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Pre-Clinical Mouse Models of Human Prostate Cancer and their Utility in Drug Discovery

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Abstract

In vivo animal experiments are essential to current prostate cancer research, and are particularly critical to studying interactions between tumor cells and their microenvironment. Numerous pre-clinical animal models of prostate cancer are currently available, including transgenic mouse models and human prostate cancer xenograft mouse models. In contrast to transgenic mouse models producing more heterogeneous cohorts of tumors, xenograft mouse models provide more controlled approaches. This unit describes the detailed procedures necessary to establish several distinct pre-clinical mouse models of human prostate cancer, including an orthotopic prostate xenograft model, an orthotopic bone metastasis model, an experimental metastasis model of intra-cardiac injection, and a vossicle model of tumor-bone interaction.

UNIT INTRODUCTION

Prostate cancer is the most common cancer among men, accounting for the second leading cause of cancer-related deaths in American men (American Cancer Society. 2008). The five-year relative survival rate of advanced stage (i.e. metastatic) prostate cancer patients is only 32%, while the survival rate of early stage prostate cancer reaches nearly 100% (Jemal et al. 2008). Common therapeutic modalities for prostate cancer include androgen ablation, conventional cytotoxic chemotherapy, radiation therapy and surgery (Loberg et al. 2005; Logothetis 2008). However, presently there is no effective cure for metastatic prostate cancer. Consequently, current prostate cancer research is increasingly focused on understanding and preventing metastasis, particularly to the skeleton. Cancer metastasis is a complex process that involves interactions between cancer cells and multiple types of cells in the microenvironment (Fidler 2003). To rigorously study the multifaceted biology of prostate cancer metastasis, pre-clinical *in vivo* models are the best available approach to comprehensively reconstitute the organ microenvironment that plays critical roles in metastasis.

The most commonly used animal models in prostate cancer research include transgenic mouse models and human prostate cancer xenograft mouse models. A number of transgenic

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mouse models of prostate cancer have been established by targeted disruption of such genes as *Nkx3.1* and *PTEN* (phosphatase and tensin homolog). As they age, mice with homozygous functional deletion of *Nkx3.1* display histopathological defects resembling prostate intra-epithelial neoplasia (PIN) (Kim et al. 2002), and prostate-specific deletion of *PTEN* gene recapitulates the natural progression of the disease from the prostatic intraepithelial neoplasia (PIN) to metastasis (Wang et al. 2003). In addition, the transgenic adenocarcinoma mouse prostate (TRAMP) model was established by transgenic expression of SV40 early genes (T and t antigens) (Greenberg et al. 1995). TRAMP mice develop high grade PIN within 12 weeks after birth that progress to poorly differentiated adenocarcinoma in 24–30 weeks (Kaplan-Lefko et al. 2003). TRAMP mice are useful to study the process of lymph node metastasis, because TRAMP mice consistently and predictably develop these metastases (100% by 28 weeks after birth) (Gingrich et al. 1996); however, tumors from these animals have neuroendocrine features, rare for most human prostate cancers (Kaplan-Lefko et al. 2003; Chiaverotti et al. 2008). Further, metastases to distant organs of greater clinical significance (especially bones) are very rare in TRAMP mice (Hsieh et al. 2007). Transgenic mouse models have provided invaluable information on the complex pathogenesis of prostate cancer, particularly with respect to deregulation of specific molecular or genetic changes. However, transgenic tumor models often require a considerable expenditure of time, effort and a large number of animals to produce statistically meaningful data, due to heterogeneity of tumor incidence and progression. Nevertheless, these models hold considerable promise in understanding aspects of prostate tumor development and for testing novel therapies.

In contrast, human prostate cancer xenograft mouse models provide extremely useful alternative approaches for understanding the biology of prostate cancer, particularly regarding specific interactions between various genetically and molecularly altered tumor cells and their organ microenvironment, as well as for evaluating efficacy of investigational new drugs and therapeutic regimens (Kim et al. 2003; Kim et al. 2003; Trevino et al. 2006; Zhang et al. 2007; Park et al. 2008). In addition, orthotopic implantation combined with subsequent harvesting at metastatic sites can generate prostate cancer cell variants of great clinical relevance to the metastasis process (Pettaway et al. 1996). In contrast to ectopic subcutaneous tumor models, orthotopic xenograft models can more accurately reconstitute an organ microenvironment that dictates the phenotypes of tumor cells, as originally proposed by Stephen Paget's "seed and soil" hypothesis and confirmed by many others (Poste and Fidler 1980; Tarin et al. 1984; 1984; Paget 1889). Therefore, human prostate cancer xenograft models complement transgenic mouse tumor models, providing valuable tools to study prostate cancer.

Recently, tumor xenograft mouse models have been expanded by directly transplanting human tissues or cells into mice, thus avoiding potential molecular and epigenetic changes that can occur after long periods (sometimes decades) of *in vitro* growth (Navone et al. 1998; Pienta et al. 2008; Garber 2009). This 'direct tumor xenograft', or "tumorgraft" approach has shown great promise in recapitulating the heterogeneity of human tumors in the laboratory (Rubio-Viqueira and Hidalgo 2009). Particularly, this approach showed greater predictability for clinical responses to several pre-clinical drugs against multiple types of solid tumors, compared to conventional cell line xenograft models (Perez-Soler et al. 2000; Kerbel 2003; Fichtner et al. 2004; Fiebig et al. 2004; Rubio-Viqueira et al. 2006). Thus, human prostate cancer cells or tissues freshly prepared from surgery or biopsy can be directly transplanted to immunocompromised mice orthotopically (intra-prostatic or intra-tibial), to establish more clinically relevant tumor models. Combined with advanced technologies such as flow cytometric analysis or sorting, direct xenograft approaches can greatly expand the usefulness of prostate cancer xenograft mouse models.

