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TITLE:

A Syngeneic Orthotopic Osteosarcoma Sprague Dawley Rat Model with Amputation to Control Metastasis Rate

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KEYWORDS:

sarcoma, osteosarcoma, orthotopic implantation, syngeneic rat model, lung metastasis, leg amputation, X-ray imaging

SUMMARY:

Here, a syngeneic orthotopic implantation followed by an amputation procedure of the osteosarcoma with spontaneous pulmonary metastasis that can be used for preclinical

investigation of metastasis biology and development of novel therapeutics is described.

ABSTRACT:
The most recent advance in the treatment of osteosarcoma (OS) occurred in the 1980s when multi-agent chemotherapy was shown to improve overall survival compared to surgery alone. To address this problem, the aim of the study is to refine a lesser-known model of OS in rats with a comprehensive histologic, imaging, biologic, implantation, and amputation surgical approach that prolongs survival. We used an immunocompetent, outbred Sprague-Dawley (SD), syngeneic rat model with implanted UMR106 OS cell line (originating from a SD rat) with orthotopic tibial tumor implants into 3-week-old male and female rats to model pediatric OS. We found that rats develop reproducible primary and metastatic pulmonary tumors, and that limb amputations at 3 weeks post implantation significantly reduce the incidence of pulmonary metastasis and prevent unexpected deaths. Histologically, the primary and metastatic OSs in rats were very similar to human OS. Using immunohistochemistry methods, the study shows that rat OS are infiltrated with macrophages and T cells. A protein expression survey of OS cells reveals that these tumors express ErbB family kinases. Since these kinases are also highly expressed in most human OSs, this rat model could be used to test ErbB pathway inhibitors for therapy.

INTRODUCTION:
Osteosarcoma (OS) is the most common primary bone tumor in children, adolescents, and young adults. The most recent advance in the treatment of OS occurred in the 1980s when multi-agent chemotherapy was shown to improve overall survival compared to surgery alone[1]. OS develops during rapid bone growth, typically occurring in long tubular bones such as femur, tibia, and humerus. They are characterized by an osteolytic, osteoblastic, or mixed appearance with notable periosteal reaction[2]. Chemotherapy and surgical resection can improve the outcome for patients with a 5-year survival for 65% of patients[2,3]. Unfortunately, high grade OS patients with metastatic disease have 20% survival. OS invades regionally and metastasizes primarily to the lungs or other bones and is more prevalent in males. The most compelling need for these young patients is a novel therapy that prevents and eliminates viability of distant metastases.

OS pre-clinical models have been reviewed[4,5,6,7] and few available immunocompetent models using amputation of orthotopic OS have been developed. In 2000, an important model was developed using BALB/c mice with orthotopic syngeneic OS and amputation[8]. Compared to this mouse model, the rat model is based on genetically outbred and 10 times larger animals leading to some advantages. The rat UMR106 model was developed from a ^{32}P induced OS in a Sprague Dawley (SD) rat, which was derived into a cell line[9]. In 2001, orthotopic implantation of UMR106-01 was first described in implanted tibias of athymic mice with rapid, consistent primary tumor development and radiological, histologic features in common with OS in humans. Pulmonary metastases developed and were dependent on orthotopic placement of UMR106 into the bone microenvironment[10]. In 2009, Yu et al.[11] established a reproducible orthotopic femur OS rat model using UMR106 cells in larger male SD rats. The successful tumor implantations and lung metastasis rate in rats without amputation were similar to the data presented here. In this study, an added amputation to the model using young rats was performed, which suggested that the timing of primary tumor removal is crucial in modeling OS, especially related to metastatic progression. With this refinement,

amputation and in vivo imaging improve this model for pre-clinical studies for novel drug assessment for OS.

PROTOCOL:

All the procedures and experiments involving rats were performed according to protocols approved by Johns Hopkins Animal Care and Use Committee.

1. The SD rat OS cell line UMR-106 cell culture protocol

1.1. Grow cells in DMEM, supplemented with 10% (v/v) FBS, penicillin (10 U/mL)–streptomycin (10 U/mL) at 37 °C in humidified 5% CO_2 atmosphere. Perform experiments using cells with passages of 2–8[12].

2. Intratibial injection of OS cells protocol

NOTE: Time-mated pregnant SD rats give birth in the animal facility and at 3 weeks of age, litters are used (since UMR 106 cell line is syngeneic to SD rats, no irradiation is needed).

2.1. Induction

2.1.1. Place the rat in a medium-sized induction chamber and induce anesthesia with 2%–3% isoflurane. Monitor the animal continuously for the depth of anesthesia by reflex to toe pinch, respiratory rate, and character.

2.1.2. Insert the nose into the nosecone. Secure with tape, if necessary.

2.1.3. Remove the hair on the right leg up to the ventral and the dorsal lower abdomen with clippers or use depilatory agent. Place the rat in a supine position.

2.1.4. Scrub the surgical area aseptically using 70% ethanol and dilute chlorhexidine acetate or dilute betadine. Begin around the area of the knee and scrub in a circular motion both proximally and distally. Repeat this step three times. No drape is used for tumor implantation.

2.1.5. Apply eye lubricant in both the eyes of the rat to prevent corneal desiccation caused by anesthesia. Place the rat on a low heat setting heating pad. Ensure that the rat has normal body temperature (37 °C) and normal respiratory rate.

2.2. Surgery

2.2.1. Turn on isoflurane at ~1.5%–2% (for maintenance). Ensure that the animal is at an adequate plane of anesthesia via lack of a toe-pinch reflex. If not, increase the isoflurane percentage to 2.5%.

2.2.2. Mark a sterile needle (approximately 22 G) at 10 mm from the tip for guidance of depth to insert.

2.2.3. Insert the needle 10 mm down into the diaphysis of the tibia by entering the bent knee in the middle of the tibial plateau, extending the needle through the metaphysis into the diaphysis using a light drill-like motion to make an opening. Remove the needle.

2.2.4. Load the cell suspension into the Hamilton syringe directly before injection into the tibia. To do this, gently mix the cells before drawing into syringe as gravity makes the cells settle in the bottom of the tube.

NOTE: Cells can be stored in a 1.5 to 2 mL tube (at room temperature) before drawing into the Hamilton syringe (100 µL) after careful mixing. Cells can be kept at room temperature for 2–3 h during the implantation procedure. Always check the cell viability of cells in the tube before and then after the implantation session. Trypan blue exclusion is the easiest method for cell viability assessment.

2.2.5. Once the bone is traversed with the first needle, insert a second (smaller diameter needle also marked at 10 mm) needle attached to the 100 µL Hamilton syringe loaded with cells. Ensure to insert the needle up to the 10 mm mark into the same hole extending into the diaphysis.

2.2.6. Gently discharge 20 µL of 75,000 OS cells suspension in PBS into the diaphysis and marrow cavity.

NOTE: The needle should not wobble in the bone and should feel secure. If the needle easily moves, the cortex may have traversed at the diaphysis. Repeat the insertion again to get a firmer placement before the injection of cells.

2.2.7. Remove the Hamilton needle from the bone.

NOTE: If a small drop of blood forms, apply light pressure. If clear fluid drop forms at the puncture site, the needle may not have been extended far enough in the bone and the tumor cell suspension may have leaked back through the hole. Record this in the notes but generally, tumor implantation will be successful. With experience, the tumor implantation procedure should take 5 min per rat. With experience, the tumor implantation of cells in the bone will become easier. Accidental injection of cells in the muscle around the bone, may not lead to the tumor microenvironment needed for pulmonary metastasis.

2.3. Recovery

2.3.1. Ensure that the rat is normothermic. Place the rat in a cage with a heating pad placed under the cage for recovery.

2.3.2. After fully awake, mobile, and breathing well, inject the rats with Buprenorphine (1.0–1.2 mg/kg SC).

NOTE: To get access to Buprenorphine, check with the institution to see options for approval through the veterinary staff to place the order.

2.3.3. Move the rats back to clean cages and monitor once a day, each week.

3. Measurement and monitoring

3.1. Measure the tumor size 9–10 days post-implantation and then every 2 days until 3 weeks post-implantation to establish a growth rate. Measure the maximum diameter of the tibia using an electronic or manual caliper. Store the data in a spreadsheet with a formula to calculate tumor volume. Measure the contralateral (not implanted tibia) as the baseline.

NOTE: The implanted hindlimb diameters are used as a surrogate for tumor size. The hind limbs are measured perpendicular to the tibia long axis at the largest diameter for two measurements, ventral/dorsal and media/ lateral on the limb. The estimated tumor volume is calculated by the formula[11]: Tumor volume (mm^3) = largest diameter (mm) x smallest diameter $(mm)^2/2$.

3.2. Consider the rats for survival amputation or chemotherapy treatment when tumors approach 15 mm in the largest dimension or 3 weeks post tumor implantation. The contralateral limb measures about 7–9 mm in most rats at this age.

