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TITLE:

Modeling Primary Bone Tumors and Bone Metastasis with Solid Tumor Graft Implantation into Bone

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SUMMARY:

Bone metastasis models do not develop metastasis uniformly or with a 100% incidence. Direct intra-osseous tumor cell injection can result in embolization of the lung. We present our technique modeling primary bone tumors and bone metastasis using solid tumor graft implantation into bone, leading to reproducible engraftment and growth.

ABSTRACT:

Primary bone tumors or bone metastasis from solid tumors result in painful osteolytic, osteoblastic, or mixed osteolytic/osteoblastic lesions. These lesions compromise bone structure, increase the risk of pathologic fracture, and leave patients with limited treatment options. Primary bone tumors metastasize to distant organs, with some types capable of spreading to other skeletal sites. However, recent evidence suggests that with many solid tumors, cancer cells that have spread to bone may be the primary source of cells that ultimately metastasize to other organ systems. Most syngeneic or xenograft mouse models of primary bone tumors involve intra-osseous (orthotopic) injection of tumor cell suspensions. Some animal models of skeletal metastasis from solid tumors also depend on direct bone injection, while others attempt to recapitulate additional steps of the bone metastatic cascade by injecting cells intravascularly or into the organ of the primary tumor. However, none of these models develop bone metastasis reliably or with an incidence of 100%. In addition, direct intra-osseous injection of tumor cells has been shown to be associated with potential tumor embolization of the lung. These embolic tumor cells engraft but do not recapitulate the metastatic cascade. We reported a mouse model of osteosarcoma in which fresh or cryopreserved tumor fragments (consisting of tumor cells plus

stroma) are implanted directly into the proximal tibia using a minimally invasive surgical technique. These animals developed reproducible engraftment, growth, and, over time, osteolysis and lung metastasis. This technique has the versatility to be used to model solid tumor bone metastasis and can readily employ grafts consisting of one or multiple cell types, genetically-modified cells, patient-derived xenografts, and/or labeled cells that can be tracked by optical or advanced imaging. Here, we demonstrate this technique, modeling primary bone tumors and bone metastasis using solid tumor graft implantation into bone.

INTRODUCTION:

Mouse models of human and animal disease are becoming increasingly popular in biomedical research. The utility of using mice in this context is that their anatomy and physiology are very similar to humans. They have a relatively short gestation period and time in post-natal life to achieve maturity, and are largely associated with a relatively low cost and ease of housing, albeit increasing costs of development or purchase are associated with greater degrees of genetic modification, immunodeficiency, and/or humanization[1]. Use of inbred strains results in a largely uniform animal population prior to study inclusion. A complete knowledge of their genome suggests a high degree of similarity to humans. Orthologous molecular targets for many disease processes have been identified in mouse genome and there is now an extensive library of mouse-specific reagents that are easily obtainable. Therefore, they provide the opportunity for relatively high-throughput analysis in a more rapid and less expensive manner when compared to larger animal models[1]. In addition, with the advent of genetic editing strategies that allows for the overexpression or deletion of certain genes either globally or in a cell type specific manner and/or constitutively or in an inducible manner, they represent a very biologically useful model system for the investigation of human and animal diseases[2].

Cancer is one field in which mouse models have great utility. Genetic mouse models of cancer rely on modulation of the expression of either oncogenes or tumor suppressor genes, alone or in combination, for cells to undergo oncogenic transformation. The injection of primary or established tumor cell lines into mice is also performed. The introduction of either cell lines or tissues from humans or other animal species, including mice, remains the most widely used model of cancer in vivo. The use of cells and tissues from dissimilar species (xenografts) in immunocompromised mice is most commonly performed[2]. However, the use of allograft tumor cells or tissues where both the host and recipient are of the same species allows for the interaction with an intact immune system when combined with the same host mouse strain in syngeneic systems[3].

Primary bone tumors or bone metastasis from solid tumors result in painful osteolytic, osteoblastic, or mixed osteolytic/osteoblastic lesions[3,4]. These tumors compromise bone structure, increasing the risk of pathologic fracture, and leave patients with limited treatment options. Primary bone tumors metastasize to distant organs, with some types capable of spreading to other skeletal sites. In breast cancer patients, bone is the most common site of first metastasis and the most frequent first site of presentation of metastatic disease[5,6]. In addition, disseminated tumor cells (DTCs) are present in the bone marrow prior to the diagnosis of, and predict the development of, metastasis in other organs[7]. Therefore, it is believed that cancer cells

present in bone are the source of cells that ultimately metastasize to other organ systems. Many mouse models of solid tumor metastasis exist that develop metastasis predominantly in the lung and lymph nodes, and depending on the tumor type and injection technique, potentially other organ systems[3]. However, mouse models of bone metastasis are lacking that dependably, reproducibly produce site specific skeletal metastasis and develop bone metastasis before even mice reach the study time point, early removal criteria from primary tumor burden or metastasis to other organs. We have reported a model of the primary bone tumor osteosarcoma that relies on the surgical implantation of a solid tumor allograft into the proximal tibia of mice[8]. Bone tumors formed in 100% of mice and 88% developed pulmonary metastasis. This incidence of metastasis exceeds what is commonly reported clinically in people (~20-50%), but is of great interest since the lung is the most common site of metastasis for osteosarcoma[9-11]. While this model is advantageous in modeling primary bone tumors, it also has great utility in modeling bone metastasis from other osteotropic solid tumors such as breast, lung, prostate, thyroid, hepatic, renal, and gastrointestinal tumors.

