# Original Article Intra-arterial injection to create bone metastasis of prostate cancer in mice

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**Abstract:** Human prostate cancer often metastasizes to the bone, but the mechanisms are not quite clear. The difficulties in studying the biology of bone metastasis are due to lack of animal models with high frequency of bone metastases. In the present study, we tested two intra-arterial injection methods, i.e., intra-caudal artery injection and intra-femoral artery injection. Mouse prostate cancer cell line MPC3-luc was injected into C57BL/6J male mice via intra-caudal artery injection (n = 8) or intra-femoral artery injection (n = 11). We found one mouse developed metastatic tumors in both hind limbs and the tail after intra-caudal artery injection. Two mice developed metastatic tumors in the hind limb after intra-femoral artery injection. Our study finds that intra-arterial (either caudal or femoral artery) injection may be a useful model in studying prostate cancer bone metastasis, however, the injection technique is difficult.

Keywords: Prostate cancer, bone metastasis, animal model, intra-femoral artery injection, intra-caudal artery injection

#### Introduction

The American Cancer Society estimates that there will be approximately 191,930 new cases and 33,330 deaths due to prostate cancer in 2020 [1]. The data showcase prostate cancer as the most common malignancy and the second most common cause of cancer-related deaths in American men. Distant metastases have been found in 35% of prostate cancer patients at autopsies, with the most frequent metastatic sites at the bone (90%), lungs (46%), liver (25%), pleura (21%), and adrenal glands (13%) [2]. Metastatic prostate cancers are usually treated with androgen deprivation therapy (ADT) and they initially respond well, however, almost all of them eventually become insensitive to ADT, thus becoming metastatic castration-resistant prostate cancers (mCRPCs). Therapies against mCRPCs are quite limited [3], hence mCRPCs are often lethal. It is not clear why prostate cancer often metastasizes to the bone in humans. Almost all animal models of spontaneous prostate cancer do not show frequent bone metastases [4], even when two or three tumor suppressor genes are deleted in the mice [5, 6]. Bone metastases have been found in 2 of 10 PBCre4: Pten<sup>f/f</sup>: Rb1<sup>f/f</sup> (double knockout) mice and one of four PBCre4: Pten<sup>f/f</sup>: Rb1<sup>f/f</sup>: Trp53<sup>f/f</sup> (triple knockout) mice [7]. Given the low frequency of spontaneous bone metastases in rodent models, xenograft approaches have been used to mimic part of bone metastasis. Tail vein injection leads to mostly lung metastases [8]. Intracardiac injection may result in bone metastases, but the frequency is still low and depends on the bone-tropism of the cancer cell lines [9, 10]. Intratibial injection is

commonly used to establish bone tumors, however, it only represents tumor growth in the bone, which has very limited usage in studying the biology of bone metastasis [4, 11]. Intrailiac artery (IIA) injection has been successfully used to establish breast tumors in the hind limb bones, sparing the lungs, brain, and other soft tissue organs that are commonly involved by intracardiac injection [12, 13]. Recently, a murine model of bone metastasis by injecting cancer cells through caudal arteries has been reported [14]. These new methods of intra-arterial injection appear promising for the studies of prostate cancer bone metastasis. The present study intended to repeat the published procedures to understand the technical challenges.

## Materials and methods

### Animals

Animal protocol was approved by the Animal Care and Use Committee of Tulane University, which was in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. A total of 19 male mice (20 to 39-week old) of C57BL/6J genetic background (Jackson Laboratory, Bar Harbor, ME) were used.

## Cell culture

MPC3 cell line was originally established in the lab of Dr. Zhenbang Chen (Meharry Medical College, Nashville, TN) from  $Pten^{-/-}$ ;  $p53^{-/-}$  double knockout mouse prostate cancer and was transfected with a firefly luciferase construct to establish MPC3-luc cell line that stably expresses luciferase protein [15]. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Genesee Scientific) supplemented with 5% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), 1% penicillin streptomycin cocktail (Mediatech, Inc., Manassas, VA), and 200 µg/ml hygromycin (Sigma), in a 37°C humidified incubator with 5% CO<sub>2</sub>.