In this unit, four different types of human prostate cancer cell line xenograft mouse models are described in detail. The orthotopic prostate cancer xenograft model (described in Basic Protocol 1) utilizes human prostate cancer cells growing in the mouse prostate for studying genetic and molecular changes in the tumor cells contributing to tumor growth *per se* as well as regional lymph node metastasis (Stephenson et al. 1992; Park et al. 2008). However, no such models have been reported to lead to spontaneous metastasis to the most frequent site for prostate cancer, the bone. Failure of these metastases may be because mice carrying orthotopic prostate cancer die of urinary obstruction before any apparent bone metastatic lesions develop, or because the microenvironment of the mouse fails to recapitulate the human microenvironment, thus failing to provide the proper “soil” for development of bone metastases. Alternatively, there are two strategies available to study metastasis to the bones and other distant organs. The second basic protocol describes an intra-cardiac inoculation model of prostate cancer cells, which will result in distant metastatic lesions (mandibles, femurs, vertebrae, ribs, adrenal glands, kidney, liver, *etc.*) within 4–6 weeks (in case of 2×10^5 PC-3 cells) (Yin et al. 1999; Padalecki et al. 2003; Schneider et al. 2005). In addition, orthotopic intra-tibial implantation of metastatic prostate cancer cells (Basic Protocol 3) produces more homogenous cohorts of tumors in bone, which are useful to study microenvironment interactions (Uehara et al. 2003; Kim et al. 2006). Lastly, ectopic implantation of vossicles (murine neonatal vertebrae isolated from mice of chosen wild-type or transgenic background) simultaneously with prostate tumor cells (described in Basic Protocol 4) provide a useful model to study tumor-bone interactions, particularly bones of different genetic alterations. Vossicle models are extremely useful to test effects of host microenvironmental changes (Koh et al. 2005; Liao et al. 2008; Pettway and McCauley 2008).

NOTE

All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals. Certain anesthetic drugs may require additional licensure and legal supervision. Aseptic surgical techniques must be employed for all surgical procedures.

BASIC PROTOCOL 1. ORTHOTOPIC PROSTATE CANCER MOUSE MODEL

Experimental techniques to establish a mouse model of orthotopic prostate cancer are detailed in this protocol. Human prostate cancer growing orthotopically in the prostate of immunodeficient mice has proven a vital tool for prostate cancer research. Orthotopic models are particularly beneficial to the study of the efficacy of novel investigational drugs on prostate tumor growth and/or regional lymph node metastasis. In pre-clinical studies using this model, expected outcomes of investigational therapeutics include reduction of tumor size and/or incidence; as well as reduced incidence of regional lymph node metastases.

Materials List

1. Male athymic mice can be purchased from commercial vendors such as the National Cancer Institute-Frederick Animal Production Area (NCr *nu/nu*) or Harlan Laboratories (Hsd athymic nude-*Foxn1^{tmu}*). Mice are used at 6 to 12 weeks of age.
2. Prostate cancer cells in culture such as PC-3, LNCaP, *etc.* (from commercial vendors such as ATCC)
 - Prepare at least 2–4 fold the required number for a given experiment. Required cell number varies greatly depending on cell type and the

purpose of experiment (e.g. duration of tumor growth). For example, PC-3MM2 (highly aggressive cell line) requires 5×10^4 cells per injection (4-week growth), and LNCaP-LN4 (less aggressive cell line) requires 5×10^5 cells per injection for producing similar tumor sizes after four weeks (both cell lines are established and gifted by Dr. Isaiah J. Fidler, the University of Texas M. D. Anderson Cancer Center) (Pettaway et al. 1996; Park et al. 2008).

- Validation experiments are necessary to optimize the injection number for individual cell lines according to the specific cell line used and experimental purposes, if not following published models. Variable numbers of cancer cells can be injected *in vivo* orthotopically and subsequently harvested at varying time points. The optimal endpoints may be determined by tumor size (approximately 0.8~1.5g wet weight and 0.5~1cm in diameter), time and/or presence of peri-aortic lymph node metastases.
3. Complete cell culture media (e.g. for LNCaP, PC-3 and their derivatives) is RPMI-1640 (Gibco brand from Invitrogen) supplemented with 10% fetal bovine serum (HyClone brand from Thermo Scientific) and antibiotics (Invitrogen).
 4. Sterile phosphate-buffered saline (PBS) and 0.25% Trypsin (Invitrogen)
 5. 0.4% Trypan blue and hemacytometer
 6. Ca^{2+} -free and Mg^{2+} -free Hanks' Balanced Salt Solution with Phenol Red Indicator (HBSS; from Invitrogen)
 7. Sterile centrifuge tubes (50ml conical tubes or Eppendorf tubes; 15ml conical tubes are not a good choice) for carrying cells to the surgery room.
 8. Anesthetics: Pentobarbital sodium (50mg/ml. NembutalR from Abbott laboratories) or Ketamine/Xylazine Mixture (Ketamine (100mg/ml):Xylazine (20mg/ml):Phosphate-buffered saline = 3:2:5) or isoflurane gas anesthetics system (e.g. XGI-8 Gas Anesthesia System from Caliper Life Sciences)
 9. Scissors (e.g. 4½ inch curved iris scissors) and forceps (e.g. 4 inch 1 × 2 teeth straight iris tissue forceps)
 10. Sterilized cotton swabs (1/10 inch × 6 inch wood shaft cotton-tipped applicators)
 11. 70% ethanol gauze and/or 10% povidone-iodine (Betadine)
 12. 1ml insulin syringes and needles (27½G × ½ inch length) (BD Medical)
 13. Wound closing surgical metal clips, stapler and remover (e.g. AutoClipR applier, AutoClipR remover and 9mm EZ Clip, BD Medical)