The rationale for the development of this model was to develop an alternative to the traditional intra-osseous injection typically into the proximal tibia or distal femur to model primary bone tumors or bone metastasis[12]. Our primary goal was to alleviate a known limitation of this technique i.e., tumor embolization of the lung. This results in the engraftment of these embolic tumor cells and "artifactual metastasis" that do not recapitulate the complete metastatic cascade from an established primary bone tumor that metastasizes to the lungs[8,13]. This would also be the situation when an established bone metastasis spreads to a distant site. In addition, this technique was, also, developed to produce a model of bone metastasis that would ensure a greater incidence of engraftment and growth of tumors in bone and at a uniform site when compared with orthotopic or intravascular injection techniques. This model has distinct advantages over these described techniques. This model involves controlled, consistent delivery of tumor cells into the bone. It, also, avoids artifactual lung metastasis following pulmonary embolization and establishes a baseline uniform study population. There is the benefit of site-specific tumors with this model without the risk of early removal criteria resulting from primary tumors or metastasis to other organs. Lastly, this model has great utility for modification, including the use of patient-derived xenografts.

The model presented has similarities to direct cell suspension injection into bone following a surgical approach followed by either injection through the cortex or delivery into the marrow cavity after making a small defect in the cortex (with or without reaming out the medullary cavity)[8,14-17]. However, the implantation of a tumor allograft makes this technique distinctly different. Therefore, the purpose of this report was to demonstrate this model of primary bone tumors and bone metastasis from solid tumors, which overcomes many limitations of previously described models. Research groups with experience in cell culture, mouse models, mouse anesthesia and surgery, and mouse anatomy are well equipped to reproduce our technique to model primary bone tumors or bone metastasis in mice.

PROTOCOL:

All described animal experiments were approved by the institutional animal care and use

committee of University of Cambridge, Cambridge, UK.

1. Preparation of cell lines

1.1. Grow cell lines in accordance with the laboratory's standard cell culture protocols for traditional cell culture or injection into mice. Standard protocols used here are growth in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin (hereafter known as complete growth medium).

NOTE: In this experiment, Abrams osteosarcoma cells are used in Balb/c *Foxn1 nu/nu* mice. For breast cancer studies, 4T1 cells in Balb/c mice and EO771 cells in C57BL/6 mice are used.

1.2. Grow cells in either vented tissue culture flasks or 6-well tissue culture plates at 37 °C in 5% CO_2.

1.3. Passage the cell line of interest and prepare the cells for injection when the cells reach a confluency commonly used with injection of these cells into mice.

2. Animals

2.1. Use Balb/c *Foxn1 nu/nu* mice at least 6-8 weeks of age for subcutaneous tumor generation to ensure that the animals are beyond the rapid growth phase and have achieved adulthood and skeletal maturity.

2.2. Use either male or female mice. Make exceptions when selecting hormone-responsive cell lines (e.g., breast cancer cells in female mice and prostate cancer cells in male mice).

2.3. For xenograft experiments, use immunodeficient athymic nude mice based on the cell line being incompatible with an intact mouse immune system under normal conditions.

2.4. For allograft experiments using murine cell lines, this is also recommended based on dissimilar mouse genetic and immune backgrounds. However, for syngeneic experiments, use animals of the same strain as the cell line of interest.

2.5. House animals at standard densities depending on the institution's husbandry policies.

3. Subcutaneous tumors

3.1. Harvest cell lines from culture by trypsinization and resuspend in sterile phosphate-buffered saline (PBS).

3.2. Assess cell viability and determine the cell density by trypan blue exclusion method. Use a hemocytometer or an automated cell counter to count the cells. A minimum cell viability of 90% is to be used for injection into mice to create subcutaneous tumors.

3.3. Adjust the cell density to inject 1-2 x 10^5 cells in a final volume of 0.1 to 0.15 mL (100 to 150 µL) of sterile PBS. Keep the cells on ice until injection.

3.4. Alternatively, pellet cells by centrifuging at 800 *x g* for 5 min. Discard the supernatant and re-suspend the pelleted cells in undiluted sterile basement membrane matrix medium to obtain 1-2 x 10^5 cells in a final volume of 0.1 to 0.15 mL (100 to 150 µL). Keep the cells on ice until further use.

3.5. Anesthetize mice to be used for subcutaneous tumor growth with isoflurane in oxygen anesthesia. Use an induction dose of 5% isoflurane in 2 L/min oxygen and a maintenance dose of 2-3% isoflurane in 2 L/min oxygen. Check for the lack of blink or pedal reflexes before proceeding further.

NOTE: Isoflurane is an inhalational anesthetic. Use isoflurane in a well-ventilated area with appropriate scavenging and free gas collection systems. Please consult with the institutional veterinary staff to develop a plan for anesthesia induction, maintenance, and monitoring, and ensure that the laboratory staff have appropriate training in anesthesia monitoring and the handling of inhalant anesthetic agents.

3.6. Remove hair from the dorsal region of the thorax or abdomen of anesthetized mice with depilating solution or with an electric clipper. Depilating solution is preferred to minimize potential trauma to the skin. Skip this step if using athymic nude mice.

