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Models of Prostate Cancer Bone Metastasis

Sun Hee Park, Matthew Robert Eber, Yusuke Shiozawa

Department of Cancer Biology and Wake Forest Baptist Comprehensive Cancer Center, Wake Forest University Health Sciences, Winston-Salem, NC, USA

Abstract

More than 180% of patients with advanced prostate cancer (PCa) experience bone metastasis, which negatively impacts overall survival and patient quality of life. Various mouse models have been used to study the mechanisms of bone metastasis over the years; however, there is currently no model that fully recapitulates what happens in humans because bone metastasis rarely occurs in spontaneous PCa mouse models. Nevertheless, animal models of bone metastasis using several different tumor inoculation routes have been developed to help study bone metastatic progression, which occurs particularly in late-stage PCa patients. This chapter describes the protocols commonly used to develop models of bone metastatic cancer in mice using different percutaneous injection methods (Intracardiac and Intraosseous). These models are useful for understanding the molecular mechanisms of bone metastatic progression, including tumor tissue tropism and tumor growth within the bone marrow microenvironment. Better understanding of the mechanisms involved in these processes will clearly lead to the development of new therapeutic strategies for PCa patients with bone métastasés.

Keywords

Bone metastasis; Bone marrow microenvironment; Mouse model; Intracardiac injection; Intraosseous injection

1 Introduction

Bone metastasis is the major cause of both increased mortality and reduced mobility of patients with advanced cancer, which is due in large part to its resilience to current treatments [1]. In fact, approximately 80% of advanced prostate cancer (PCa) metastasizes to bone, yet the mechanisms of its selectiveness and growth in bone remain unclear [2]. What is clear however, is that the mechanisms of metastatic tumor growth within the bone must be better understood before the development of effective therapies is possible. Although in vitro cell culture systems allow for a reductionist approach to achieving parts of this goal, our best tools in this pursuit are animal models that recapitulate the bone metastatic progression we observe in human patients. However, just like the ideal treatments for bone metastatic PCa, the ideal animal models of bone metastatic PCa remain elusive.

A large number of animal models have been developed to aid in the study of the molecular pathways of PCa metastasis, to varying degrees of utility. For instance, orthotopic models of primary human PCa and transgenic models of spontaneous PCa in mice have been beneficial for the study of PCa progression specifically within the primary site [3–6]. However,

studying bone metastasis using these models has been challenging in part due to their low frequencies of spontaneous skeletal metastasis [4, 6–8]. In addition, most of the experimental animals will die from complications involving the primary tumor long before any overt bone lesions develop [4, 6–8]. Therefore, distinct approaches from these to effectively recapitulate the process of bone metastasis are needed. There are several xenograft and syngeneic animal models that allow us to study bone metastatic progression utilizing conceptually simple, but technically difficult injection strategies. This chapter describes detailed procedures for two of these strategies: the intra-cardiac and intraosseous routes of injection.

As with the orthotopic and transgenic models, the intracardiac and intraosseous models have limitations. The intracardiac route of injection infuses high numbers of cancer cells directly into the blood stream, which bypass the lungs and ultimately settle and grow into tumors at other common metastatic sites, including bone [9–11]. Therefore, the intracardiac injection strategy is useful in studying the cellular and molecular mechanisms of metastatic site tropism; however, the sites where tumors grow can be unpredictable in this model. On the other hand, intraosseous injections allow the implantation of tumor cells directly into a specific site of interest, such as the femur or tibia [12]. Since these tumors grow locally by design, the intraosseous injection is a very reliable and reproducible model to study the interaction between tumor cells and the bone marrow microenvironment, resulting in subsequent tumor growth. However, this model does not enable us to address the mechanisms of the dissemination process. Moreover, both of these models allow for the detection and analysis of bone metastases in a relatively short period of time; however, neither are capable of addressing the early stages of metastasis that occur in the primary tumor.

In this chapter, we describe the routinely used protocols of both the intracardiac and intraosseous injection in mice. Neither model is fully adequate to explain the complete mechanisms of bone metastasis, but each provides a means to study the unknown drivers of PCa bone metastatic progression, resulting in the development of effective therapeutic strategies for the PCa patients who suffer from bone metastases.