Steps and Annotations

1. Intra-prostatic injection is an open-abdominal surgery. During the entire procedure, animals should be kept in specific pathogen-free conditions (e.g. in a designated surgery room or laminar flow hood) and proper surgical aseptic technique should be applied to all surgical procedures.
2. Anesthetize animals:
 1. For Pentobarbital Sodium (NembutalR), 0.5mg per 1g body weight should be administered intra-peritoneally (i.p.). To minimize anesthesia-related mortality, weigh animals individually and adjust dosage accordingly.

2. For Ketamine/Xylazine/PBS mixture, administer 3 μ l per 1g body weight intraperitoneally (i.p.)
 3. 2% isoflurane mixed with 98% oxygen in the induction chamber. Animals should be kept under anesthesia through nose cone during surgery. Gas anesthesia should be performed in a properly ventilated area.
 4. To prevent blindness due to xerophthalmia during general anesthesia, proper eye protection should be applied (e.g. application of ophthalmic ointment or sterile petroleum jelly with cotton swabs).
 5. Prior to surgery, ensure depth of anesthesia by lightly pinching the foot where the mouse should give no response. If there is a response (e.g. twitch or jerk), wait longer or administer another dose of anesthetics. It should be noted, however, that additional anesthetic dosing may increase anesthesia-related mortality. Isoflurane inhalation anesthesia (through a nose cone) is preferred when additional anesthesia is required.
3. Prepare prostate cancer cell suspension for implantation.
 1. Cell viability decreases rapidly after detachment, and cells should be injected as quickly as possible (preferably within one hour after trypsinization). For best results, an assisting investigator should prepare cells while anesthesia is being administered to the mice. In addition, animals should be grouped for each cell preparation to be injected within as short a period of time as possible such that cells are not in suspension for long periods.
 2. Wash cells with PBS once, and trypsinize (usually within 5 minutes).
 3. Enumerate live cells by trypan blue exclusion assay with the aide of a hemacytometer.
 4. Put desired number of cells in centrifuge tubes. For example, PC-3MM2 (highly aggressive cell line) requires 5×10^4 cells per injection (4-week growth), and LNCaP-LN4 (less aggressive cell line) requires 5×10^5 cells per injection for producing similar tumor sizes after four weeks (Pettaway et al. 1996; Park et al. 2008).
 5. Pellet cells by centrifugation (1000 $\times$ g for 3 minutes).
 6. Aspirate supernatant and resuspend pellet in desired volume of HBSS. Injection volume per mouse is 50 μ l.
 - Volume loss during this process needs to be considered (for example, dead space in syringes). Thus, optimally 2–4 fold of the required cell suspension volume should be prepared.
 7. Bring cells to the surgery room while keeping on ice.
 4. Surgical implantation of tumor cells
 1. Place a mouse in a supine position. Immobilization is not necessary, but can be performed according to investigator's preference. Use surgical adhesive tape (e.g. 3M Micropore Surgical Tape).
 2. Clean the lower abdomen by 70% ethanol swab or 10% povidone-iodine (Betadine).

3. Make midline skin incision (approximately 1.5–2cm in length) with scissors. Lower end of incision begins 5–8mm superior to the external genitalia (Figure 1A).
4. Make midline peritoneal incision (same length as the skin incision) with scissors.
5. Identify urinary bladder (Figure 1A).
 - Urinary bladder can be easily identified as a yellow-light brown spherical organ, located directly under the incision. Size varies greatly among individuals depending on volume of urine.
6. With two sterile cotton swabs in both hands, externalize urinary bladder.
 - Avoid twirling internal organs by direct touching with cotton swabs. Lightly pressing on both sides of the incision will gently protrude the bladder (Figure 1A).
7. Further extending the bladder antero-inferiorly will result in externalization of seminal vesicles on both sides (left and right).
 - Use a wet cotton swab to avoid tissue damage.
 - Seminal vesicles are a pair of white saclike organs, and clearly distinct.
8. Arrange both seminal vesicles (left and right) and urinary bladder (middle) as shown in Figure 1B. Press in between the seminal vesicles lightly (antero-inferiorly), and locate the rectum.
9. Carefully dissect the rectum (by pushing postero-superiorly with a wet cotton swab) (Figure 1D).
10. Dorsum of prostate gland will appear. Murine prostate gland appears as two distinct lobes (left and right).
11. Load syringe with cell suspension.
 - Agitate well before each loading.
 - Cell suspension should be kept on ice during the entire procedure.
12. Inject 50µl of cell suspension *slowly* (Figure 1C). Identify a clear bulla formation in the dorsum of the prostate.
 - Bulging bulla while injecting indicates correct positioning of needle tip.
13. When retracting the needle, press lightly on the injection site with a cotton swab and hold for 20–60 seconds, to prevent regurgitation.
14. Bring internal organs back into the peritoneum with a cotton swab.
15. Close wound with surgical metal clips.
 - Closing of skin only (instead of clipping peritoneum together) will be sufficient, unless incision is excessively long (more than 3cm).