3.7. Clean the prepared area with a 70% ethanol swab prior to injection of the cell suspension.

3.8. Use a 1 mL tuberculin syringe with a 27 G needle to inject cells subcutaneously over the dorsal region of the thorax or abdomen, not to be impacted by movement of the shoulder blades. Alternatively, inject the cells subcutaneously as a suspension in commercially available extracellular matrix.

NOTE: Injection in commercially available extracellular matrix will limit migration of the cell suspension in the subcutaneous space because these matrices solidify at room temperature.

3.9. Recover the mice on a heating pad in individual cages until ambulatory. Mice can then be placed in their normal cages with clean, dry bedding.

3.10. Monitor the size of the subcutaneous tumor overlying the dorsal thorax or abdomen with a caliper and measure body weights weekly to ensure that the subcutaneous tumors do not ulcerate or mice meet early removal criteria as established by the institutions' animal care and use committee. A maximum tumor size of 15 mm in any dimension is recommended to reduce the risk of skin ulceration or central tumor necrosis.

NOTE: Consult local guidelines to determine maximum permissible tumor size/volume.

3.11. Euthanize mice bearing subcutaneous tumors after three to four weeks according by CO_2 inhalation followed by cervical dislocation. Follow institution's acceptable policies for mouse euthanasia.

3.12. Harvest subcutaneous tumors using aseptic surgical technique. Sterilize the skin overlying the tumor as before with 70% ethanol after removal of the hair (if applicable). Incise through the skin overlying the tumor with a #15 scalpel blade (with or without a scalpel blade handle). Sharply dissect the tumor from the surrounding attached soft tissues with a pair of sterile surgical scissors.

3.13. Place tumor in 6-well tissue culture plates containing complete growth medium and mince into multiple small fragments of pre-determined size (~ 0.6 mm x 0.6 mm x 0.6 mm – 0.25 mm^3 up to 1 mm x 1 mm x 1 mm – 1 mm^3) with a #15 scalpel blade (with or without a scalpel blade handle).

3.14. Maintain tumor fragments in sterile complete growth medium at room temperature until the time of intratibial implantation. For cell lines that carry luciferase or fluorescent reporter genes, use ex-vivo bioluminescent or fluorescent imaging to confirm tumor viability ahead of intratibial implantation into mice.

3.15. For cryopreservation, place multiple fragments in the same cryovial in complete growth medium supplemented with 20% FBS and 10% dimethyl sulfoxide (DMSO). Freeze gradually using a commercial cryopreservation system at –80 °C and store long-term in liquid nitrogen. Preserve tumor fragments for subsequent analysis, but not for future implantation, by snap freezing using liquid nitrogen immersion. Store these frozen tumor fragments for long-term at –80 °C.

NOTE: It has been previously reported that snap frozen tumors will not engraft and grow in vivo[8].

4. Surgical implantation of subcutaneous tumor fragments

4.1. Bring fresh or cryopreserved fragments of subcutaneous tumor to room temperature in complete growth medium prior to surgical implantation.

4.2. Anesthetize mice of the strain of interest using isoflurane in oxygen anesthesia as described in Section 3. Check for the lack of pedal reflexes before proceeding. Administer subcutaneous buprenorphine at a dose of 0.02-0.05 mg/kg to provide peri-operative analgesia. This can be repeated every 6-8 h in the post-operative period, if needed.

4.3. Remove hair on the right knee joint and proximal tibia of the hindlimb with depilating solution to minimize the potential trauma to the skin.

4.4. Scrub the prepared area with surgical antiseptic. Scrub first with a 70% ethanol swab and then scrub with alternating chlorhexidine and saline scrub.

4.5. Visualize the proximal tibia as the region just distal to the knee joint while flexing and extending the joint.

4.6. Create a 3-4 mm incision at the level of the proximal tibia on the medial aspect of the limb with a #15 scalpel blade (with or without a scalpel blade handle). Incise through the skin and subcutaneous tissue to expose the medial cortex of the proximal tibia.

4.7. Apply gentle pressure with the tip of a 25 G needle, while also rotating the tip, to create a small hole in the medial cortex of the proximal tibia. Make this hole approximately 2 mm distal to the knee joint at a point equidistant between the cranial and caudal tibial cortexes. Select the needle size depending on the size of the tumor fragments.

4.8. Use sterile forceps to pick up and insert the tumor fragments into the medullary cavity of the proximal tibia. Use a 27 to 30 G needle to manipulate the tumor fragment into the medullary canal. Depending on the size of the tumor fragments, implant a minimum of 0.5 mm^3 total tumor volume into each tibia. This may require implantation of 1 or more tumor fragments depending on the size of tumor fragments created.

NOTE: Modifications to prevent or limit displacement of the graft outside of the bone would be placement of bone wax or bone cement in the bone defect or either gel foam or a subcutaneous fat graft over the hole in the bone.

4.9. Appose the skin edges with sterile liquid tissue adhesive or a single skin suture. Do not use wound clips in this site. Take caution if using fluorescence imaging in the post-operative period, since both tissue adhesives and suture have the potential to fluoresce.

4.10. Recover the mice on a heating pad in individual cages until ambulatory.

5. Serial and end point assessment

5.1. Anesthetize mice using isoflurane in oxygen anesthesia as described previously.

5.2. Evaluate tibial tumor growth non-invasively by either weekly digital radiography, bioluminescence, or fluorescence imaging (if using cells expressing luciferase or a fluorescent reporter gene). Caliper measurements of the limb at the site of implantation can also be performed in awake mice.