4. Doxorubicin intravenous administration

4.1. Anesthetize the rats with 2% isoflurane. Prepare the skin over jugular vein with three surgical washes using betadine and alcohol as described[13].

4.2. Under careful dissection, visualize the right or left jugular vein. Insert the needle into the overlying muscle and then direct toward the rat's head into the jugular vein lumen as it is visualized in the jugular vein anterior to the pectoralis muscle.

4.2.1. When the needle is inserted into the jugular vein, gently draw blood into the syringe to ensure proper insertion. It is possible to believe that the needle is through the vein, but the needle is under the vein and not in the lumen. If the jugular vein becomes too small for injections when blunt dissecting to expose the jugular vein, use the other jugular vein for the injection.

4.3. Inject doxorubicin (2 mg/kg) slowly over 1 min in a volume of 100–150 µL. The solution can be visualized intravenously in the jugular vein during delivery.

4.4. Inject similar volumes of normal saline in control rats.

4.5. Remove the needle and apply gentle pressure on the vein with a sterile gauze.

4.6. Close the skin incision using 3–4 wound clips. Remove the clips at 7–10 days post injection.

NOTE: Rats do not usually try to remove metal clips but will bite and remove sutures. The jugular injection method is preferable to tail vein injections for doxorubicin since any drug that leaks outside the vessel causes necrosis of tail that may require amputation.

5. Hind limb amputation protocol

5.1. Induction

5.1.1 Place the rat in a medium-sized induction chamber and induce anesthesia with 2%–4% isoflurane. Monitor the rat continuously for the depth of the anesthesia.

NOTE: The induction chamber is scavenged to a charcoal canister and any other gases are removed by a down-draft table used for surgery.

5.1.2 Insert the nose into a nosecone. Secure with tape, if necessary.

NOTE: This procedure is done on a down-draft table to scavenge excess volatile gases (i.e., isoflurane).

5.1.3 Remove the hair on the right leg up to the ventral and the dorsal lower abdomen with clippers or use depilatory agent. Place the rat in a supine position.

5.1.4 Scrub the surgical area aseptically using 70% ethanol and dilute chlorhexidine acetate or dilute betadine. Prepare the skin for surgery from middle of the calf to skin area just above the hip joint of the right lower abdomen. Scrub the leg proximal and the distal area circumferentially. Repeat this step three times.

5.1.5 Apply the eye lubricant in both the eyes of the rat. Ensure that the rat has normal body temperature (37 °C) and normal vital signs. Monitor and regulate body temperature using a heating pad that is connected to a rectal temperature probe monitor.

5.2. Surgery

5.2.1. Turn on isoflurane at 1.5%–3% (maintenance). Monitor for anesthetic depth, including reaction to toe pinch, respiratory rate, and character. Adjust the rate of isoflurane as needed to maintain an appropriate plane of anesthesia.

NOTE: Respiratory rate while under anesthesia should be between 50–100 breaths per minute. Deep, infrequent breaths are an indication that the rat is too deeply anesthetized.

5.2.2. Open sterile instrument packs and drapes and don sterile gloves. Take care to maintain sterility through the duration of the procedure. Basic sterile surgical instruments needed include, scalpel blade holder, forceps, hemostats, needle holder, scissors, and wound clip applier.

5.2.3. Pull the leg of the rat through the vent of the sterile surgical drape. Ensure that the animal is at an adequate plane of anesthesia via toe-pinch reflex.

5.2.4. Using a scalpel blade or surgical scissors, make a circumferential, cutaneous incision just proximal to the stifle (knee joint).

5.2.5. Deglove the hind limb using gauze or blunt dissection to expose the femoral artery and vein on the ventral-medial surface of the hind limb.

5.2.6. Ligate the vessels using 4-0 absorbable sutures at the level of the mid-femur and transect distally.

5.2.7. Clamp the vein distally to reduce leakage during muscle dissection.

NOTE: Circumferential musculature will be transected distal to the level of femoral artery vessel ligation and muscles elevated from the femur to the level of the coxofemoral joint.

5.2.8. Using blunt dissection, abduct the hip joint with lateral outward rotation.

5.2.9. Find the head of the femur and disarticulate it from the acetabulum. Cut any remaining tissue keeping the leg attached to the body.

5.2.10. Give a splash block to the acetabulum and sciatic nerve with approximately 6 mg/kg Ropivacaine.

5.2.11. Close the musculature over the acetabulum using a simple interrupted suture (4-0, absorbable suture).

NOTE: Additional 0.5% bupivacaine or lidocaine (0.15–0.2 mg total) may be injected in several sites along the closed muscle layer (local infiltration/splash block).

5.2.12. Oppose and close the edges of the skin using wound clips placed apart every 5–10 mm.

5.3. Recovery

5.3.1. Place the rat in a clean recovery cage with heat support using a heating pad placed under the cage.

NOTE: To prevent hyperthermia, the heating pad should not be in direct contact with the animal and should not exceed 40 °C.

5.3.2. Monitor the animal until it fully recovers and is normothermic (37.5–39 °C).

NOTE: The animal should not be left unattended until fully conscious and sternally recumbent and moving easily around the cage.

5.3.3. After fully awake, mobile, and breathing well, inject the rats with Buprenorphine (1.0–1.2 mg/kg SC).

NOTE: Giving Buprenorphine in an anesthetized rat may impair recovery.

5.3.4. Administer 10 mL of warm lactated Ringer's solution subcutaneously between the shoulder blades.

5.3.5. Move the rats back to the clean cage and reunite with conspecifics.

5.3.6. Monitor all the animals twice daily for the next month for signs of pain and distress, including piloerection, hunched posture, or inappetence or signs of incision site infection, including erythema, purulent discharge, or wound dehiscence.

NOTE: To date we have not observed clinical signs (such as infections) following post-operative recovery in any of the animals. Only one rat had a dehiscence with sutures, after which wound clips were used to close wounds with no further problems.

5.3.7. Administer buprenorphine or meloxicam to animals presenting clinical signs of pain at published doses in consultation with a laboratory animal veterinarian.

5.3.8. Administer antibiotics (i.e., cephalosporin) to animals presenting clinical signs of pain at published doses in consultation with a laboratory animal veterinarian.

NOTE: Any animals exhibiting protracted signs of pain or distress that do not improve with analgesics or animals exhibiting signs of infection that do not respond to antibiotics need to be humanely euthanized.

6. **Imaging with X-ray**

6.1. After tumor implantation, image the tibias and lungs non-invasively to detect tumor growth using X-ray with a machine designed for rodents.

6.2. Anesthetize the rats as previously done.

6.3. Take images at 3x magnification for 6 s at 25 kV.

6.4. Process the film using the X-ray processor. Radiographs can be digitally scanned as well.

7. **Necropsy procedure**

7.1. Euthanize the rats with CO_2. Confirm death via lack of heartbeat and immediately draw 3 mL blood from the heart for serum or plasma samples.

7.2. Open the thorax and abdomen for examination.

7.3. Isolate the trachea and cannulate with a (18 G) catheter. To secure the catheter in the trachea, tie a silk suture around both the trachea with the catheter.

7.4. Connect the infusion catheter to a 3 or 5 mL syringe. Infuse formalin or saline to gently inflate the lung lobes for better histology specimens. On infusion, the lungs will puff up and enable a better visualization of pulmonary metastases.

7.5. Examine, dissect, and weigh all the selected thoracic and abdominal organs (such as liver, kidneys).

7.6. Fix the organs in formalin for histopathology or freeze on dry ice, 2-methylbutane, or liquid nitrogen.

7.7. For evaluation of protein expression using western blot, make lysates of frozen tissues. Antibodies that react with rat tissues are detailed[13].

8. **Immunohistochemistry**

8.1. Process the primary OS tissue, embed in paraffin, and section it for immunohistochemical staining.

8.2. Retrieve the antigen after deparaffinization using citrate buffer (pH 6.0). Incubate in 0.3% H_2O_2 in methanol for 30 min to quench endogenous peroxidase.

8.3. Block the 5 µm thick paraffin sections using normal serum.

8.4. Incubate with primary anti-CD68 and CD3 antibodies (see table) overnight at 4 °C.

8.5. Rinse the sections in PBS and incubate them in HRP polymer using a detection kit.

NOTE: Immunostaining was developed with diaminobenzidine as a chromogen.

9. Western blotting

9.1. Lyse the UMR-106 cells in 200–300 µL of lysis buffer[14] to perform standard gel electrophoresis and western blotting.

9.2. Use 4% to 12% Bis–Tris gels.

9.3. Incubate in anti-ErbB2, anti-ErbB4, anti-EGFR, anti-ERK, β-actin or anti-mouse β2-AR primary antibody (see table) and horseradish peroxidase-linked secondary antibody.