5. Bring animals back to clean cage and keep under warming lamp or on heating pad. Monitor animals constantly until complete recovery from anesthesia.
6. Monitor animals regularly until the end of experiment, according to the approved IACUC protocol.
 1. Surgical metal clips must be removed after two weeks.
 2. Lethargic mice and mice carrying unusually large tumors (outliers) must be euthanized and removed from the experiment, according to the protocol.
 3. A simple and easy method of monitoring is to observe skin color and texture; activity; and constant urination and defecation while holding a mouse for examination or treatment. Significantly reduced body weight often indicates a lethargic condition.
 4. Tumor-bearing animals should be in good health except for presence of tumors until the end of experiment.
 5. Experienced investigators can palpate when tumors grow to 5mm in diameter (at the time of day-10 to -14, for the case of 5×10^4 PC-3MM2 cells injection). Tumor incidence of PC-3MM2GL cells is 100%.
7. Experimental treatment of animals
 - Depending on the proposed mechanism of anti-tumor effects (e.g. anti-metastatic and/or anti-proliferative; effective on early-stage tumor vs. later stage tumor), the starting point of experimental treatment can be determined. For example, potential anti-metastatic drugs can be used after establishment of certain size tumor.
 - Dosage and route of administration (e.g. intra-peritoneal injection, oral-gavage, intra-venous injection, *etc.*) must be determined previously by pre-clinical pharmacokinetic and pharmacodynamic studies.
 - Reduction of tumor size by an experimental treatment can be 40–60% of the untreated control tumors (measured by wet tumor weight), as demonstrated by Park *et al.* using PC-3MM2GL cells and a Src family tyrosine kinase inhibitor (Dasatinib from Bristol-Myers Squibb Oncology Co.) (Park et al. 2008)
8. Necropsy and pathologic examination
 - Animals should be euthanized by an IACUC-approved method (e.g. CO₂ asphyxiation, anesthetic drug overdosing, *etc.*)
 - Subsequently to immobilization, make a vertical incision of entire abdominal and thoracic length with surgical scissors.
 - Grossly examine entire abdominal organs and thoracic cavity for tumor nodules and/or pathologic changes.
 - Excise prostate tumors with scissors. Measure wet tumor weight and size. Photograph if desired. Tumor tissues can be fixed and processed for further histological analysis. The most common method is fixation in 10% buffered formaldehyde (Fisher brand 10% Formalin from Fisher Scientific) followed by embedding in paraffin. Alternatively, tumors can be snap-frozen in freezing media (e.g. OCT solution from Sakura Finetek) and liquid nitrogen.

- Examine peri-aortic lymph nodes for metastases. Lymph node metastasis can be defined as white and solid enlargement (>1mm) of lymph node (for example, see Park et al., 2008). Representative enlarged lymph nodes must be histopathologically confirmed for the presence of metastatic tumor cells.
- Statistical analysis of tumor size comparison can be performed using Mann-Whitney *U* test. If a parametric test is applicable, use the Student *t* test. For comparisons of tumor or lymph node metastasis incidence, the Fisher's exact test can be used.

BASIC PROTOCOL 2. EXPERIMENTAL METASTASIS MODEL OF PROSTATE CANCER BY INTRA-CARDIAC TUMOR CELL INOCULATION

Currently, no *in vivo* models of spontaneous metastasis other than to the regional lymph nodes from orthotopic prostate xenograft tumors have been reported. The most common alternative method to mimic hematogenous metastasis is to introduce tumor cells directly into the systemic circulation. This model system is specifically useful to study interactions of the tumor and its metastatic microenvironment. Basic Protocol 2 describes details about establishing an intra-cardiac prostate tumor model.