5.3. In addition to the traditional monitoring of tumor-bearing mice (body weight, activity level, respiratory rate, grooming, posture, mentation, and behavior) monitor mice weekly for signs of hind limb lameness, swelling, and surgical site infection.

5.4. Monitor the skin surgical wound for the first 10-14 days for excessive redness, swelling, draining, and wound dehiscence until the skin wound is healed. After 4-5 weeks, evaluate mice in accordance with the study outcome evaluation either alive or following euthanasia.

REPRESENTATIVE RESULTS:

A positive result would be associated with tumor engraftment and progressive tumor growth over time. Depending on the tumor type, intraosseous tumor growth may be associated with progressive hind limb lameness, but many tumors do not cause lameness despite signs of attendant bone disease. Successful engraftment was documented with advanced imaging, whereby there would be progressive radiographic, μCT, or μMRI changes in the proximal tibia associated with the bone phenotype of the cell line of interest (osteolytic, osteoblastic, or mixed osteolytic/osteoblastic metastasis) (**Figure 1**)[8]. In our previous report, the cortical defect created for tumor graft implantation was visible in the proximal tibia 1 week after implantation (**Figure 1A**). By week 2, there was visible osteolysis and bone remodeling adjacent to the cortical defect. From weeks 2-5, there was progressive bone destruction and the formation of new bone associated with tumor engraftment and growth (**Figure 1B-E**). For cell lines with reporter genes, bone changes would be accompanied by increases in fluorescence or bioluminescence imaging outputs over time. Acute lameness may be an indicator of impending or actual pathological bone fracture, necessitating immediate and careful radiographic evaluation of the mouse, and possibly early removal from the study. Depending on the tumor cell line being studied, bone destruction may occur rapidly, well ahead of metastasis. This is of specific importance in studies involving primary bone tumors, since mice may have to be removed from the study prior to the development of clinically-relevant metastasis, namely to the lung. In these cases, surgical amputation of the tumor-bearing limb is recommended to permit the study of minimal residual disease and subsequent metastasis when using primary bone tumors, as we have reported previously[4,8]. While complete hind limb amputation in people is not typically performed, this is standard of care in veterinary medicine, of which the similarities in the clinical and molecular characteristics of human and canine osteosarcoma are well-documented. Hind limb amputation in mice is, therefore, relevant for many animal cell lines. In addition, amputation is a viable treatment method in mice since limb-sparing procedures used in people are not achievable in small rodents such as mice. In mice bearing primary bone tumors, inappetence, progressive weight loss, general poor body condition and difficulty breathing (increased rate or effort) may signal the development of tumor metastasis to the lung and other organs. Confirmation by bioluminescent imaging, μCT, or μMRI imaging is recommended to confirm metastasis, and affected animals should be euthanized.

A negative result, most likely resulting from the lack of tumor engraftment, should be suspected if there is no evidence of progressive changes (osteolytic, osteoblastic, or mixed osteolytic/osteoblastic lesions, depending on the cell line being studied) on radiography or μCT examination of the implanted tibia. For cell lines carrying reporter genes, the lack of an increase in fluorescence or bioluminescence signaling over time by optical imaging would also support a conclusion that the tumor has failed to engraft. Lack of or poor engraftment could be associated with surgical site infection. However, this would exhibit specific clinical signs such as redness, swelling, or discharge most commonly in the early post-operative period (first 1-2 weeks after

surgery). Animals could potentially also be febrile and display a lack of activity or hunched posture in the early post-operative period due to surgical site infection. Harvesting and maintaining the allografts under sterile conditions during preparation and proper sterile preparation of the limb, sterile intraoperative technique, and complete wound closure will minimize the likelihood of a post-operative surgical wound complication resulting in infection and failure of tumor engraftment.

In our previous report[8], when including both the reported pilot and definitive studies, 16 out of 16 mice (100%) implanted with osteosarcoma fragments progressed to osteolytic bone lesions and 14 out of these 16 mice (88%) developed metastasis. All metastases were observed to the lung and diagnosed by histology as early as 3 weeks post-implantation[8]. Additional animals necropsied at 1 (n=2) and 2 (n=2) weeks after implantation did not reveal any evidence of lung metastasis.

FIGURE LEGENDS:

Figure 1: Serial radiography. Serial radiography (in order) performed weekly at (**A**) 1, (**B**) 2, (**C**) 3, (**D**) 4, and (**E**) 5 weeks after intratibial implantation of a subcutaneous tumor allograft using a primary bone tumor cell line (osteosarcoma). Over time, there was progressive bone destruction and the formation of new bone associated with tumor engraftment and growth. This figure has been modified with permission from ref[8].

DISCUSSION:

This report documents our model to create primary bone tumors or bone metastasis following the intratibial implantation of a tumor allograft. We believe that there are several critical steps in this process. A safe anesthetic plane should be established for both subcutaneous injection of the tumor cell suspension and intratibial placement of the resultant tumor fragments. There should be sterile preparation of the surgical site for both removal of the subcutaneous allograft and intratibial placement of the allograft. Tumor allograft fragments should be created uniformly for subsequent intratibial implantation. An appropriately sized defect in the proximal tibia should be created for implantation of tumor fragments. The surgical skin wound should be closed completely to reduce the risk of post-operative wound complications. The last critical step is that if extracellular matrix is used as part of the tumor cell suspension for subcutaneous injection, the use of manufacturer recommended handling instructions should be done to avoid solidification of the suspension prior to injection. Proper sterile preparation of the limb, sterile intraoperative technique, and complete wound closure will minimize the likelihood of a post-operative surgical wound complication.