2 Materials

2.1 Animals

4–8-week-old immunodeficient mice (BALB/c nu/nu and CB17 SCID) or immunocompetent mice (C57BL/6) can be used (Jackson laboratory) for xenograft or syngeneic mouse models, respectively.

2.2 Cell Lines

1. Listed below are several popular cell lines used as models of PCa. There are many factors that must be considered when selecting the best cell line for any given study, but an exhaustive list of these factors and cell lines is beyond the scope of this chapter. However, a small review of considerations can be found in Table 1.

- Human PCa cell lines: PC-3, PC-3M, DU145, LNCaP, LNCap C4–2, LNCaP C4–2B, LuCaP23.1, and LuCaP35.
 - Mouse PCa cell line: RM1.
 - Dog cell line: Ace-1.
2. Cells can be transduced to stably express firefly luciferase to allow for the quantification of in vivo tumor growth in the bone using bioluminescence imaging (BLI) with the IVIS system (see Subheading 3.5). Detailed protocols for the generation of lentiviral particles and transduction of firefly luciferase into cancer cells have been described many times elsewhere [13] (see Note 1).
 3. For the intracardiac inoculation model of PCa, a highly metastatic cancer cell line (e.g., PC-3 and DU145) typically gives rise to multiple metastases, whereas very few or no metastases will be detected from a poorly metastatic cancer cell line (e.g., LNCaP). For the intraosseous injection model, bone metastases should be detected in the bone between 4 and 6 weeks after tumor injection, but this time frame is also cell line dependent.

2.3 Reagents

1. 0.05% Trypsin-EDTA (Gibco, #25300054).
2. Dulbecco's Phosphate-Buffered Saline (PBS) without Calcium and Magnesium (Gibco, #14190250).
3. Roswell Park Memorial Institute (RPMI) 1640 (Gibco, #11875119) or Dulbecco's Modified Eagle Medium (DMEM) (Gibco, #11995073): Add 100 U/ml penicillin-streptomycin (Gibco, #15140122), and 10% (V/V) fetal bovine serum (FBS) (Sigma-Aldrich, #F2442).

2.4 Equipment and Tools

1. Sterile tissue culture flasks or dishes (Thermo Scientific, #130190 and #130182).
2. Sterile 15 ml or 50 ml sterile conical tubes (Corning, #352098).
3. Sterile 1.5 ml microcentrifuge tubes (Fisherbrand, #05–408–129).
4. Sterile 40 µm cell strainers (Fisherbrand, #22–363–547).
5. 0.4% Trypan Blue Solution (Gibco, #15250061).
6. Hemacytometer (Hausser Scientific Partnership, #1475).
7. Electric hair clipper or chemical hair remover (Nair).
8. Cotton-Tipped Applicators (Fisherbrand, #23–400–106).
1 ml syringes (Becton Dickinson, #309628) with 27G needles, ½ in. (Becton Dickinson, #305109).
9. 0.5 ml insulin syringes with 28G needles, ½ in. (Becton Dickinson, #329461).
10. Povidone-iodine, 5% (Betadine).

11. Alcohol Swabs (Fisherbrand, #06–669–62).
12. Isoflurane.
13. Oxygen tank.
14. Isoflurane/oxygen-based anesthesia system fitted with an induction chamber and a nose cone for mice.
15. Ophthalmic ointment.
16. Heating pad.
17. 0.2 μ m syringe filter unit (Corning, #431222).
18. 40 mg/ml D-Luciferin (Perkin-Elmer, #122799) in PBS.
19. In vivo imaging system (Xenogen IVIS, Perkin-Elmer).