Materials

1. Male athymic mice can be purchased from commercial vendors such as the National Cancer Institute-Frederick Animal Production Area (NCr *nu/nu*) or Harlan Laboratories (Hsd athymic nude-*Foxn1^{tmu}*). Mice are used at 6 to 12 weeks of age.
2. Metastatic prostate cancer cells in culture such as PC-3 (from ATCC)
 - Expand the culture to produce at least 2–4 fold of necessary cell number.
 - Cells labeled with luciferase gene are greatly useful to follow metastasis *in vivo*. Lentiviral transfer of luciferase gene and subsequent selection and verification of stable clones should be performed in advance. Luciferase-labeled cancer cells can be purchased from Caliper Life Sciences or generated individually by transducing cells with lentiviral vectors (Naldini et al. 1996; Ory et al. 1996) and luciferase reporter vectors (pGL4-*luc2* vectors from Promega).
3. *In vivo* bioluminescence imaging system (e.g. Xenogen IVISR-200 from Caliper Life Sciences, Inc) and luciferin substrate (from Caliper Life Sciences, Inc. or Promega)
4. Complete cell culture media (e.g. for LNCaP, PC-3 and their derivatives, RPMI-1640 from Gibco brand from Invitrogen) supplemented with 10% fetal bovine serum (HyClone brand from Thermo Scientific) and antibiotics (Invitrogen).
5. Sterile phosphate-buffered saline (PBS) and 0.25% Trypsin (Invitrogen)
6. 0.4% Trypan blue and hemacytometer.
7. Ca²⁺-free and Mg²⁺-free Hanks' Balanced Salt Solution with Phenol Red Indicator (HBSS; from Invitrogen).
8. Sterile tubes (50ml conical tubes or Eppendorf tubes; 15ml conical tubes are not a good choice) for carrying cells to the surgery room.

9. Anesthetics: Pentobarbital (e.g., NembutalR) or Ketamine/Xylazine Mixture (Ketamine (100mg/ml):Xylazine (20mg/ml):Phosphate-buffered saline = 3:2:5) or isoflurane gas anesthetics system (e.g. XGI-8 Gas Anesthesia System from Caliper Life Sciences)
10. 70% ethanol gauze (BD Medical)
11. 1ml syringes and needles (27½G × ½ inch long; regular or short bevel) (BD Medical)
12. Surgical adhesive tape (e.g. 3M Micropore Surgical Tape)
13. Fine-point marker pen

Steps and Annotations

1. Although intra-cardiac injection is minimally invasive, animals should be kept in specific pathogen-free conditions during the entire procedure (e.g. in a designated surgery room or laminar flow hood).
2. Anesthetize animals (as described in Basic Protocol 1, Step # 2)
3. Prepare cell suspension in Ca²⁺-free and Mg²⁺-free HBSS (as described in Basic Protocol 1, Step # 3)
 1. Injection volume per mouse is 100µl.
 2. Intra-cardiac injection cell number is different from those used for other models (e.g. orthotopic prostate injection, intra-tibial injection, *etc.*) For PC-3 cells, 2×10^5 cells in 100 µl HBSS are suggested (Schneider et al. 2005).
4. Intra-cardiac inoculation of tumor cells
 1. Place mouse in a supine position.
 2. Tape down both upper limbs. Both limbs should be stretched perpendicular to the midline (Figure 2A).
 3. Place surgical adhesive tape horizontally across the abdomen to prevent body movement during injection. Care should be taken not to press hard and displace internal organs.
 4. Intra-cardiac injection is a blind technique (i.e. injection point on the heart is not visualized) and hence maintaining consistency of topographic anatomy (i.e. immobilization by taping) is very important.
 5. Clean the skin over thorax by 70% ethanol swab.
 6. Mark the inferior-most point of xyphoid process and jugular notch with a marker pen (Figure 2A).
 7. Mark a point evenly dividing two points on jugular notch and the xyphoid process.
 - If SCID (Severe Combined Immunodeficiency) mice are used, visualization will be hindered by fur. In such case, a thoracic skin midline incision can be made with scissors or the fur can be removed by shaving or depilation. Incision must be closed with surgical metal clips (e.g. AutoclipR) after injection.

8. Injection point is 1–2mm left of the midline on the mouse. (Figure 2A)
9. Load syringe with cell suspension.
 - An air bubble should remain in the syringe (as depicted in Figure 2B) to allow blood pulsating. (See step 12 below)
 - With a needle attached, dip the tip of needle in the suspension, and take back the plunger to 200µl graduation.
 - Cell suspension should be kept on ice during entire procedures.
 - Agitate cell suspension well before each loading.
10. Insert needle *vertically* to the injection point.
 - Hand must rest firmly on the table or the other hand.
 - Extra care must be taken to confirm that the long axis of the syringe is perfectly perpendicular to injection site.
 - Depth of needle insertion is approximately 2mm.
11. If tip of the needle is correctly placed in the left ventricle, pulsation of bright-red blood should be visible in the needle hub and/or in the cell suspension.
 - If pulsation of blood is not observed, the needle tip is not in the heart. Retract and try again. Change needle, because blood clotting inside the needle prevents blood pulsation.
12. Start injection. It is very important that cell suspension (100µl) be *very slowly* injected over 40–60 seconds.
 - To avoid positional change of injecting hand during injection, firm hand rest must be maintained.
13. Press the injection site with a cotton swab while retracting the needle and hold for 15 seconds to insure hemostasis.
5. Bring animals back to clean cage and keep under warming lamp or on a heating pad. Monitor animals continually until complete recovery from anesthesia.
6. Animals should be imaged by *in vivo* bioluminescence to confirm tumor cells in the systemic circulation within 24 hours after injection.
 - If tumor cells are correctly injected in the arterial circulation through the left heart ventricle, bioluminescence signaling can be observed in the whole body (for example, see Figure 5A and B).
7. Visualization of metastatic tumor growth is commonly performed by *in vivo* bioluminescence.
 1. At each time point, inject 150µg/g luciferin substrate (stock solution 40mg/ml in PBS) i.p.
 2. While waiting for 12 minutes, anesthetize animals in 2% isoflurane mixed with 98% oxygen in induction chamber. Place mice in IVIS platform while keeping mice under anesthesia through a nose cone.

