethanol
- 1 × 3 min deionized water

c. Incubate the unstained slides in the Basic Staining Buffer for 20 min in an oven at 37°C.

d. Stain the slides in TRAP Staining Solution for 30-45 min in an oven at 37°C. Monitor the color change every 15 min until the TRAP-positive area turns red.

e. Counterstain the slides with hematoxylin for 10 min, rinse with deionized water twice, and incubate with PBS for 2 min.

f. Coverslip slides with Immu-Mount for analysis. TRAP staining will be dissolved by ethanol. If Permount is desired, then incubate the slides directly in xylene for 10 min and repeat twice to remove water.

Data analysis

A. X-ray images

1. Review X-ray images of mouse tibia and determine tumor-induced osteolytic and osteoblastic lesions in a double blind manner.
2. Evaluate the degree of osteolysis as in Figure 1.

Note: X-ray is not a quantitative method, so there is no well-accepted scoring system for animal data.

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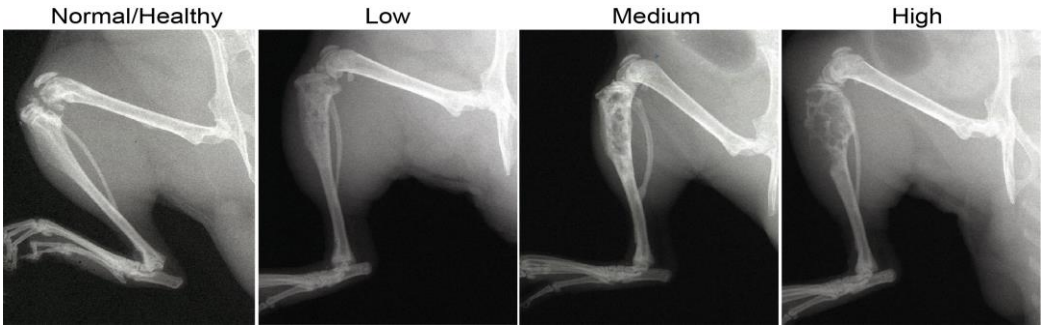

Figure 1. X-ray images of tibia bearing 5-week PC-3 tumors.

The degree of bone destruction is shown at the top of each image.

B. Analyze tumor area in H&E stained slides

1. Scan whole H&E stained tissue slides with an Olympus Hamamatsu NanoZoomer whole slide scanner at 40× magnification.
2. Use the Freehand Region tool in the NDP.view2 software to calculate total area (Figure 2B) and tumor area (Figure 2C) in tibial tumor samples. Typically, cancer cells have varied nuclei and more mitotic figures.
3. Calculate the ratio of tumor area to total area.
4. Show representative images with higher resolution as desired (Figure 2).

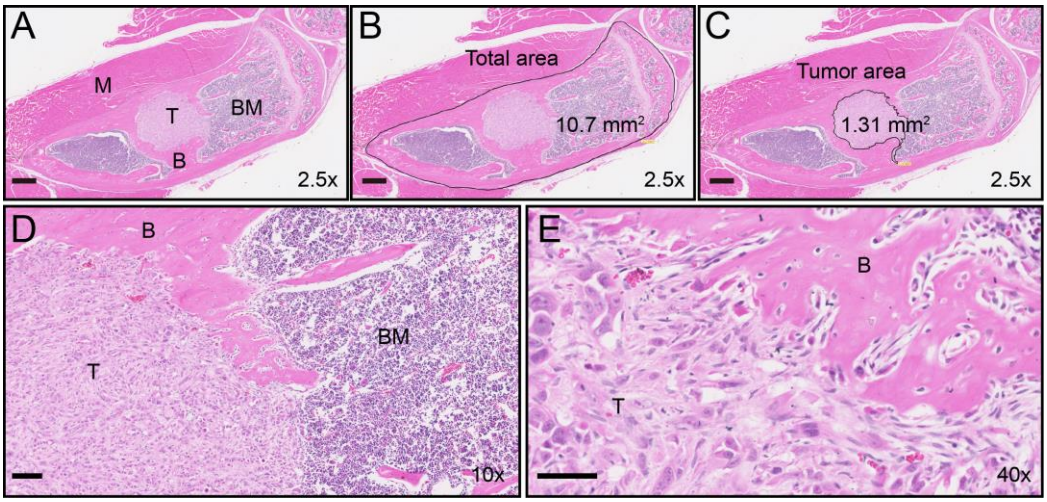

Figure 2. H&E staining in 5-week tibial tumors.