3 Methods

3.1 Preparing the Inoculum

1. Passage the cells 1–2 days prior to harvest so that they are about 70–80% confluent on the day of inoculation.
2. Perform the following cell preparation steps in a laminar flow hood using a proper aseptic technique.
3. Grow the cells on 10 cm dishes and harvest as follows: aspirate the growth media; gently wash with PBS; add 1 ml of 0.05% Trypsin-EDTA; and incubate at 37 °C for 1–3 min to detach the cells.
4. Inactivate the trypsin by adding 4–5 ml of fresh growth media.
5. Pass the cells through a 40 μ m cell strainer to exclude any large cell aggregates.
6. Count the total number of live and dead cells with a hemocytometer using the trypan blue exclusion assay; and calculate both cell concentration and viability. Cell viability should be >90% prior to inoculation.
7. Calculate the number of cells required for the experiment; and transfer at least twice the required amount of cell suspension to a new 15 ml conical tube (see Note 2).
8. Spin the cell suspension in a swinging bucket centrifuge at 4 °C (100–400 $\times g$ for 3 min).
9. Aspirate the supernatant; resuspend the cell pellet in an appropriate volume of PBS, and transfer to a sterile microcentrifuge tube (see Note 2).
10. Place the cell suspension (inoculum) on ice and bring immediately to the surgery room (see Note 3).

3.2 Preparing the Mice for Surgery (See Note 4)

1. Place and arrange all necessary equipment and materials in a laminar flow hood.

2. Turn on the anesthesia machine, and allow the induction chamber to fill completely with 2% isoflurane/98% oxygen.
3. Place a mouse in the induction chamber, and wait until it is anesthetized and no longer moving (~3–5 min).
4. Transfer the mouse to the surgery platform, and insert its head (nose) into the nose cone supplied with a constant flow of 2% isoflurane/98% oxygen for the duration of the procedure and ensure depth of anesthesia before operating by performing a toe pinch (Fig. 1a and see Note 5).
5. Apply ophthalmic ointment, and remove any hair with electrical clippers or chemical hair remover, if needed.

3.3 Intracardiac Injection

This section describes the protocol for injecting tumor cells directly into the left ventricle of the heart. Once in circulation, the tumor cells will disseminate to various tissues depending on the cell line used. We have found the PCa cell lines recommended above often form tumors in the long bones of mice. Other sites of metastasis include mandibles, brain, spine, lymph nodes, and adrenal glands. Tissue tropism, time to tumor formation, and frequency are cellline and mouse strain dependent and as such will require individual characterization.

1. Place the mouse in a supine position (Fig. 1a).
2. Disinfect the chest area with povidone iodine followed by an alcohol swab.
3. Push and withdraw the plunger of an insulin syringe until the plunger moves without resistance; slowly load 120 μ l of inoculum; and mix by gentle flicking until the cell suspension is homogenous. Retain a small air bubble between the plunger and inoculum.
4. Hold the syringe in your dominant hand.
5. Use the index and middle finger of your non-dominant hand to gently hold down the limbs of the mouse, being sure to secure and position it flat on its back (Fig. 1b).
6. Locate the injection point 1–2 mm from the midline on the animal's left halfway between the clavicle and xiphoid process (Fig. 1b). A heart beat can often be seen at this point.
7. Insert the needle perpendicular into the injection point about 7–8 mm deep; then carefully move your non-dominant hand to the plunger; and pull up very slightly without moving the position of the needle. If the needle is in the correct position a small pulse of bright red blood will flush into the syringe (Fig. 1c and see Note 6).
8. Slowly inject the cell suspension (~30 s for 100 μ l). During the injection, slightly aspirate the plunger several times and check for a pulse of blood to confirm that the needle is still correctly placed in the left ventricle.
9. Gently and slowly remove the needle from the injection site.

10. Place the mouse in a clean cage on a heating pad until fully recovered. Monitor the mouse until movement, breathing, heart rate, and eating and drinking return to normal.
11. Measure in vivo luciferase activity by BLI using the IVIS system within 2 h to confirm successful systemic tumor cell inoculation (Fig. 3a and see Note 7).

3.4 Intraosseous Injection (Intratibial or Intrafemoral)

Here, we describe two ways to perform intraosseous injections: tumor cells injected directly into the mouse tibia or femur. Intraosseous injections are invasive and as such the injection channels can be observed in histological sections. We, therefore, recommend that any studies that may be affected by a physical defect in the bone should also contain sham injected mice as negative controls.