## Intra-arterial injection of MPC3-luc cells

MPC3-luc cells were grown to near confluence on the day of surgery and harvested in phosphate-buffered saline (PBS). Mice were initially anesthetized with 3% isoflurane at an induction chamber flow rate of 0.8 L/min. Mice were then positioned supine on the surgical table with maintenance of 2-2.5% isoflurane through a nosecone. Mice received buprenorphine at a dose of 0.1 mg/kg body weight via subcutaneous injection immediately before and after surgery. Intra-caudal artery injection was done according to the reported procedure [14] with minor modifications. The proximal tail was sterilized with 70% ethanol and then betadine solution. Because it was difficult to see the caudal artery through the dark skin of C57BL/6J mice, a 0.5-1 cm longitudinal incision was made at the proximal tail with a scalpel. Then, a 3-mm transverse incision was made at the two ends of the incision. The skin flap was bluntly elevated to expose the caudal artery. 100 µl MPC3luc cell suspension (containing 2.9×10<sup>5</sup> cells) was injected into the caudal artery within 10 seconds using a 31 G needle (instead of 29 G needle [14]) attached to a 1cc Tuberculin syringe. A 10× Leica S9D Stereomicroscope (Leica Microsystems Inc., Buffalo Grove, IL) was used to visualize the caudal artery during injection. After withdrawing the needle, the injection site was pressed for 2 minutes to stop bleeding. The skin flap was sutured with 5/0 chromic gut suture (CP Medical, Norcross, GA, USA). Initially, we tried to do intra-iliac artery injection according to the previous reports [12, 13]. We found that the intestines interfered with exposure of the iliac artery, thus we turned to intrafemoral artery injection without opening the abdominal cavity. Mice were anesthetized and laid down in supine position. The right inguinal region was shaved and disinfected with 70% ethanol and betadine solution. A 1-cm incision was made below and along the inguinal ligament. The femoral artery was exposed through blunt dissection of the subcutaneous tissue. A 10× Leica S9D Stereomicroscope was used to visualize the femoral artery. 100 µl MPC3-luc cell suspension (containing 1×10<sup>6</sup> cells) was injected into the femoral artery within 5 seconds using a 31 G needle attached to a 1cc Tuberculin syringe. After withdrawing the needle, the injection site was pressed for 5-10 minutes to stop bleeding. The skin was sutured with 5/0 chromic gut suture. After surgery, the animals were observed daily for wound healing and activities.

## In vivo bioluminescent imaging

Starting 7 days post-surgery, tumor growth was assessed using IVIS Lumina XRMS *In Vivo* 



**Figure 1.** Intra-caudal artery injection resulted in metastatic tumors in the hind limbs and tail. A. Illustration of intra-caudal artery injection. B. Bioluminescent imaging of a crippled mouse (insert) at 18 days after injection; arrows indicate the tumors and positive imaging signals. C. X-ray showed bone erosions in the femurs and tail bone (arrows) of the mouse with tumors (left mouse); another mouse without tumors was used as a control to show normal bone images (right mouse). D. Necropsy revealed the tumors formed in both thighs and the tail (arrows).

Imaging System (PerkinElmer, Waltham, MA) as described previously [15]. D-luciferin potassium salt (catalogue #MB000102-R70170, Syd Labs Inc., Natick, MA) was resuspended in PBS to a final concentration of 20 mg/ml. Mice were dosed with 10  $\mu$ L/g luciferin substrate intraperitoneally and waited for 10 minutes to allow for distribution of substrate prior to image acquisition. Animals were anaesthetized with 3% isoflurane with a 1 L/min induction chamber flow rate and maintained at 2% isoflurane at a 0.5 L/min imaging chamber flow rate. Bioluminescent imaging parameters were prioritized as exposure time = 3 minutes, binning = medium, and *f* stop = 1. X-ray was taken immediately after bioluminescent imaging. After completion of imaging, mice were replaced in the cage in a sternal position and observed for 10-15 minutes until becoming conscious. Endpoint was determined in consulting with a veterinarian, including the following criteria: palpable tumor > 1.5 cm in diameter, inability to eat or drink, inability to ambulate (i.e. when animal failed to move when approached), loss of 15% body weight during the course of weekly weighing, or loss of bioluminescent signals on imaging or no signals for 4 weeks.

#### Histology

Mouse tissues with tumors were isolated subsequent to euthanasia and fixed overnight in 4% paraformaldehyde at 4°C and kept in 70% ethanol. Since bone was present in the tissues, the samples were demineralized for two weeks at room temperature on an orbital shaker in the Immunocal reagent (Decal Chemical Corp., Tallman, NY). Once samples were demineralized, they were serially dehydrated in increasing percentages of ethanol (75%, 85%, 95%, and 100%)

for approximately one hour at each percentage. Tissue samples were then cleared in 100% xylene for 3-5 minutes. Samples were then incubated in a 50/50 mix of xylene and paraffin wax for 10 minutes at 60°C and embedded in 100% paraffin wax for 30 minutes at 60°C and left to cool at room temperature overnight. Three-µm tissue sections were cut and processed through two washes in 100% xylene and then rehydrated in decreasing concentrations of ethanol (100% twice, 95% twice, 85% once, and 75% once) for 5 minutes each. Sections were washed extensively in tap water before staining with hematoxylin and eosin.



**Figure 2.** Histology of metastatic tumors. A. Left leg showing tumor tissues invading the tibial plateau; of note, there is a tumor mass above the patella on the left upper corner; green arrows indicate the broken bone edges; yellow arrow indicates the tumor tissue. B. Right leg showing tumor tissues invading the femoral condyle; there is a tumor mass above the patella on the right upper corner; green arrows indicate the broken bone edges; yellow arrow indicates the tumor tissue. C. The tail showing tumor tissues invading the tail bone; green arrow indicates the broken tail bone; yellow arrows indicate the tumor tissues. D. Normal knee from a mouse without metastasis showing normal structures including femoral condyle and tibial plateau.