9.4. Add the chemiluminescent substrate. Expose the membranes to X-ray film.

NOTE: β-actin levels are used as loading controls.

REPRESENTATIVE RESULTS:

Immunocompetent SD outbred rats are used for these OS studies, which offers an animal model with an intact immune system. We have used the UMR106 cell line from ATCC, developed from cells that were initially isolated from an OS from a SD rat. We implanted the

cells into SD rats, thus providing a syngeneic model for OS. UMR106 cells are implanted into the tibia of 3-week-old male and female SD rats, simulating a pediatric OS model. Moreover, the orthotopic implantation of UMR106 cells directly into the tibia metaphysis/diaphysis gives a relevant tumor microenvironment.

When implanting tumor cells, a needle must be inserted correctly through the tibial plateau **(Figure 1)** at the correct angle (parallel to the bone shaft) extending the needle tip approximately 10 mm into the central cavity of the bone. With this procedure, 95% (52/55) of rats developed tumors in tibias distal to the knee. With tibial injection experience, 100% of rats developed tumors. In a group of rats that were not amputated, the average tumor volumes in males was 504 mm^3 at 3 weeks and 1195 mm^3 at 5 weeks post-implantation. In females, tumor volumes average at 285 mm^3 at 3 weeks and 495 mm^3 at 5 weeks post-implantation.

Two cohorts of rats were compared, including those with amputation (23 rats) **(Figure 2)** and those without amputation (29 rats). Both cohorts were euthanized at 7 weeks post implantation to examine tumor metastasis to the lungs. In the amputation group (3/23) rats developed pulmonary metastases. These three rats died or were euthanized within 24 h of surgery due to post surgery complications. Two rats died from prolonged anesthesia as the surgeon was learning the method. One rat developed a dehiscence and was euthanized the following day. Lungs of these three rats were evaluated and three small metastases (>1 mm) were found histologically. The surviving 20 rats did not have lung metastases 7 weeks post-implantation. This indicated that 3 weeks post-implantation amputations are adequate to decrease the number of rats with pulmonary metastasis. In a second group of 29 rats that did not have the amputation procedure, 26/29 rats had lung metastases consistent with the previously published data[11]. We saw no pattern in the size or number of metastases in these rats. Most rats have more than 10 grossly visible 2–7 mm diameter metastases that were easily sampled during necropsy. Occasionally, rats had even larger metastases of up to 10 mm in diameter. It is important to implant UMR106 cells with a low passage number, as the studies demonstrated that the cells with 10 or higher passage number become more aggressive and metastasize as early as 2–3 weeks post-implantation. The reason for the nature is not known but the speculation is that the cells in culture could develop mutations that favor metastasis.

In addition to the amputation surgery, another refinement of the methods included the X-ray imaging for tumor surveillance or at necropsy. This method allows the researcher to confirm bone tumor invasion in rats under anesthesia. The planar radiography method can also be used on recently amputated limbs or formalin fixed limbs. The method is fast (5 min per rat) and inexpensive ($2–5/rat) compared to Computed Tomography (CT). For in vivo monitoring, it requires the rats to be anesthetized during imaging. **Figure 3** demonstrates the detailed morphology seen by X-ray imaging of two previously amputated limbs. This method illuminates the osteolytic and osteoblastic nature of these tumors. Note the disruption of normal bone cortical architecture of the tibia and fibula in both examples (white arrows). **Figure 4** illustrates the radiographic morphology of lungs with and without metastases. Imaging by X-ray can quickly reveal to the laboratory, the need for euthanasia to prevent unnecessary spontaneous deaths.

Primary and metastatic tumors to the lung are histologically similar to human OS exhibiting both osteolytic and osteoblastic tumor morphology. In the rat OS, both osteolytic and osteoblastic tumor morphology is confirmed by histopathology of amputated limb in **Figure 5** and **Figure 6**. Note that the cortical bone is absent in this example and the adjacent bone is also replaced or fortified by new woven bone (exostoses) that is oriented perpendicular to the existing shaft of the cortex. Islands of immature osteoid (amorphous extracellular material) are shown within the tumor example. Additionally, the microscopic morphology of the lung metastases, some with mineralized bone, and tumor vascular emboli are shown in **Figure 7**.

Limb amputation with OS increases survival in rats. Rats can die spontaneously due to pulmonary metastasis housed for longer than 7 weeks post implantation. The use of amputation can allow the further investigation of standard or targeted cancer therapy in this model. Lengthening the time between tumor implantation and amputation will increase the incidence of metastasis.

Doxorubicin is a chemotherapeutic agent used to treat OS in humans. In rats, doxorubicin can be given via jugular injections[13] or a catheter[15] as described here. The jugular injection does require 5–10 min per rat but assures the delivery of the dose in the exposed vein. Overall, jugular injections are much more reproducible compared to the rat tail vein injections. If doxorubicin leaks into the dermis during tail vein injections, necrosis of the tail can occur and prevent further treatments. In this study, five rats were treated with 2 mg/kg dose of doxorubicin and euthanized 48 h post injection to investigate cell death in the tumors as shown in **Figure 5A,B**.

Five control rats treated with saline were also evaluated to select antibodies that can be used to immunostain immune cells in rat OSs. Here, two antibodies were tested for immune reactivity. For immunohistochemistry studies, tumors were fixed in formalin for 48–72 h and then moved to 70% ethanol to reduce protein cross-linking that occurs in formalin. Immunohistochemistry was performed for immune cell infiltrates in primary OS tumors and immunostained for macrophages (CD68) and T cells (CD3). **Figure 8** shows two examples of immunostains of immune cell infiltrates within the tumor microenvironment.

The potential targets for therapeutic intervention were also explored. After amputation of limbs with tumors, rat OS samples were frozen for future protein isolation. In this study, we discovered that UMR106 cells express the ErbB family pathway proteins. Western blots performed on UMR106 cell protein lysates demonstrate the expression of ErbB2, EGFR, ErbB4, and other proteins that interact with these pathways (**Figure 9**).

FIGURE AND TABLE LEGENDS:

Figure 1: Tibia with tumor implantation needle inserted.

Figure 2: Tibia during amputation procedure with skin removed (**A**), exposed femoral artery and vein (**B**), with muscle elevated from the femur (**C**), and in a rat 3 weeks post amputation surgery (**D**).

Figure 3: **X-ray radiograph of right legs after amputation (ex vivo) from two rats with OS.** Note the osteolytic and osteoblastic nature of the tumor.

Figure 4: **X-ray images of the rat lungs.** (**A**) with no lung metastases. (**B**) with OS pulmonary metastasis. (**C**) correlation to gross pathology of metastases in an inflated lung.

Figure 5: (**A**) Histopathology of OS with 90% of cells showing cell death in the tibia tumor 48 h after a dose of 2 mg/kg doxorubicin. (**B**) Tumor cell death (arrow) in tibial primary OS at 48 h after a dose of 2mg/kg doxorubicin. Note the top right and left corner has viable cells. (**C**) OS invasion in cortical bone.

Figure 6: (**A**) Histopathology of OS that has replaced the bone marrow cells and infiltrated into the cortices of the tibia. Notice the accompanied reactive new bone growth as it is layered outside and perpendicular to the pre-existing cortex. (**B**) Higher power examination of OS tumor cells adjacent to an island of bone. (**C**) Higher power examination of OS cells embedded in pink to blue extracellular matrix (osteoid).

Figure 7: (**A**) Multiple pulmonary metastases in rat with tibia tumor implantation. (**B**) Tumor OS cells in an embolus in small pulmonary artery branch vessel adjacent to a bronchiole below the vessel. (**C**) Some pulmonary metastases contain islands of bone while other metastases are more cellular. (**D**) Higher power of metastases with OS cells admixed with islands of mineralized bone.

Figure 8: **Immunohistochemistry of (A) CD68 immunostaining macrophages and (B) immunostaining T cells showing CD3 positive cells in OS in the tibia.**

Figure 9: ErbB pathway proteins expressed in UMR106 OS cells from tibia tumors. Lysates from primary tumors were examined for protein expression from ErbB family signal transduction pathway, including ErbB2, EGFR, ErbB4, AKT, ERK1/2, and β2-adrenergic receptors with actin as a loading control.

DISCUSSION:

Rats with OS tibial implants develop measurable tumors by 3 weeks post-implantation. If limbs with tumors are amputated 3 weeks post-implantation, the incidence of lung metastasis is reduced significantly. OSs are both osteolytic and osteoblastic. Rats without amputation develop lung metastases that are multiple and variably sized, observed by radiography or at necropsy by 7 weeks post-implantation. EGFR, ErbB2, and ErbB4 are expressed in rat UMR106 OS, similar to human OS[16,17,18]. CD3 T cells and macrophages are easily detected in OS by immunohistochemistry methods. Jugular vein injections are preferred to tail vein for delivery of chemotherapy doxorubicin, a drug given to OS patients. The method described here is a complete coxofemoral amputation. This procedure is a refinement and could be considered to replace the tumor removing surgical method (femoral osteotomy) where the bone is cut leaving a stump for the patient[8]. The study suggests a complete limb removal to reduce the likelihood of post-surgical pain and complications.