1. Place the mouse in a supine position.
2. Disinfect the hind limb with povidone iodine followed by an alcohol swab.
3. Using thumb and index finger of your non-dominant hand, hold the ankle of the leg to be injected, and bend the knee (Fig. 2a).
4. With your dominant hand, insert a 27G needle attached to an empty syringe (drilling needle) into the appropriate area of the knee joint to generate a hole for tumor cell inoculation.
 - For the intratibial injection, insert the drilling needle through the patellar tendon and joint space into the tibial plateau. Using a gentle drilling motion and pressure toward the tuberosity, bore a hole into the tibia (Fig. 2b).
 - For the intrafemoral injection, insert the drilling needle near the patellar groove of the distal femur and bore a hole using a gentle drilling motion and pressure toward the femoral shaft (Fig. 2c).
5. Gently move the needle back and forth to confirm the needle is in the right space. If the needle tip is in the correct position, the syringe will be secure on its own. Faxitron X-ray images may be taken at this step to confirm the correct positioning of the needle within the trabecular bone.
6. Remove the drilling needle.
7. Load an insulin syringe with the inoculum as mentioned before.
8. Place the needle in the hole bored by the drilling needle, and slowly inject 10 μ l of the inoculum (see Note 7).
9. Remove the needle while pressing the injection site with a cotton swab. Alternatively, drilled holes can be sealed with bone wax or dental amalgam to prevent cancer cell migration from the medullary cavity.

10. Place the mouse in a clean cage on a heating pad until fully recovered. Monitor the mouse until movement, breathing, heart rate, and eating and drinking return to normal.

3.5 Imaging

This section describes the protocol for measuring BLI signal in the mice using a IVIS system.

1. The monitoring of tumor growth is commonly done by BLI using the IVIS system, which is a well-established technique used to locate and track metastatic foci within living animals. This system is sensitive enough to detect photons emitted from luciferase-expressing cells deep within the bones and other tissues of live mice. We recommend imaging once or twice a week after injection.
2. PCa bone metastatic lesions can also be detected by small animal diagnostic imaging (microcomputed tomography (micro CT), magnetic resonance imaging, or Faxitron cabinet X-ray systems). While BLI imaging is useful to detect the metastatic sites of the animal, micro CT or Faxitron X-ray systems can be used to detect the type of bone lesion (osteoblastic or osteolytic) as well as the location of metastatic lesions inside bone. If both BLI and other imaging techniques are not available, histopathological examination of metastatic nodules after necropsy is the best approach.
3. Prepare a stock solution of D-luciferin in PBS (40 mg/ml); and sterilize using a 0.2 μ m syringe filter unit.
4. Using an insulin syringe, inject the appropriate dose of d-luciferin into the intraperitoneal cavity (150 μ g/g), and then place the mouse back in its cage.
5. After 6 min, transfer the mouse to an induction chamber connected to 2% isoflurane mixed with 98% oxygen until fully anesthetized (~3–5 min).
6. Transfer the mouse to the IVIS cabinet with its face in a nose cone and its body on its side or supine/prone.
7. Acquire bioluminescence images (Fig. 3).

4 Notes

1. The most important thing to consider when generating a luciferase expressing cell line is the method of selection. If gene alteration of your cell line is a desired downstream application, choose a method of antibiotic selection for the luciferase gene that does not overlap with the gene of interest or gene alteration method. Alternatively, dual expression of luciferase and a fluorescent protein reporter allows for routine and quick purification by fluorescence-activated cell sorting (FACS). This is a convenient selection method only if a FACS machine is easily accessible.
2. The number of cells to be injected depends on the metastatic ability of the cell line and the inoculation model. The recommended number of cells for each

inoculation route is below. It is important to prepare 2–4 times the required number of cells for the experiment due to the loss of inoculum in the syringe dead space.