Features of the implanted tumor and the surgical technique are critical for successful and reproducible results for incorporation into your study population. Initially, it should be verified that the tumor xenograft/allograft is implanted directly into the medullary cavity of the tibia, making sure that the tumor tissue is fully inside the bone. Modifications to prevent or limit displacement of the graft outside of the bone would initially be placement of bone wax or bone cement in the bone defect or either gel foam or a subcutaneous fat graft over the hole in the bone. An alternative would be to insert the tumor allograft through a hole on the lateral aspect

of the proximal tibia, created under the cranial tibial muscle. The presence of the cranial tibial muscle would then serve as a biological cover over the bone defect, limiting potential displacement of the tumor allograft. However, this is a more aggressive surgical approach with the potential for neuromuscular trauma on the lateral aspect of the proximal tibia. Care should be taken to make sure that the tumor allografts are created to be of uniform size prior to implantation. That being said, tumor allografts should not exceed 1 mm in any dimension, since larger tumor sizes in any biological system are less likely to engraft than smaller grafts when there is not a direct, immediate vascular supply provided to the graft at the time of implantation. We have reported successful results with a final implanted tumor volume of 0.5 mm^3 but anticipate combined tumor fragment volumes up to 1 mm^3 will achieve successful results. However, the maximum implantable tumor fragment volume has not been determined. As compared with cryopreserved tissue, fresh tumor xenografts and allografts are more likely to survive, although we have not observed a problem with the practice of using carefully cryopreserved tumor allografts. Snap-frozen allografts should not be implanted. In addition, dissection of the capsule and outer portion off of the subcutaneous tumor will prevent any contribution from this layer of the tumor, which is largely fibrous and vascular with a significant immune infiltrate rather than consisting largely of tumor cells proper. An alternative method to creating tumor allografts from the larger tumor mass directly with a scalpel blade is by using a small biopsy needle that creates a cylindrical tumor "core" that can then be cut to a predefined length prior to implantation. Use of a biopsy needle or core biopsy punch may be beneficial in creating uniform tumor fragments for implantation, for after two uniform dimensions (width and depth) have been created by the biopsy process, they can then be cut to an appropriate length prior to implantation. If reporter genes such as luciferase or fluorescent proteins are being used in the implanted tumor cells, evaluation of tumor fragments at the time of implantation or 1 week after implantation will indicate relative contribution from tumor cells in the implanted tumor fragments, as well as viability.

As with any procedure, there are limitations in addition to our reported benefits of this technique. Metastasis is a multi-step process which requires a cell from its primary location to successfully execute invasion into and migration through the extracellular matrix, intravasate the tumor's blood supply, survive in circulation until it arrives to its final metastatic destination, extravasate into the organ proper and then either stay in a dormant state or proliferate to create a metastatic lesion, and modification of the normal tissue architecture to become a clinically detectable metastasis[18]. Currently used mouse models of bone metastasis rely largely on either direct orthotopic injection of tumor cells into the normal site of the primary tumor, intravascular injection into the left ventricle of the heart or peripherally into the tail veins (lateral or dorsal caudal veins), or direct intra-osseous injection of the tumor cell suspension[3]. However, recent reports trying to improve bone metastasis models have advocated injection into the illiac, caudal, or femoral arteries[19-21]. All of these methods incorporate one or more steps within the bone metastatic cascade, except direct intra-osseous injection or in our case implantation of a tumor allograft, which only models end tissue modification. Therefore, this is one inherent limitation of our technique. An additional limitation is that as currently described, it requires propagating the allografts in an intermediate mouse host, which requires usage of additional mice and results in the implantation of a tumor that is not 100% pure tumor cells. The allograft will possess some

stromal contribution provided by tumors growing in a subcutaneous location. A potential alternative to minimize this subcutaneous stromal contribution would be the injection of cells subcutaneously in a biological substrate such as an extracellular matrix or scaffold. Alternatively, orthotopic injection into the primary site of the tumor, followed by tumor removal and graft preparation could be performed.