#### Results

Eight mice were used for intra-caudal artery injection (Figure 1A). Only one mouse was found crippled at 18 days after injection (Figure **1B**) and was euthanized. Prior to euthanasia, bioluminescent imaging showed signals in both hind limbs and the tail (Figure 1B). X-ray showed bone erosions in both femurs and the tail bone (Figure 1C). Necropsy revealed tumors in both thighs and the tail (Figure 1D). Histological examination showed that the tumors invaded both knee joints with erosions in the tibia (Figure 2A) and the femur (Figure 2B). The tumor in the tail also invaded the tail bone (Figure 2C). The other seven mice did not show any abnormal activities or positive imaging signals for 4 weeks and were euthanized. Necropsies revealed no tumors and histological examination showed normal tissues (**Figure 2D**).

Eleven mice were used for intra-femoral artery injection (Figure 3A). One mouse presented positive imaging signals in the right thigh at 14 days after injection, which continued to 21 days (Figure 3B) with a palpable tumor mass. X-ray showed a dense mass in the right thigh (Figure 3C), which was confirmed by necropsy (Figure 3D) after euthanasia. Histological examination showed tumor tissues adjacent to the femur (Figure 4A and 4B), with thickening of the cortical bone (Figure 4A) and without obvious bone erosions. Another mouse presented positive imaging signals at 14 days after injection, which increased from 21 days to 28 days



**Figure 3.** Intra-femoral artery injection resulted in metastatic tumors in the hind limb. A. Illustration of intra-femoral artery injection. B. Bioluminescent imaging of a mouse at 21 days after injection; arrow indicates positive imaging signals. C. X-ray showed a dense mass in the right thigh. D. Necropsy revealed a tumor formed in the right thigh.

(Figure 5A). X-ray showed suspicious bone erosion in the right femur (Figure 5B). Necropsy revealed a tumor in the right thigh (Figure 5C and 5D). Histological examination showed a tumor mass near the eroded bone (Figure 6A-D). The other nine mice did not show any abnormal activities or positive imaging signals for 4 weeks and were euthanized. Necropsies revealed no tumors and histological examination showed normal tissues (data not shown).

#### Discussion

Although human prostate cancer frequently metastasizes to the bone, the mechanisms are still not quite clear [16]. Lack of any animal

models to study the biology of bone metastasis is a critical challenge in this research field, as spontaneous primary prostate cancers in the rodents usually do not develop bone metastases. The recent models using intra-iliac artery or intra-caudal artery injection appear very useful, however, the success rate of such intraarterial injection was not discussed in the reports [12-14]. Therefore, we tried to repeat the intra-arterial injection in our laboratory, in order to establish a mouse model of prostate cancer bone metastasis in immunocompetent mice.

In our hands, a total of 19 mice were used (8 for intra-caudal artery injection and 11 for intra-femoral artery injection). Only 3 mice developed metastatic tumors in the hind limbs. Many factors may contribute to this low success rate. For example, MPC3-luc cell line may not be very aggressive in colonization after intravascular inoculation. However, we noted that both intra-caudal artery and intra-femoral artery injections are technically challenging due to the small sizes of caudal artery and femoral artery in mice. Both arteries

are smaller than the diameter of a 31 G needle that is the smallest needle usable in the injection approaches. It is very difficult to insert the needle into the artery, and when insertion is successful, injection may break the artery. Leakage upon needle withdrawal also increases the chance of failure. The 10× stereomicroscope is very helpful in visualizing the artery. The performer's skill is very important, which could be the reason for the success in the reported studies [12-14]. The performer (L.Z.) in the present study had been practicing gynecologic surgery for 10 years and had intensively practiced the intra-arterial injection skills in mice before working on this project. However, she still felt that it was very difficult to perform



**Figure 4.** Histology of metastatic tumors. A. Right leg showing tumor tissues (black arrow) adjacent to the femur where the cortical bone was thickened (green arrow). B. Right leg showing tumor tissues (black arrow) adjacent to the femur (green arrow).



**Figure 5.** Intra-femoral artery injection resulted in metastatic tumors in the hind limb. A. Bioluminescent imaging of a mouse at 28 days after injection; arrow indicates positive imaging signals (right mouse); a mouse without any signals was shown on the left. B. X-ray showed a suspicious bone erosion

in the right femur of the mouse with positive imaging signals (right mouse), compared with the left mouse without any imaging signals. C. Necropsy revealed a tumor (arrow) formed in the right thigh. D. The hind limb with a tumor (arrow) was harvested for histological examination.

the injection. In comparison between intra-caudal artery injection and intra-femoral artery injection, we felt that intra-caudal artery injection causes less trauma to the animal, as we observed some animals with necrosis of the leg due to disruption of femoral artery after intra-femoral artery injection. In conclusion, our study finds that intra-arterial (either caudal or femoral artery) injection may be a useful model in studying prostate cancer bone metastasis, however, the injection technique is difficult.

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**Figure 6.** Histology of metastatic tumors. A-D. Right leg showing tumor tissues (black arrow) adjacent to the femur where the cortical bone was eroded (green arrow). Of note, the tumor tissues were separated from the bone, which was likely caused during the processing of the sample.

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#### Disclosure of conflict of interest

None.

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