There are a number of critical steps in this protocol. First, it is important to note the passage of tumor cells and to use lower passage of cells for the studies to keep the model consistent

from experiment to experiment. The older passage cells become more aggressive with time in culture. Second, using the needle of appropriate size and the Hamilton syringe will help in correctly injecting the cells in tibia at a very small volume of 20 µL, a volume determined as optimal and did not cause leakage. Third, the surgeon needs to initially practice disarticulation when doing necropsies on similar aged rats to learn the mechanics of the procedure. Fourth, for the success of amputation, maintain thermoregulation and limit the surgery time. An experienced surgeon can complete the amputation in 15 min.

It was observed that implantation of cells in the tibia greatly improved when a larger bore needle was used to make the initial opening followed by the insertion of a smaller bore Hamilton syringe needle. This protects the Hamilton syringe from breakage and dulling over time. Hamilton syringes can have volumes as small as 10 µL. The 1 mL tuberculin syringes would not be accurate enough for the implantation of 20 µL. The same Hamilton syringe was used for all the rats implanted on a day but were washed between the surgical procedures of each rat. Avoid autoclaving the Hamilton syringe syringes as they are prone to breakage. At the end of the procedure, wash it with saline (10 times) and then with 100% ethanol (10 times) and let it dry with plunger removed to store.

Skin and subcutaneous sutures were initially used to close the incision, yet one rat was found with dehiscence the day after the surgery. The use of wound clips and surgical glue to close the incision improved the method. With this refinement, no other rats had any such post-surgery complication. The inclusion of the radiography of the lungs by X-ray refines this model to demonstrate lung metastasis in rats allowing for euthanasia that is timely and prevents unexpected deaths. X-ray images allow us to determine the osteolytic and osteoblastic nature of these rat OSs, similar to human OSs.

A moderate level of surgical expertise is necessary to perform the amputation procedure. The most difficult step is the dissection into the musculature to locate the coxofemoral joint. Magnification and good lighting are important during this step. Surgical expertise can be achieved with practice on animals that have been euthanized. After about 10 rats, the surgeon should be confident to amputate a limb with OS from a live rat under anesthesia.

Existing methods to remove limbs with sarcomas in mice and rats are based on removing the tibia by cutting the femur bone and musculature mid-shaft and leaving the stump[8]. Although, this may be useful for some investigations, in this study, the complete leg removal was attempted. The procedure was found to be satisfactory and offered no post-surgical complications. In rats with a hind limb stump, there could be more post-surgical skin, muscle, or nerve pain. Leaving a stump, rats could reach around and access the surgery site. Rats do very well post-amputation and ambulate well in the cage with one hindlimb.

Advantages of full limb amputation include removing the primary tumor before it becomes too large and painful for the rat. Importantly, removal of the primary tumor will help control primary tumor metastasis to the lung. Rats with amputation can be further studied in order to test efficacy of novel therapeutics on circulating tumor cells in the blood or in the micrometastases in capillaries of the lungs or other bones.

There is a substantial need for development of new cancer therapeutics for OS and other

sarcomas, especially therapeutics that have drug activity against metastatic progression. Compared to the novel therapeutics developed for other cancers, therapeutics for OS have unfortunately not progressed in many decades. Responding to this problem, a meeting of key leaders and experts in OS and metastasis convened to develop guidelines for improved OS drug development[19]. As per the suggestions of the panel, studies were set out to improve the rat pre-clinical model, a lesser-known model of OS. In summary, amputation and imaging refines rat pre-clinical model for further use by the sarcoma research community. The amputation procedure will allow for improved patient survival for multiple months enabling evaluation of efficacy of novel treatments on micrometastases or dormant tumors or to test for toxicity of treatments with a model with better longevity.

In summary, we provide the advantage of this OS model. Immunocompetent SD outbred rats are used to provide a syngeneic model with implanted UMR106 OS cell line isolated from a SD rat OS. The primary and metastatic tumor is histologically similar to OS in humans. Juvenile male and female rats are used for UMR106 tumor implantation studies modeling pediatric sarcoma. Orthotopic placement of implanted cells is done directly into the tibia for a relevant tumor microenvironment. The primary tumor metastasizes to the lung and the metastases can be monitored by in vivo imaging with X-ray method. The rat OS expresses proteins in common with human OS, such as ErbB2. Compared to the dog OS, rat model allows for larger numbers of animal to be used simultaneously. Rats are 10 times larger than mice for ease of tibial injections, surgery, imaging, blood draws, and biopsy. The longevity of rats is more assured with amputation and this model can combine neoadjuvant therapy, amputation and adjuvant therapy allowing for improved patient survival enabling evaluation of efficacy of treatments on micrometastases or dormant tumors. Off target toxicity evaluation can also be assessed in this model where rats can be treated with cancer therapeutic such as doxorubicin and monitored long-term for doxorubicin induced cardiac toxicity or recurrence of OS. This would allow for the testing of cardio-protection agents in a model with OS.

ACKNOWLEDGMENTS:

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DISCLOSURES:

No disclosure to declare.

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Figure 1 .

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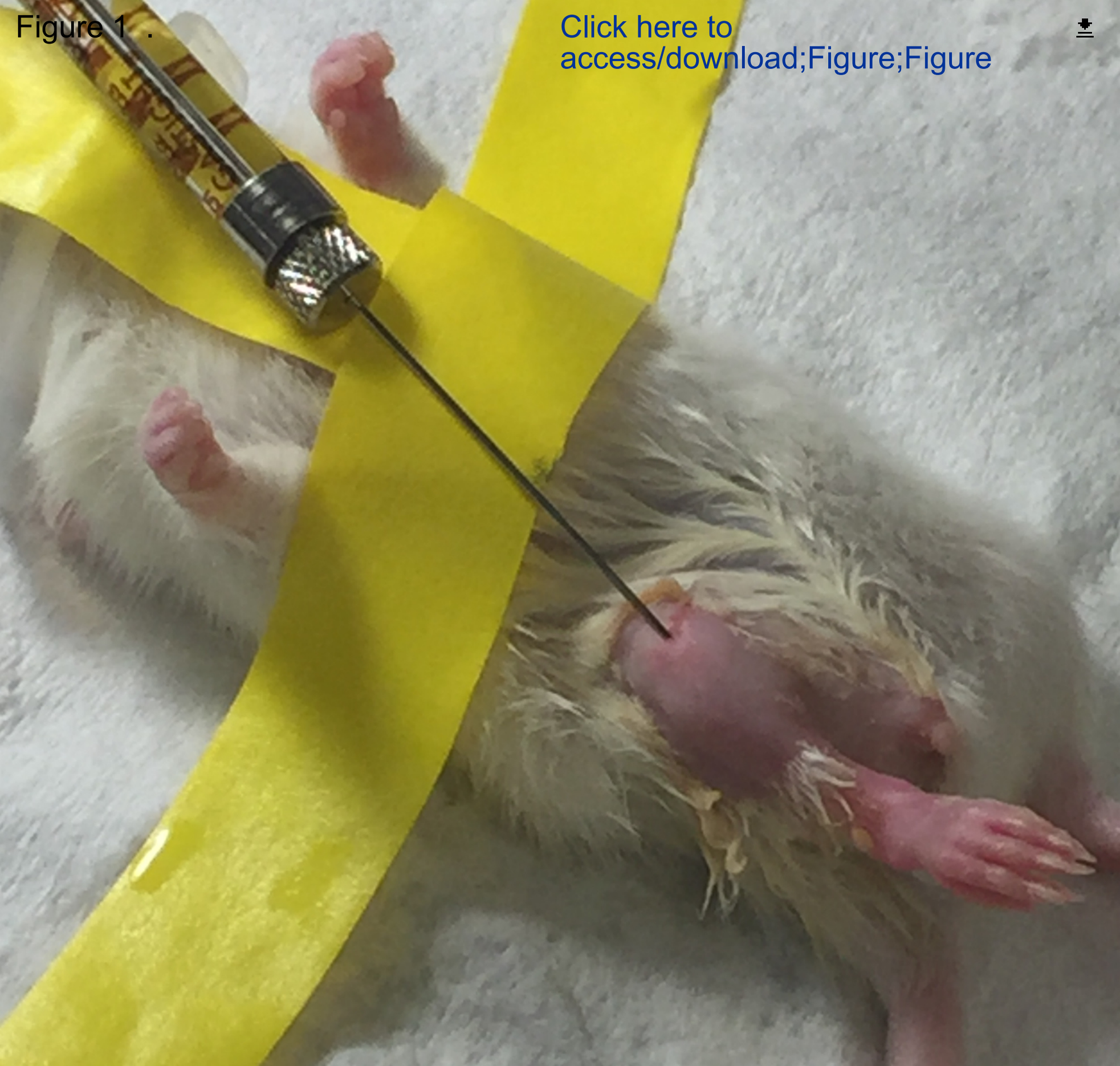