The method outlined in this report has key advantages when compared to existing methods of modeling primary bone tumors or bone metastasis following the direct intra-osseous injection of tumor cells. As discussed, we and others have observed direct embolization of the lungs and occasional immediate death following intratibial injection of a cell suspension[8,13]. This results from a pressurized injection of tumor cells into the marrow cavity, after which tumor cells enter the peripheral blood supply through bone marrow vascular sinusoids and are carried to the lung by venous return. These events are comparable mechanistically to that observed with pulmonary or venous thromboembolism associated with pressurized placement of implants into the marrow cavity associated with total joint arthroplasty in people and animals. We and others hypothesize that this embolic phenomenon to the lungs may be cell-line specific (unpublished observations). However, very detailed steps have been reported to limit the creation of these embolic artifactual metastases[12]. Initially, there should be adequate trypsinization and creation of a uniform single cell suspension for injection, which should be stored on ice to prevent cell clumping. The needle should be properly placed through the proximal tibial growth plate, after which small injection volumes (~10 µL) and resistance-free injections into the marrow cavity should be performed. Despite this, tumor cell delivery to the lungs, and also to other organs, can be readily observed by bioluminescence imaging following intra-osseous and intravascular injection techniques, suggesting that it is possible that cells generating this bioluminescent signal in the lungs and other organs could give rise to distant metastasis (unpublished observations)[3]. We have found that another benefit of this technique is that a single tumor grows in a uniform bone site within the study population, reducing animal-to-animal variability. This enables the establishment of a uniform study population and ready comparison between treatment groups. This is in contrast to bone metastasis that develop following orthotopic or intravascular injection, where it is possible for cells to metastasize to different skeletal sites and grow with different kinetics, making comparisons between study groups challenging. Tumor growth in one anatomical site following implantation of our tumor allografts also avoids the possibility of mice being removed due to metastasis to other organ systems or primary tumor burden, as can be seen with intravascular and orthotopic injection techniques. In addition, we have observed a 100% engraftment following tumor allograft implantation, which supports a uniform study population and avoids unnecessary use of additional mice to achieve appropriate sample size numbers. This also overcomes the limitation of incomplete and inaccurate injection following percutaneous injection of tumor cell suspensions into bone.

This technique has a large degree of versatility which has resulted in modifications that are currently being used in our research and has the potential for great utility for future applications. Initially, while we employ the proximal tibia as our intra-osseous implantation site, other common sites of primary bone tumors or bone metastasis can readily be used, including but not limited to the distal femur and proximal humerus. However, the surgical approach to these sites

as well as to areas such as the pelvis and spine require a gradually more extensive surgical approach when compared to the proximal tibia. In addition, sites such as the pelvis, spine, skull, radius, and ulna are not benefited by adequate bone stock for tumor implantation. For bone-invasive malignances such as with oral tumors, placement of the tumor allografts adjacent to bone or within the oropharynx may also recapitulate the progression of invasive tumors[3,22]. In vitro and in vivo experiments often employ co-culture or co-injection techniques to determine the effects of different cell types on a cell type of interest. Therefore, the potential exists for injection of two or more cell types simultaneously to create the subcutaneous tumors, which can then be implanted into bone using this technique. Genetically modified cells can be used alone or in combination with other normal or genetically modified cells to create the tumor allografts. With the growing interest in precision medicine and creating in vitro and in vivo models of human cancer, patient-derived xenograft transplantation into mice is gaining popularity. Recently, a model of breast cancer bone metastasis has been described where patient-derived xenografts implanted orthotopically metastasized to human bone discs implanted subcutaneously in nude mice[23]. From this, patient-derived xenografts could be directly implanted into bone using our model, without the need for an intermediate host for subcutaneous propagation. This would allow for the rapid evaluation of tumor kinetics and ability to evaluate multiple treatment combinations potentially from just a single patient biopsy, not requiring a large tumor mass for allograft preparation. The increasing use of organoids in cancer research could also be employed in our model[24]. This would require needle or pipette injection of these cell aggregates through the hole in the tibial cortex. We would, therefore, recommend sealing of the cortical defect (as discussed above) to limit the possibility of organoid migration outside of the bone and marrow cavity. As we have performed, cells can be labelled and tracked by optical (bioluminescence or fluorescence) or advanced (digital radiography, µCT, or µMRI) imaging allowing for the assessment of tumor growth over time. This also has the benefit of minimizing animal numbers since the same animals are imaged over time.