3. Obtain bioluminescence signal images overlaid on a grey picture by running Living Image software on a computer attached to the IVIS machine.
8. Development of metastatic lesions and analysis of experimental outcomes are discussed in the Commentary section.
9. Few practical alternative approaches exist to detect metastatic lesions *in vivo*, other than molecular imaging techniques or small-animal diagnostic imaging (computed tomography or magnetic resonance imaging). If bioluminescence or other imaging techniques are not available, histopathological examination of metastatic nodules subsequent to necropsy is the best approach.
10. Necropsy and pathologic examination
 1. Animals should be euthanized by an IACUC-approved method (e.g. CO₂ asphyxiation, anesthetic drug overdosing, *etc.*)
 2. Subsequent to immobilization, make a vertical incision of entire abdominal and thoracic length with surgical scissors.
 3. Grossly examine entire abdominal organs and thoracic cavity for tumor nodules and/or pathologic changes.
 4. Excise metastatic tumors with scissors. Measure wet tumor weight and size. Photograph if desired. Tumor tissues can be fixed and processed for further histological analysis. The most common method is fixation in 10% buffered formaldehyde (Fisher brand 10% Formalin from Fisher Scientific) followed by embedding in paraffin. Alternatively, tumors can be snap-frozen in freezing media (e.g. OCT solution from Sakura Finetek) and liquid nitrogen.
 5. For bone metastatic lesions, excise bones (hind limb bones, vertebrae and/or ribs) and strip off muscles. Take photographs or make a radiographic image, if necessary. Fix bones in 10% buffered formaldehyde solution (Fisher brand 10% Formalin from Fisher Scientific) for 24 hours. Subsequently, decalcify bones by agitating in 10% EDTA solution for 14 days with frequent solution changes. Bones then can be embedded in paraffin and sectioned for histological examination. Multiple alternative fixation/decalcification methods exist depending on the desired method of antigen retrieval for immunohistochemical staining.

Note

1. It is strongly recommended to practice the surgery and injection procedures before the planned experiment. For training purposes, any conventional mouse strain can be used. Visualization of ribs (by thoracic midline skin incisions) facilitates the correct position of needle. The needle passes through the third inter-costal space (i.e. between the third and fourth ribs; note that the first rib is embedded under the clavicle thus not visible). Instead of mock injection, draw blood and confirm that the blood is drawn from the left ventricle (i.e. bright-red oxygenated arterial blood, distinctive from darker venous blood of the right ventricle).
2. The injection technique described above uses anatomic landmarks (midline, xyphoid process and jugular notch) that are relatively easy to identify and to maintain consistency. However, there are several alternative approaches reported by multiple investigators. For example, oblique insertion of needle through the second inter-costal space is also a commonly used technique (for a detailed

description, see Arguello et al., 1992). In addition, oblique insertion of the needle through the diaphragm has also been reported. With or without horizontal incision below lower margin of rib cage, the needle is inserted parallel to the midline (from frontal view), and immediately lateral to the xyphoid process, penetrating the diaphragm and base of the heart (Pettaway et al. 1996).

BASIC PROTOCOL 3. ORTHOTOPIC BONE TUMOR MODEL OF METASTATIC PROSTATE CANCER

This protocol details the techniques to establish orthotopic prostate tumors growing in the tibiae of athymic mice. Orthotopic tumors in the skeleton are useful to determine efficacy of investigational drugs affecting bone microenvironment and/or metastatic prostate tumor cells. Expected outcomes of experiments using this model include reduction of tumor size and improvement of bone architecture (determined by bone histomorphometry, *etc.*)

Materials

1. Male athymic mice can be purchased from commercial vendors such as the National Cancer Institute-Frederick Animal Production Area (NCr *nu/nu*) or Harlan Laboratories (Hsd athymic nude-*Foxn1^{tmu}*). Mice are used at 6 to 12 weeks of age.
2. Metastatic prostate cancer cells in culture such as PC-3 (ATCC). Expand the culture to produce at least 2–4 times necessary cell number for injection.
3. Complete cell culture media (for PC-3, RPMI-1640 media, Gibco brand purchased from Invitrogen) supplemented with 10% fetal bovine serum and antibiotics (HyClone brand from Thermo Scientific).
4. Sterile phosphate-buffered saline (PBS) and 0.25% Trypsin (Invitrogen)
5. 0.4% Trypan blue and hemacytometer
6. Ca²⁺-free and Mg²⁺-free Hanks' Balanced Salt Solution with Phenol Red Indicator (HBSS; from Invitrogen)
7. Sterile tubes (50ml conical tubes or Eppendorf tubes; 15ml conical tubes are not a good choice) for carrying cells to the surgery room.
8. Anesthetics: Pentobarbital (e.g., NembutalR) or Ketamine/Xylazine Mixture (Ketamine (100mg/ml):Xylazine (20mg/ml):Phosphate-buffered saline = 3:2:5) or isoflurane gas anesthetics (2% isoflurane mixed with 100% oxygen)
9. 70% ethanol gauze. (BD Medical)
10. 1ml syringes and needles (27½ G × ½ inch long). (BD Medical)
 - For more accurate volume injection, HamiltonR syringes (e.g. Microliter Syringe Models No. 702, 705 or 725 with Luer-tip termination) with regular hypodermic needle (27 ½ G × ½ inch) can be used. Priming the needle with regular 1ml syringe before attaching to Hamilton Syringe will help removing trapped air bubbles.
11. Small animal X-ray imaging system (FaxitronR)

Steps and Annotations

1. Animals should be kept in specific pathogen-free conditions during the entire procedure (e.g. designated surgery room or laminar flow hood), and aseptic technique must be employed for all steps.