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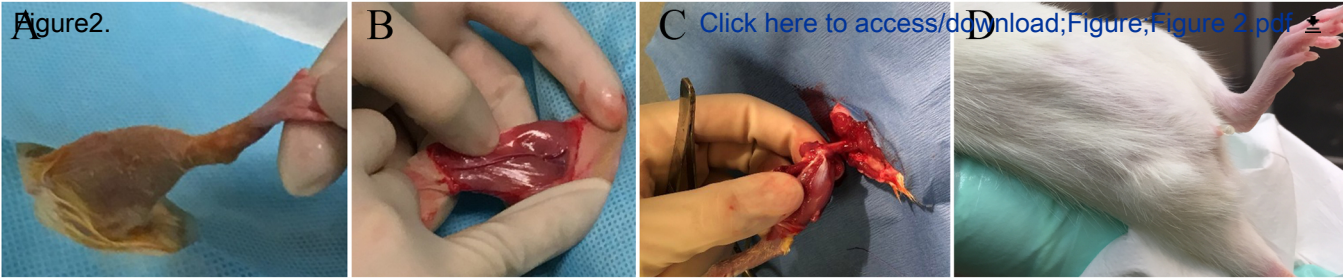

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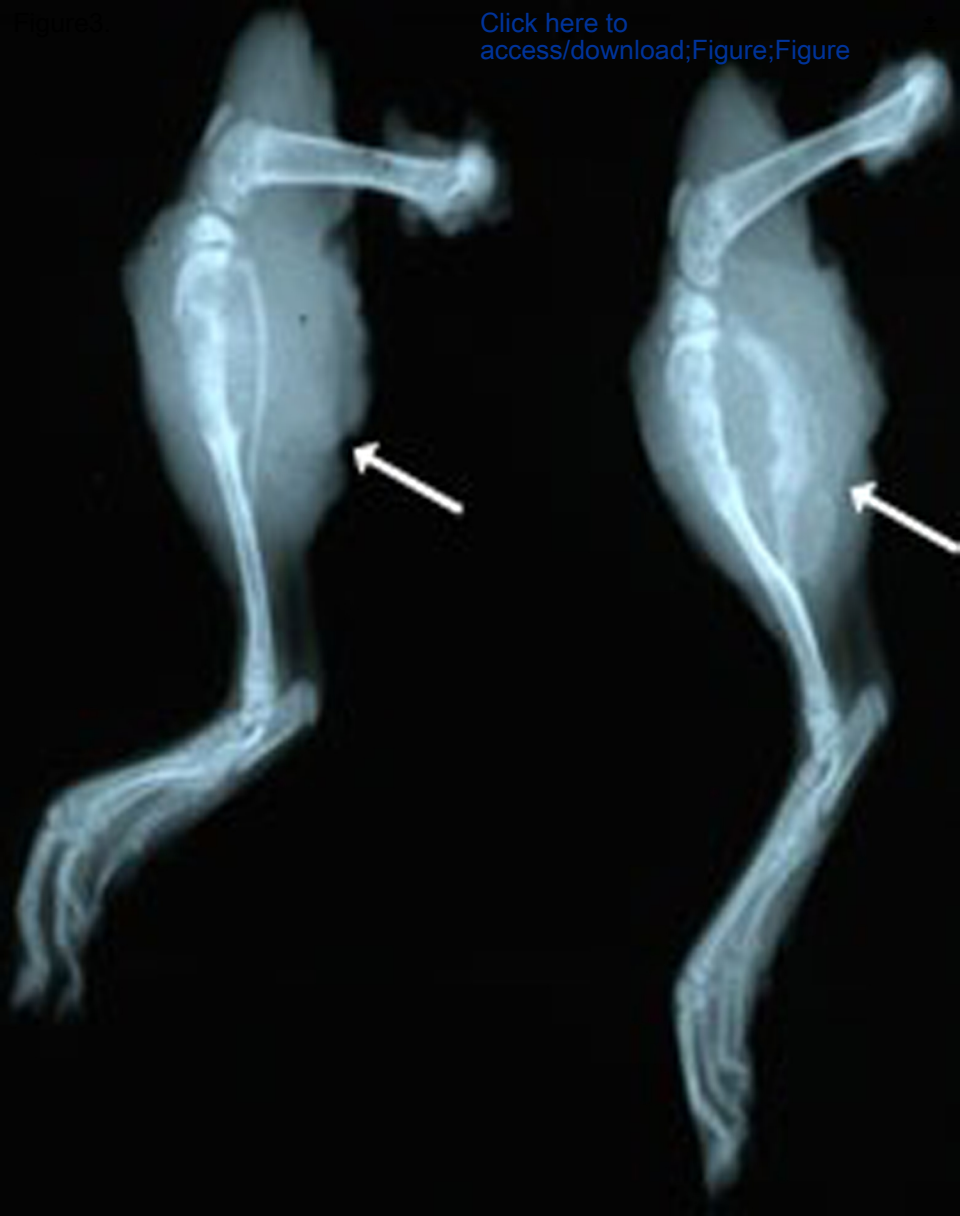