In summary, this report demonstrates our technique modeling primary bone tumors and bone metastasis using solid tumor graft implantation into bone.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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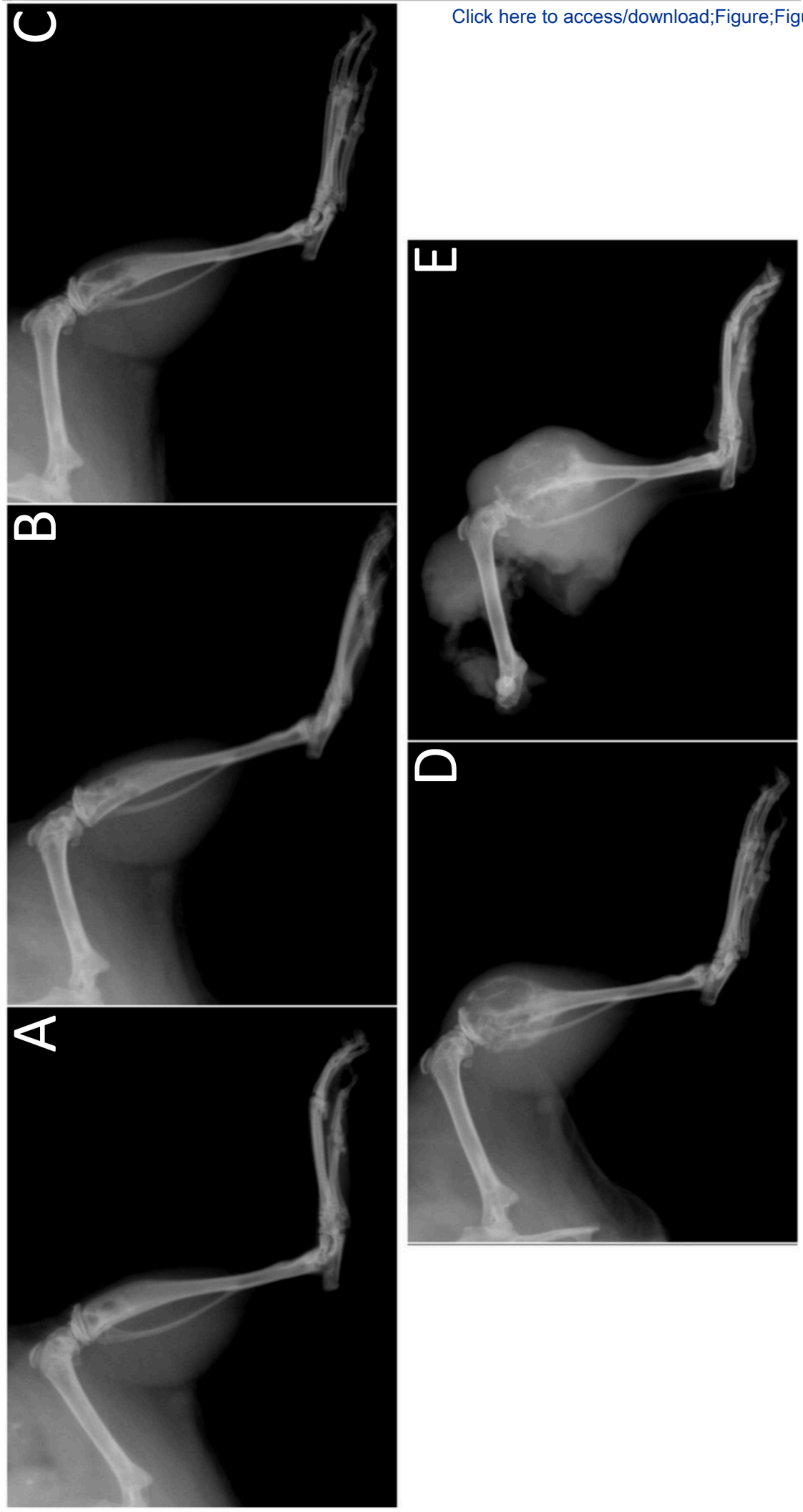

Name of Material/Equipment	Company	Catalog Number
#15 scalpel blade	Henry Schein Ltd.	75614
6-well tissue culture plates	Thermo Fisher Scientific	10578911
Abrams osteosarcoma cell line	Not applicable	applicable
Anesthesia machine with isoflurane vaporiser and oxygen tank(s)	VetEquip	901805
Animal weighing scale	Kent Scientific	SCL- 1015
BALB/c nude mouse (nu/nu)	Charles River Ltd.	NA
Bone cement	Depuy Synthes	160504
Bone wax	Ethicon	W31G
Buprenorphine	Animalcare Ltd.	N/A
Carbon dioxide euthanasia station	N/A	N/A
Cell culture incubator set at 37 °C and 5% carbon dioxide	Heraeus	Various
Chlorhexidine surgical scrub	Vetoquinol	411412
Cryovials (2 ml)	Thermo Scientific Nalgene	5000-0020
D-luciferin (Firefly), potassium salt	Perkin Elmer	122799
Digital caliper	Mitutoyo	500-181-30
Digital microradiography cabinet	Faxitron Bioptics, LLC	MX-20
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	1371171000
Dulbecco’s modified Eagle’s medium	Thermo Fisher Scientific	11965092
Ethanol (70%)	Sigma Aldrich	2483
Fetal bovine serum	Thermo Fisher Scientific	26140079
Forceps, Dumont	Fine Science Tools, Inc.	11200-33
Freezer (– 80 °C)	Sanyo	MDF-794C
Hemocytometer	Thermo Fisher Scientific	11704939
Hypodermic needles (27 gauge)	Henry Schein Ltd.	DIS55510
Ice	N/A	N/A
Iris scissors	Fine Science Tools, Inc.	14084-08
Isoflurane	Henry Schein Ltd.	1182098

IVIS Lumina III bioluminescence/fluorescence imaging system	Perkin Elmer	CLS136334
L-glutamine	Thermofisher scientifc	25030081
Liquid nitrogen	British Oxygen Corporation	NA
Liquid nitrogen dewar, 5 litres	Thermo Fisher Scientific	TY509X1
Matrigel® Matrix GFR, LDEV-Free, 5 ml	Corning Life Sciences	356230
Microcentrifuge	Thermo Fisher Scientific	75002549
Mr. Frosty freezing containiner	Fisher Scientific	10110051
NAIR Hair remover lotion/oil	Thermo Fisher Scientific	NC0132811
Penicillin/streptomycin	Sigma-Aldrich	P4333
Scalpel handle, #7 Short	Fine Science Tools, Inc.	10007-12
Small animal heated pad	VetTech	HE006
Stereomicroscope	GT Vision Ltd.	H600BV1
Sterile phosphate-buffered saline (PBS)	Thermo Fisher Scientific	10010023
Tissue adhesive (sterile)	3M Corporation	84-1469SB
Trypan blue	Thermo Fisher Scientific	5250061
Trypsin-EDTA	Thermo Fisher Scientific	25300054
Tuberculin syringe (1 ml with 0.1 ml gradations)	Becton Dickinson	309659
Vented tissue culture flasks, T-75	Corning Life Sciences	CLS3290