- Intracardiac injection: 1×10^5 – 1×10^6 cells/100 μ l of PBS per mouse
 - Intraosseous injection: 5×10^3 – 5×10^5 cells/10 μ l of PBS per mouse
3. It is important to perform the injections immediately after the preparation of the inoculum to maintain cell viability. We recommend that the inoculum is stored on ice for no longer than 2 h prior to injection.
 4. All experimental mice should be monitored regularly and according to the procedures for the care and use of laboratory animals required by an Institutional Animal Care and Use Committee (IACUC). The experimental mice should be diligently euthanized if any clinical sign meets the humane endpoints, including rapid weight loss and any condition interfering with daily activities, such as eating or drinking, ambulation, or elimination.
 5. To test if the mouse is properly anesthetized, pinch the paw skin between the toes of one hind paw. The mouse is fully anesthetized when it does not respond to this stimulus and the procedure can begin. However, if the mouse responds in any way to the stimulus, it is not fully anesthetized and must be placed back in the anesthesia induction chamber.
 6. If the needle is properly inserted in the left ventricle, bright red blood (oxygenated arterial blood) will be pumped back into the cell suspension and pulsation should be visible from the air bubble remaining in the syringe. If the needle is not placed in the left ventricle, darker venous blood from the right ventricle can be detected. If there is no blood pumping back or dark red blood observed, retract, and re-insert the needle, but no more than three attempts should be carried out within 24 h.
 7. If the tumor cells were correctly injected into the left ventricle, bioluminescence signals will be observed throughout the whole body. Otherwise, signals will be concentrated mainly in the chest area near the injection site (Fig. 3a).

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Fig. 1.

The intracardiac injection, (a) Visualization of an anesthetized mouse with its body in the correct position for the intracardiac injection and its face in a nose cone supplied with a constant flow of 2% isoflurane mixed with 98% oxygen, (b) The upper limbs of the mouse are gently held down with the index and middle fingers to secure the torso of the mouse. The location of the injection point, 1–2 mm from the midline on the animal's left, is shown as a white dot. (c) A side view of the mouse with a 28G insulin syringe inserted in the left

ventricle. Correct needle insertion is evidenced by the mixing of bright red blood with the inoculum in the syringe

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Fig. 2.

The intraosseous injection, (a) Correct positioning and visualization of the left leg of a mouse is shown. The ankle of the leg is tightly held to bend the knee to position both femur and tibia for the injection. Clear visualization of the knee joint (red dotted circle) is shown, (b) A 27G needle attached to a 1 ml syringe is inserted into the proximal tuberosity of tibia through the joint with drilling motion to generate a hole for intratibial tumor inoculation, (c) Visualization of the correct manipulation of the leg for the intrafemoral injection. A 27G needle attached to a 1 ml syringe is inserted into the patellar groove of the femur toward the shaft. The needle-syringe is in line with the long axis of tibia and femur (white arrow)

Fig. 3. Bioluminescence imaging of the tumor inoculated mice, (a) A mouse was imaged with bioluminescence imaging (BLI) 2 h after luciferase expressing cancer cell inoculation into the left ventricle. (Left) BLI signals detected throughout the whole body are indicative of a successful intracardiac injection, and (Right) intense signals limited to the thoracic region are indicative of injection failure, (b) Detection of skeletal metastases 4 weeks after intracardiac tumor injection of luciferase-expressing PC-3 cells, (c) Visualization of tumor

growth in the femur by BLI 4 weeks after intrafemoral injection of luciferase-expressing PC-3 cells

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Table 1

Prostate cancer cell lines used for skeletal metastasis mice model

Animal	Cell lines	IO growth	IC growth	Type if bone metastasis	Mouse strain	References
Human	PC-3	+	+	Osteolytic	BALB/c	[14–16]
	PC-3M	+	+	Osteolytic	nu/nu	[17]
	DU145	+	–	Osteolytic		[16]
	LNCap	+	+	Mixed	or	[15]
	LNCap C4–2	+	+	Mixed	CB17 SCID	[5,15,18]
	LNCap C4–2B	+	+	Mixed		[5,15,19]
	LuCaP23.1	+	–	Osteoblastic		[12]
	LuCaP35	+	–	Osteolytic		[12]
	Ace-1	+	+	Mixed	BALB/c	[20]
	RM1	+	+	Mixed	C57BL/6	[21,22]

IO Intraosseous, IC Intracardiac