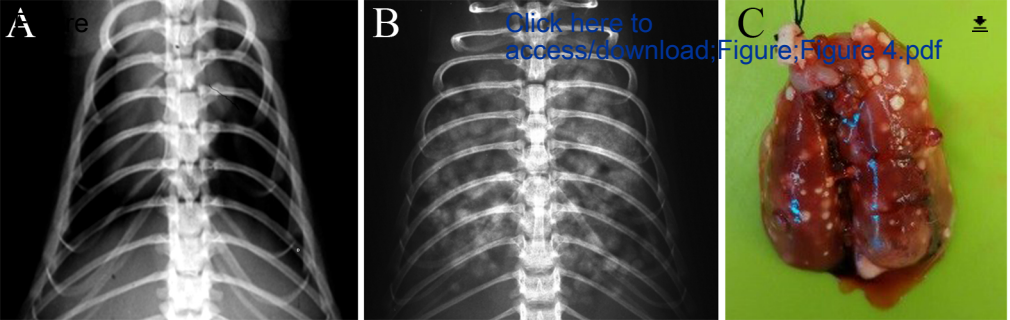
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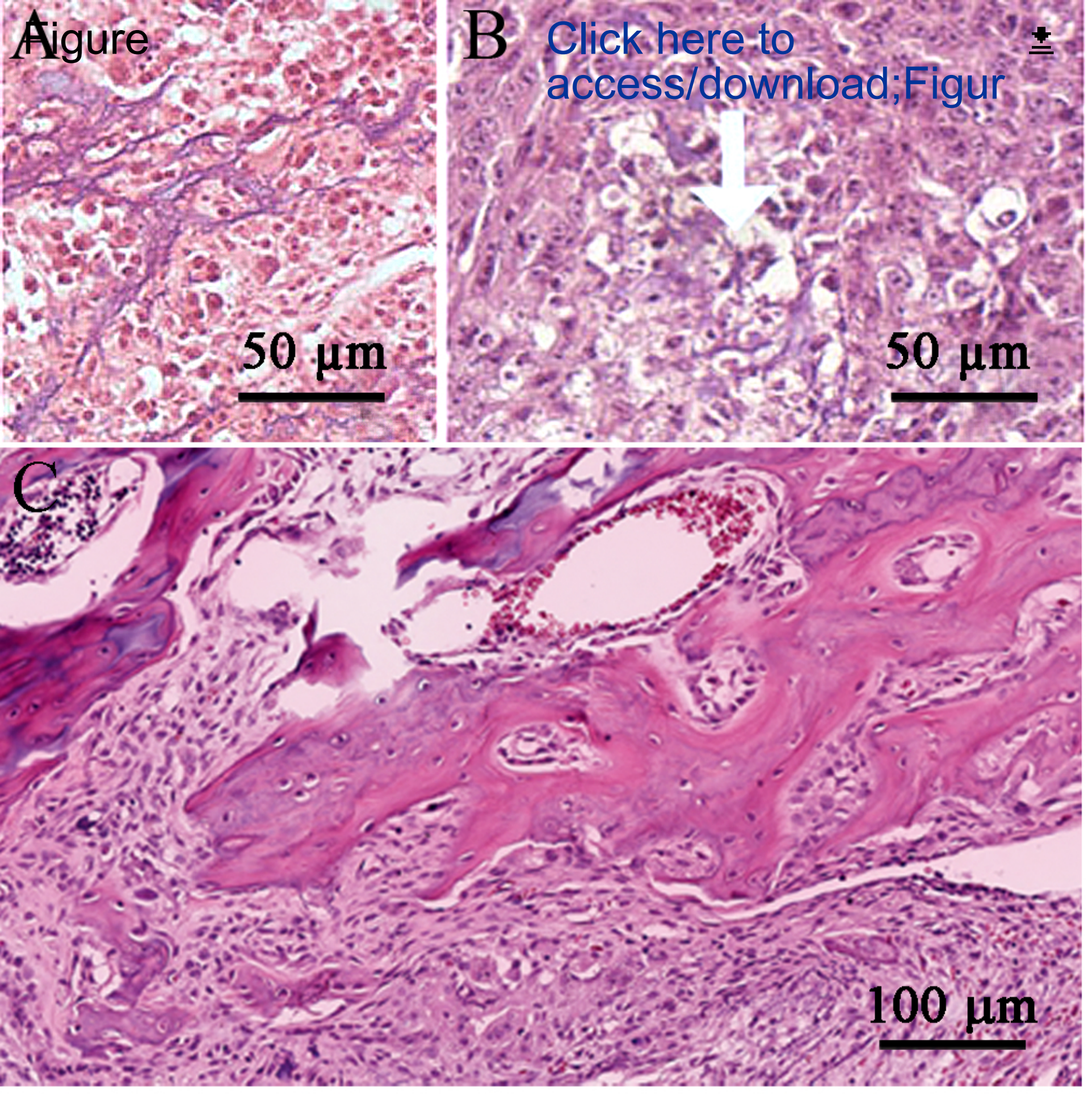
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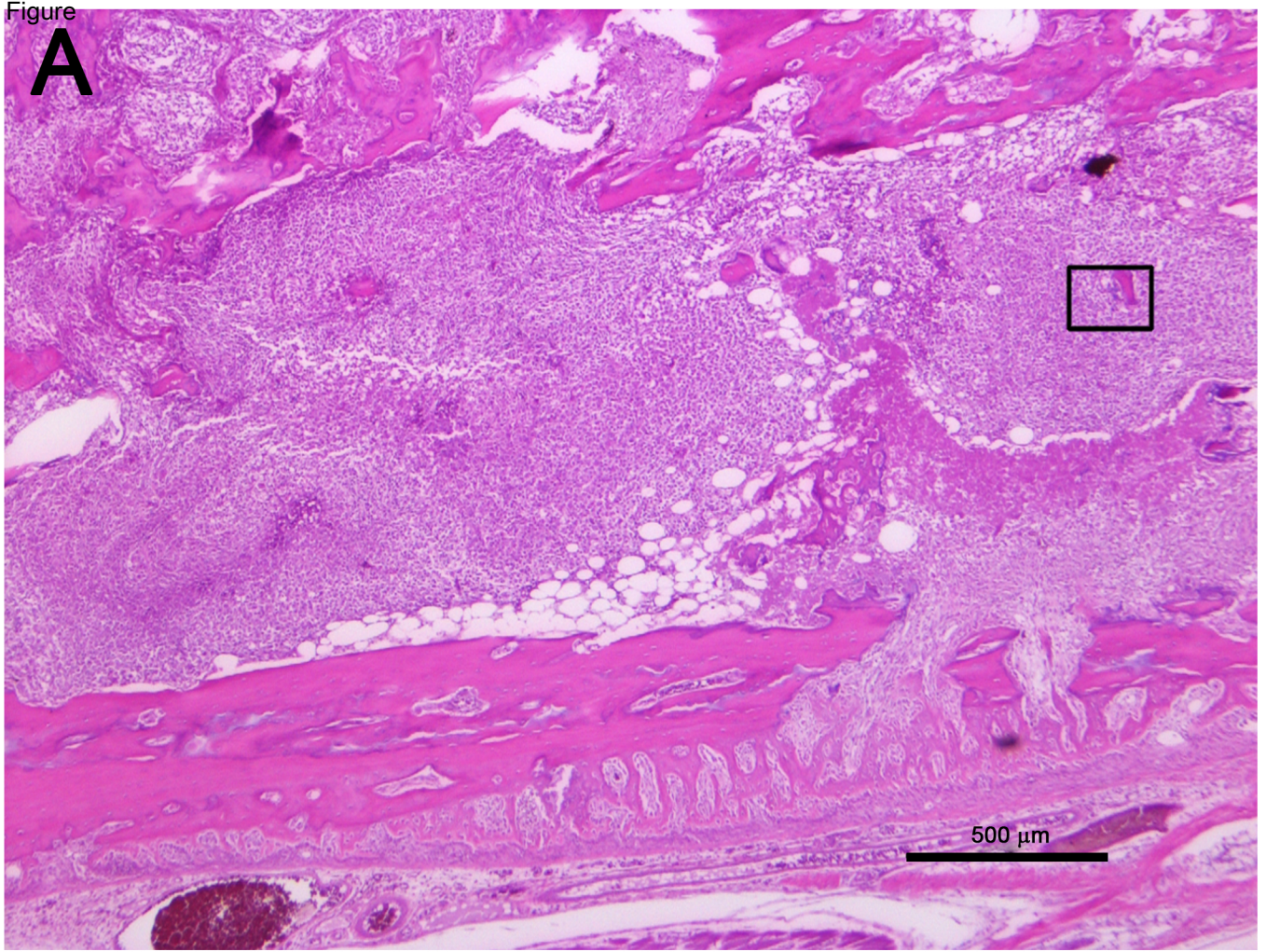