Shown are representative images of 2.5× (A-C), 10× (D), and 40× (E) magnifications of a PC-3 tibial tumor sample. Scale bars in A-C: 500 μm. Scale bar in D: 100 μm. Scale bar in E: 50 μm. T, tumor tissue. B, bone tissue. BM, bone marrow. M, muscle tissue.

C. Analyze osteoclast differentiation in TRAP stained slides

1. Scan whole TRAP stained tissue slides with an Olympus Hamamatsu NanoZoomer whole slide scanner at 40× magnification.
2. Use the Freehand Line tool in the NDP.view2 software to calculate the length of the interface

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between tumor and bone in tibial tumor samples. If multiple interfaces exist in the samples, calculate the sum of lengths.

3. Count the occurrence of TRAP positive cells at the tumor and bone interface.
4. Calculate the average occurrence of TRAP positive cells at the tumor and bone interface.
5. Show representative images with higher resolution as desired (Figure 3).

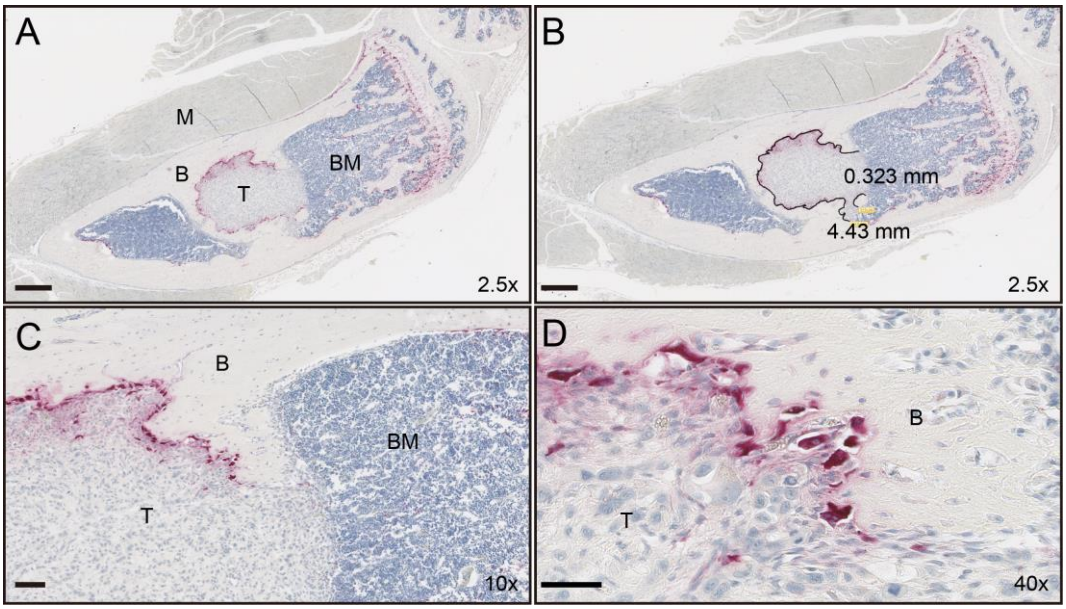

Figure 3. TRAP staining in 5-week tibial tumors.

Shown are representative images of 2.5× (A, B), 10× (C), and 40× (D) magnifications of a PC-3 tibial tumor sample. Scale bars in A-B: 500 µm. Scale bar in C: 100 µm. Scale bar in D: 50 µm. T, tumor tissue. B, bone tissue. BM, bone marrow. M, muscle tissue. The lengths of tumor and bone interface were calculated in B using NDP.view2.

Recipes

1. Basic Staining Buffer for TRAP staining (store at room temperature up to 6 months)
   a. Sodium acetate anhydrous 9.2 g
   b. L-(+) tartaric acid 11.4 g
   c. Adjust pH to 4.7-5.0 by adding acetic acid
   d. Add deionized water to a total volume of 1 l
2. TRAP Staining Solution (make freshly before use)
   a. Basic staining buffer for TRAP staining: 200 ml
   b. Fast Red Violet LB salt: 120 mg
   c. Napthol AS-MX phosphate: 20 mg (dissolved in 1 ml ethylene glycol monoethyl ether)
3. 10% EDTA
   Dilute 1,000 ml 14% EDTA by adding 400 ml deionized water.

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Competing interests

The authors declare no conflict of interest.

Ethics

The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Emory University with a protocol number of #PROTO201700737, which is validated from 7/12/2018 to 6/30/2024.

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