2. Anesthetize animals (as described in Basic Protocol 1, Step # 2).
3. Prepare cell suspension in Ca^{2+} -free and Mg^{2+} -free HBSS (as described in Basic Protocol 1, Step # 3).
 1. Injection volume per mouse is 20 μl .
 2. Intra-tibial injection cell number is different from those used for other models (e.g. orthotopic prostate injection, intra-cardiac injection, *etc.*) Numbers will vary based on cell type and purpose of experiment. For example, $0.2\sim 1.5 \times 10^6$ cells in 20 μl HBSS have been used in past experiments (Kim et al. 2003; Li et al. 2008).
 3. Variable numbers of cancer cells can be injected intra-tibially and tumor growth can be followed for extended time periods by plain radiographs at appropriate times post inoculation. Optimal endpoints can be determined by desired tumor size depending on the purpose of experiment.
4. Intra-tibial implantation of tumor cells
 1. Place mouse in a supine position.
 2. Clean injection site with 70% ethanol swab.
 3. Using thumb and index finger (by opposition motion), hold ankle (i.e. medial and lateral malleoli) as shown in Figure 3A.
 - Correctly holding the animal is of great importance for subsequent steps. Tips of both fingers should tightly grip the medial and lateral malleoli.
 4. Rotate ankle (tibia and fibula) laterally and bend the knee (in anatomical terms, combinatorial movements of plantar flexion-lateral rotation of lower hind limb-flexion of knee joint) (Figure 3A, Arrow).
 - In the correct position, anterior crest of tibial body is clearly visible through the skin (Figure 3A, Solid lines).
 - Wetting skin with 70% ethanol will increase visibility.
 5. With a needle attached to 1ml syringe, align syringe needle with the long axis of the tibia.
 6. Percutaneously through the knee joint, insert needle and place the needle tip on the proximal tuberosity of tibia (Figure 3B).
 7. Start drilling by rotating syringe (half to 3/4 turn) (Figure 3B, Arrow).
 - Take extra care to maintain alignment of the long axis of the syringe with the long axis of tibia while making rotational movement of syringe (Figure 3B, Solid line).
 - If the needle is in the correct position, simultaneous rotational movement of tibia can be palpated while drilling.
 - Do not use force to advance the needle. Force should be used only for rotating action.
 8. If the needle tip has advanced to the correct position, release the syringe and the syringe will stay still. (Figure 3C)
 - At this step, X-ray images can be taken to confirm correct position of the needle. The tip of needle must be within bony

trabeculae near growth plate, not in the bone marrow cavity unless the cells are specifically intended to be injected into the bone marrow.

9. Retract the drilling needle.

- The needle becomes blunt after one (or a few) drillings. Use new needles for each drilling.

10. Load the syringe with the cell suspension.

- Use a new syringe needle; do not use the same needle used for drilling.

11. Place the injection syringe needle in the exact previously drilled position.

12. Inject slowly 20 μ l cell suspension.

- Strong resistance indicates correct needle position. Tumor cells should be injected into the trabecular bone, not into the bone marrow space, unless the experiment is specifically intended to study tumor growth in the bone marrow.

13. When retracting the injection syringe, press the injection site with a cotton swab for 20–30 seconds.

5. Bring animals back to a clean cage and keep under warming lamp or on a heating pad. Monitor animals until complete recovery from anesthesia.

6. Tumor growth can be followed up by Faxitron X-ray images or bioluminescence if cells express an appropriate construct.

1. For $0.2\sim 1.5 \times 10^6$ PC-3 cells, bone tumor incidence is nearly 100%.
2. Morbidities such as impaired mobility, necrosis, lethargy, *etc.* require euthanasia.

7. Experimental treatment of animals

1. Depending on the proposed mechanism of anti-tumor effects (e.g. targeting tumor cell proliferation vs. targeting cells in the microenvironment such as endothelial cells and/or bone cells), the parameters of experimental treatment (e.g. starting point, duration, frequency, *etc.*) can be determined.
2. Dosage and route of administration (e.g. intra-peritoneal injection, oral-gavage, intra-venous injection, *etc.*) must be determined previously by pre-clinical pharmacokinetic and pharmacodynamic studies.
3. Reduction of tumor size by an experimental treatment can be to 10% of the un-treated control tumors (measured by wet tumor weight), as demonstrated by Kim *et al.* using multidrug-resistant PC-3MM2 cells and a platelet-derived growth factor receptor inhibitor (imatinib from Novartis) in combination with paclitaxel (Kim et al. 2006).

8. Necropsy and pathologic examination

1. Animals should be euthanized by an IACUC-approved method (e.g. CO₂ asphyxiation, anesthetic drug overdosing, *etc.*)
2. If necessary, take X-ray images (FaxitronR) of both tumor- and control-hind limbs before harvesting tumors.