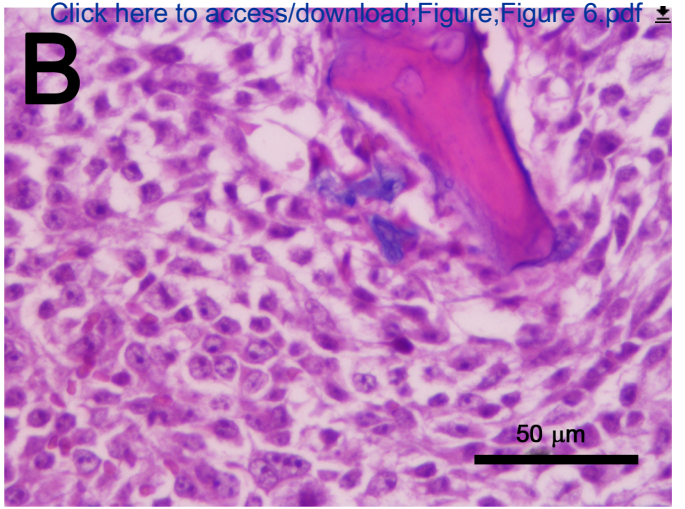

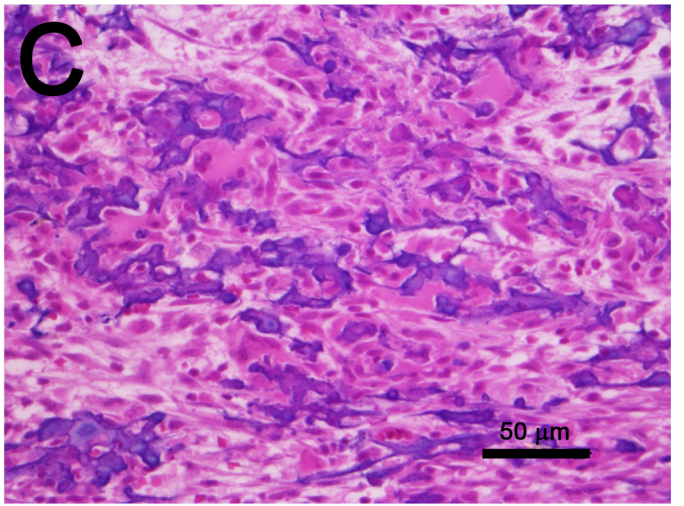

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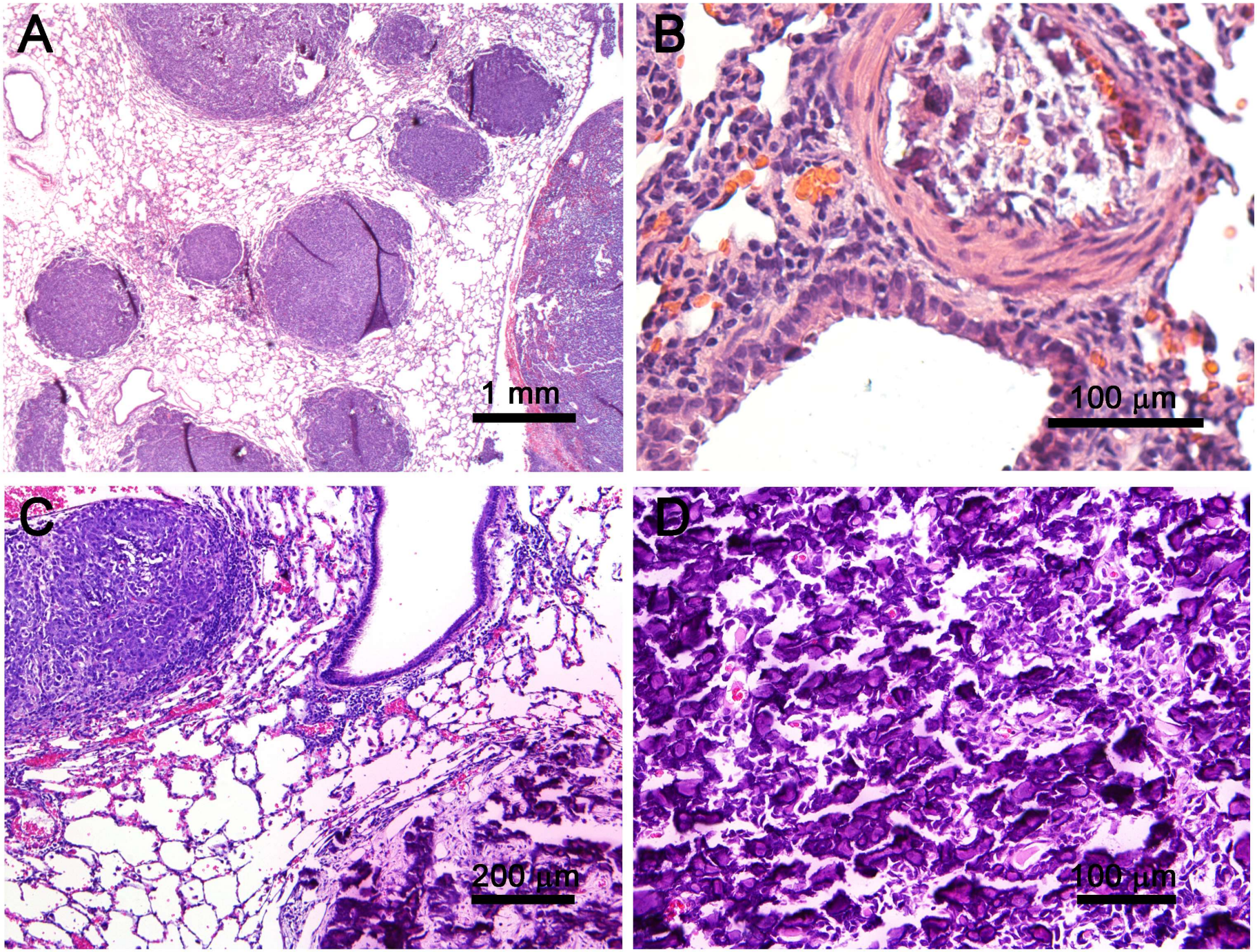

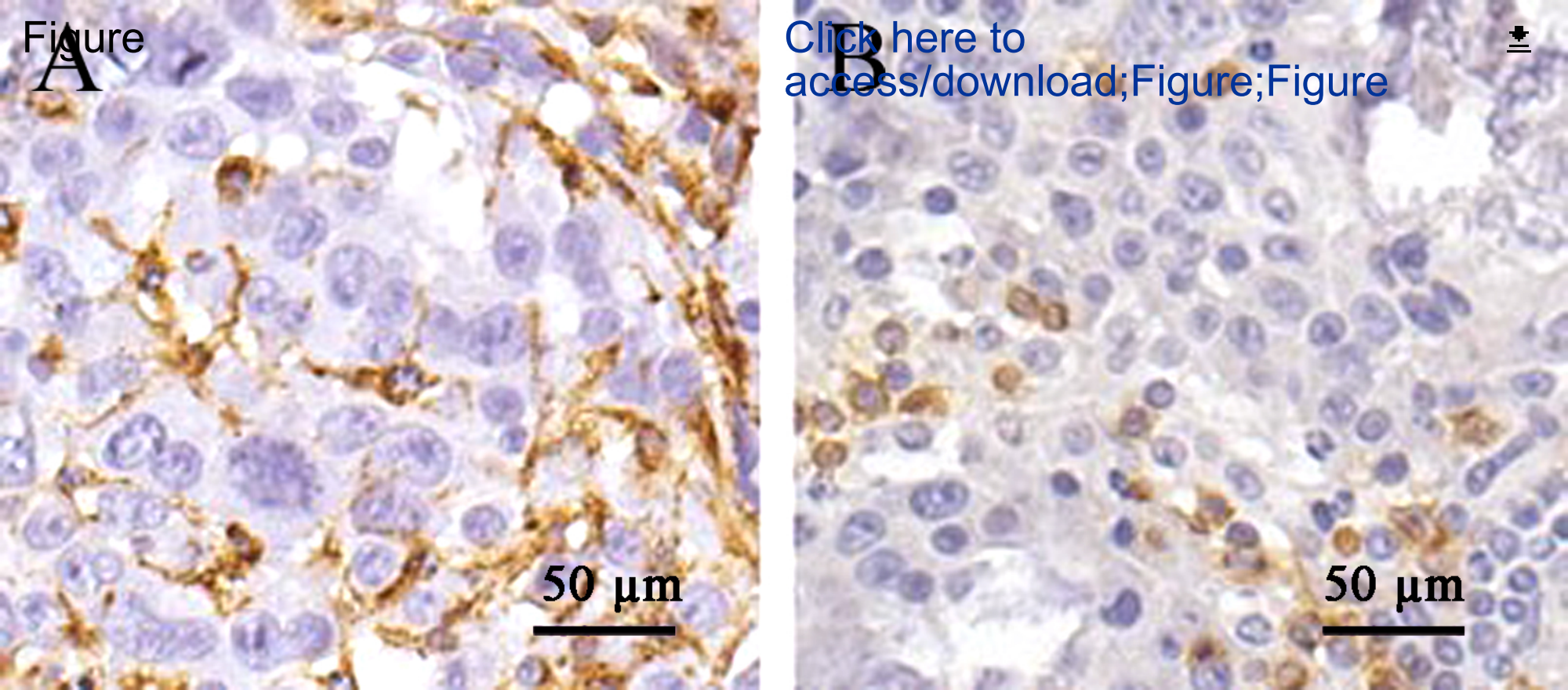
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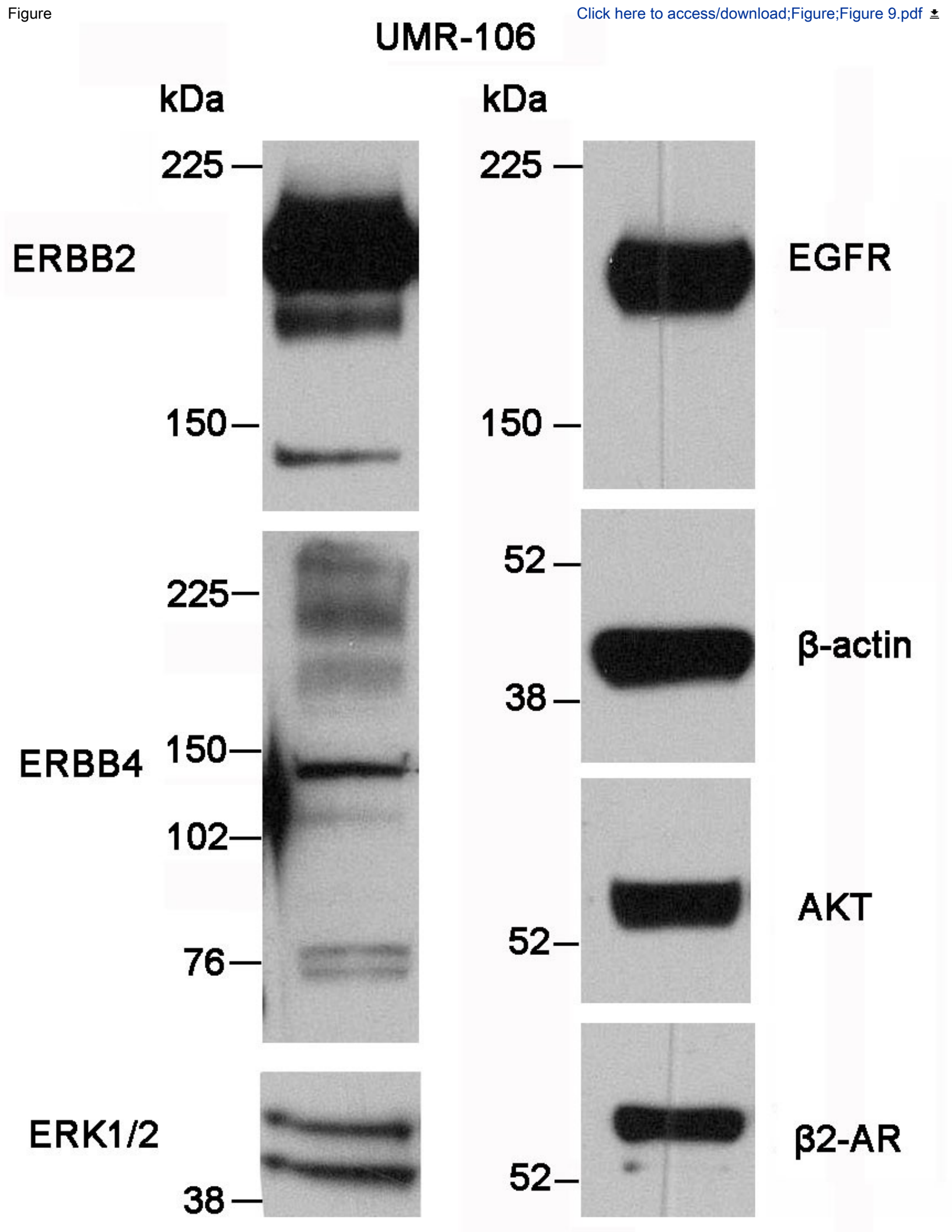