Comments/Description
None
Used for mincing tumor pieces. Can also be used for cell culture
None
None
None
6-8 weeks of age. Male or female mice
Optional use instead of bone wax
Optional
Buprecare 0.3 mg/ml Solution for Injection for Dogs and Cats
Should be provided within animal facility
None
None
Optional if cryopreserving tumor fragments
Optional if cell line of interest has a bioluminescent reporter gene
Can be manual
Optional to evaluate bone response to tumor growth
Optional if cryopreserving tumor fragments
None
None
None
None
Optional if cryopreserving or snap freezing tumor fragments
Can also use automated cell counter, if available
May also use 25G (DIS55509) and 30G (Catalog DIS599) needles
Ideally small pieces in a container for syringe and cell suspension storage
None
None

Optional if cell line of interest has bioluminescent or fluorescent reporter genes
None

Optional if cryopreserving or snap freezing tumor fragments
Optional if cryopreserving tumor fragments
Optional. Also available in 10 ml size (354230)
Pellet cells at 1200 rpm for 5-6 minutes
Optional if cryopreserving tumor fragments
Can alternatively use an electric clipper with fine blade
None
User preference as long as it accepts #15 scalpel blade
None
None
Use for injections and also as part of the surgical scrub, alternating with chlorhexidine
Can alternatively use non-absorbable skin suture (6-0 size)
None
Use 0.05%-0.25%
Slip tip preferred over Luer
Can also use smaller or larger flasks, as needed

Dr. Bajaj,

The authors would like to thank the reviewers for their further comments and suggestions, in addition to you for your editorial comments.

Initially, all of your editorial comments/recommended changes have been made, namely the highlighting the 2.75 pages of text for video scripting in green in the revised manuscript.

All of the reviewer comments/edits are addressed below and can be observed in the revised manuscript as being highlighted in yellow text.

Editorial Comments:

Comment 1: The editor has formatted the manuscript to match the journal's style. Please retain and use the attached version for revision.

We have used the attached version for this current revision.

Comment 2: Please address all the specific comments marked in the manuscript.

This has been performed.

Comment 3: Once done please ensure that the highlight is no more than 2.75 pages including headings and spacings.

This has been performed.

Comment 4: Please proofread the manuscript well.

This has been performed.

Comment 5: Please check with your funding source regarding PMC deposition. We do not deposit articles into PubMed Central on behalf of the authors. However, authors can self-deposit into PMC if required by their funding source.

We will self-deposit in PMC after publication in JoVE

Reviewer #1:

Comment 1: The authors addressed most of the issues.

We thank you for your comments that strengthened the manuscript.

Comment 2: Lack of procedure video is still an issue.

The video will be made following acceptance of the JoVE manuscript.

Reviewer #2:

Comment 1: Please proofread the MS, especially the newly added material. I noted on line 352 first word, "or" should be "off". Please double check the rest of the MS for errors also.

We have made this correction to “of” and thoroughly proofread the manuscript.

Comment 2: In the protocol itself, I saw no mention of using bone cement or something to ensure allograft fragments remain in the tibia. This was listed in the discussion, but one would think something is needed to ensure the tumor fragments do not pop out.

This is a great point to incorporate earlier in the “Protocol” section, of which we have now included “Modifications to prevent or limit displacement of the graft outside of the bone would initially be placement of bone wax or bone cement in the bone defect or either gel foam or a subcutaneous fat graft over the hole in the bone.”

Comment 3: Can this procedure also be used in the femur? Would there be any reason why it could not? There was no mention of this in the discussion.

We had mentioned in the second to last paragraph of the Discussion “Initially, while we employ the proximal tibia as our intra-osseous implantation site, other common sites of primary bone tumors or bone metastasis can readily be used, including but not limited to the distal femur and proximal humerus. However, the surgical approach to these sites as well as to areas such as the pelvis and spine require a gradually more extensive surgical approach when compared to the proximal tibia. In addition, sites such as the pelvis, spine, skull, radius, and ulna are not benefited by adequate bone stock for tumor implantation. For bone-invasive malignances such as with oral tumors, placement of the tumor allografts adjacent to bone or within the oropharynx may also recapitulate the progression of invasive tumors.[3,16]”

No additional changes were made.

Comment 4: This question was not addressed from my first review - would the authors please provide a "take-rate" on their fragment implants, i.e. out of how many mice were implanted with the fragment method described here, how many progressed to a) tumor growth in the tibia, and b) metastasis (metastases) to other organs (list which organs)? Please provide specific numbers (i.e. 8 out of 8 mice, 3 out of 8 mice, etc.). If osteosarcoma tumor fragments were used, state something like '8 out of 8 mice implanted with osteosarcoma fragments progressed to bone lesions, and 4 out of those 8 mice progressed to metastasis with 2 of the 4 exhibiting lung and 3 of the 4 exhibiting brain metastases'. This can also be listed in a table.

We have added the following to the Representative Results section of the manuscript. This can also be seen in the Introduction section of the manuscript.

“In our previous report, when including both the reported pilot and definitive studies, 16 out of 16 mice (100%) implanted with osteosarcoma fragments progressed to osteolytic bone lesions and 14 out of these 16 mice (88%) developed metastasis. All metastasis were to the lung and diagnosed by histology as early as 3 weeks post-implantation. Additional animals necropsied at 1 (n=2) and 2 (n=2) weeks after implantation did not reveal any evidence of lung metastasis.”

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