Table of Materials

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Name of Material/Equipment	Company	Catalog Number
AKT	Cell Signaling TECHNOLOGY	4685S
absorbable suture	Ethicon	J214H
β-actin	SANTA CRUZ BIOTECHNOLOGY	sc-47778
β2-AR antibody	SANTA CRUZ BIOTECHNOLOGY	sc-569
Bis–Tris gels	Thermo Fisher	NP0321PK2
Buprenorphine SR Lab	ZooPharm	IZ-70000-201908
CD3 antibody	Dako	#A0452
CD68 antibody	eBioscience	#14-0688-82
Chemiluminescent substrate	cytiva	RPN2232
CL-Xposure film	Thermo Fisher	34089
Complete Anesthesia System	EVETEQUIP	922120
diaminobenzidine	VECTOR LABORATORIES	SK-4100
Doxorubicin	Actavis	NDC 45963-733-60
EGFR antibody	SANTA CRUZ BIOTECHNOLOGY	sc-03
ERBB2 antibody	SANTA CRUZ BIOTECHNOLOGY	sc-284
ERBB4 antibody	SANTA CRUZ BIOTECHNOLOGY	sc-283
ERK antibody	SANTA CRUZ BIOTECHNOLOGY	sc-514302
eye lubricant	PHARMADERM	NDC 0462-0211-38
Hamilton syringe (100 µL)	Hamilton	Model 1710 SN SYR
horseradish peroxidase-linked secondary antibody	cytiva	NA934
HRP polymer detection kit	VECTOR LABORATORIES	MP-7401
HRP polymer detection kit	VECTOR LABORATORIES	MP-7402
isoflurane	BUTLER SCHEIN	NDC 11695-6776-2
isoflurane vaporizer	EVETEQUIP	911103
UMR-106 cell	ATCC	CRL-1661
X-ray	Faxitron	UltraFocus
X-ray processor	Hope X-Ray Peoducts Inc	MicroMax X-ray Processor
wound clips	BECTON DICKINSON	427631

Comments/Description
replaced by β2-AR (E-3): sc-271322
replaced by EGFR (A-10): sc-373746
replaced by Neu (3B5): sc-33684
replaced by ErbB4 (C-7): sc-8050
Hope Processors are not available in USA anymore

JOHNS HOPKINS
MEDICINE
SCHOOL OF MEDICINE

Department of Molecular and Comparative Pathobiology

Re: Rebuttal letter.

December 12, 2020

Dear Dr. Nguyen,

We appreciate the review of our manuscript" **A syngeneic orthotopic osteosarcoma Sprague Dawley rat model with amputation to control metastasis rate"**. The manuscript was revised based on the following reviews.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.
2. Please provide an email address for each author.
3. Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:

a) For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.

b) Discuss maintenance of sterile conditions during survival surgery.

c) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

d) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all

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commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Eppendorf; Hope Micro-Max X-ray processor; Faxitron Mx20 etc
5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
6. 1: Please cite a reference (passaging, sub-culturing, trypsinization, counting etc) for the cell culture part.
7. 2.1.1: what signs do you look for when monitoring depth of anesthesia?
8. 2.1.5: please specify the vital signs and temperature (both body and of the heating pad) so readers can replicate your protocol.
9. Please include steps (even if you cite references) stating that western blotting can be performed.
10. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.
11. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.
12. Please do not abbreviate journal names in the reference list.
13. Please include the molecular marker in the western blot (Figure 8).
We addressed all suggestions and made corrections following the paper's policy and the suggestions.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This model describes a model for osteosarcoma in the rat, which can be used for studying osteosarcoma metastasis. This is clinically highly relevant, given the devastating consequence of osteosarcoma metastases in human subjects. The fact that it is a syngeneic model is especially important for testing immunotherapy based drugs in a model system. The protocol described is very

detailed and well documented and many useful practical tips are provided.

Major Concerns:

Major concerns

1. A major hallmark of osteosarcoma is the production of osteoid. However the histological pictures in Fig 5 and 6 do not show any clear osteoid. Could a more representative picture of tumor histology be shown?

We added new histopathology photographs as suggested.

2. Could the authors provide documentation on the size of the metastases in amputated vs. non-amputated animals? Is there a marked difference?

No pattern in the size or number of metastasis was observed and this note was added to the manuscript.

3. It is intriguing that late passage UMR106 cells are more aggressive upon implantation. The authors may speculate why this is observed.

The reason for this is not known but we can only speculate that cells in culture could develop mutations that favor metastasis. This was added to the manuscript.

Minor Concerns:

The authors say that there is tumor invasion in the cortical bone in Fig 5C. But this is actually more likely to be woven bone occurring after healing of the wound made for orthotopic injection.

We agree with you and provided new histopathology photographs to show lytic areas in cortex with a woven bone reaction.

Reviewer #2:

Manuscript Summary:

The article describes a protocol for inducing the metastatic osteosarcoma model in Sprague-Dawley rats. The procedure and the results presented in the paper are convincing. The steps of the procedure are clearly explained, and most of the critical steps are highlighted. However, I have some concerns regarding some points of the procedure and did not find several important details. Please find my concerns below.

Major Concerns:

Isoflurane concentration in these experiments is unusually low. From my experience, it is hard to induce

anesthesia with 2-3% isoflurane. Also, 1.5% for maintenance during invasive surgery is quite a low concentration. No data on the gas is provided. There is also no data on scavenging systems used. It would be highly recommended to clearly state that using scavenging system is essential. The authors do not address the issue of sterile technique in surgeries. They should explain whether they observed infections and how to identify them. I did not also find any mention of the administration of antibiotics.

The procedure should define humane endpoints.

The necropsy procedure should include confirmation of animal's death.

The list of materials and equipment is very short and not very detailed; for example, isoflurane vaporizer, scavenging system, equipment for monitoring of the animals, most of the basic materials for surgeries, surgical tools, sutures etc.

The description of results is mixed with interpretations and description of future directions. I would suggest rewriting this section and including some information from this section in the Discussion.

We appreciate the suggestions and incorporated all into the revised manuscript.

Minor Concerns:

Line 59 - The authors state that they hypothesize that this model is pre-clinically relevant. However, they do not test this hypothesis. After this statement, they refer to the model's characteristics or well-known data to support this hypothesis. I would suggest modifying this sentence and stating clearly the aim of the study.

Line 61 - I would suggest to clearly state that UMR106 cells originate from SD rats.

Line 68 - "this rat model is poised" - I would suggest rewriting this sentence

Line 69 - I would suggest rewriting this sentence - this is not really a conclusion

Minor spell check is required.

Line 89 - "leading to some advantages" - please be more specific

Line 237 - please provide a route of administration

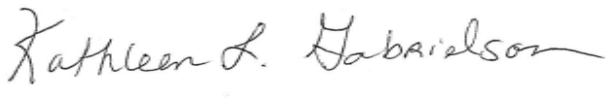
Line 252 - please refer to radiography rather than to the equipment's name (the same concerns other references to this technique). The details of the machine should instead be put in the materials table.

Line 255 - no anesthesia procedure described

We have followed each suggestion made by the reviewers and editor and our manuscript is much improved. We revised the abstract, introduction, methods, results, legends and discussion. We have also added more panels to figures with new photographs of both bone and lung cancer in response to the reviews. We also added molecular weight markers to the western blots.

We sincerely appreciate the opportunity to resubmit our manuscript.

Sincerely,



Kathleen Gabrielson, DVM, Ph.D.
Associate Professor
Molecular and Comparative Pathobiology, Pathology and Oncology