3. Place the mouse in a supine position, and stretch both hind limbs.
4. Cut both legs with a single-edge prep razor blade (Fisher Scientific) at the level of mid-femoral or inguinal area. Both legs should be cut at the same level.
5. Measure weight of both hind limbs individually, and calculate tumor weight by subtracting control leg weight from tumor leg weight.
6. Strip off muscles and skin for histological preparation. Disarticulate the foot if necessary.
7. Fix tissues in 10% buffered formaldehyde (Fisher brand 10% Formalin from Fisher Scientific) for 24 hours, followed by decalcification (e.g. in 10% EDTA solution for 14 days with occasional buffer change). Adequate fixation and decalcification techniques must be used for antigen retrieval for immunohistochemical staining.
8. Embed in paraffin and section for histology.

BASIC PROTOCOL 4. VOSSICLE MODEL FOR STUDY OF TUMOR-BONE INTERACTIONS

To determine the effects to genetic alterations of the host microenvironment, implantation of tumor cells in the genetically modified host mice can be one of the best approaches. Alternatively, vertebrae from the transgenic mice can be implanted into athymic hosts with tumor cells. This vossicle model is a valuable approach to determine the effects of genetic alterations in the microenvironment on tumor development.

Materials

1. For the vossicle donor, postpartum day-4 neonatal mice of chosen background (e.g. transgenic or wild-type)
2. Sterile 60mm dishes. (BD Falcon)
3. Scissors (e.g. 4½ inch curved iris scissors), forceps (e.g. 4 inch 1 × 2 teeth straight iris tissue forceps), Metzenbaum scissors (e.g. curved 4 inch), scalpel holder and No. 15 surgical blades (Fisher Scientific).
4. Wound closing surgical metal clips, stapler and remover (e.g. AutoClipR applier, AutoClipR remover and 9mm EZ Clip) (BD Medical).
5. Male athymic mice of 6–12 weeks age, for host mice (from commercial vendors such as the National Cancer Institute-Frederick Animal Production Area (NCr *nu/nu*), Harlan Laboratories, and Charles River Laboratories.
6. Metastatic prostate cancer cells in culture such as PC-3 (ATCC). Expand the culture to produce at least 2–4 times the necessary cell number for injections.
7. Complete cell culture media (for PC-3, RPMI-1640 media, Gibco brand from Invitrogen) supplemented with 10% fetal bovine serum (HyClone brand from Thermo Scientific) and antibiotics (Invitrogen).
8. Sterile phosphate-buffered saline (PBS) and 0.25% Trypsin
9. 0.4% Trypan blue and hemacytometer
10. Ca²⁺-free and Mg²⁺-free Hanks' Balanced Salt Solution with Phenol Red Indicator (HBSS; from Invitrogen)

11. Sterile tubes are used for carrying cells to the surgery room (50ml conical tubes or Eppendorf tubes should be used, 15ml conical tubes are not a good choice).
12. Anesthetics: Pentobarbital (e.g., NembutalR) or Ketamine/Xylazine Mixture (Ketamine (100mg/ml):Xylazine (20mg/ml):Phosphate-buffered saline = 3:2:5) or isoflurane gas anesthetics (2% isoflurane mixed with 100% oxygen)
13. 70% ethanol gauze. (BD Medical)
14. HamiltonR syringes (e.g. Microliter Models No. 75 or 701 with Luer-tip termination, purchased from Hamilton Co.) and regular hypodermic needles (27½G × ½ inch) (BD Medical)

Steps and Annotations

1. Vertebral Isolation (Vossicle)
 1. Euthanize mice (decapitation, CO₂ asphyxiation, *etc*).
 - Euthanasia method must be justified and clearly stated in the IACUC protocol (see above).
 2. Disinfect back of mice with 100% ethanol spray.
 3. Incise skin with scissors along the entire length of back.
 4. Dissect lumbar vertebrae (by cutting just under the ribs and above pelvis, as depicted in Figure 4A, arrows), and strip off muscle and internal organs (Figure 4B).
 5. Transfer vertebrae to sterile PBS in a 60mm dish.
 6. Carefully dissect the vertebrae with a No. 15 surgical blade and cut to separate single vertebra (vossicle) (Figure 4B, dotted lines).
 - Carefully but firmly hold vertebrae with forceps when isolating single vertebra. One vossicle donor mouse can produce five to six vossicles.
 7. Transfer isolated vossicles into fresh PBS in a new 60mm dish until implantation.
 8. Isolated vossicles must be implanted to the host immediately. Properly coordinate donor and recipient before starting experiment (e.g. one donor mouse can produce five to six vossicles, thus anesthetize five recipient mice while preparing vossicles from one donor mouse).
2. Prepare cell suspension in Ca²⁺-free and Mg²⁺-free HBSS (as described in Basic Protocol 1, Step # 3).
3. Surgical implantation of vossicles
 - Anesthetize host animals (athymic mice; as described in Basic Protocol 1, Step #2).
 - Clean the dorsal surface of mice with 70% ethanol gauze.
 - Make two small midline incisions (approximately 1cm long) on the back (at the levels of forelimbs and hind limbs).
 - Create subcutaneous pouches by dissecting with Metzenbaum scissors. Pouches should be deep enough for vossicles to be transplanted into the flank (indicated as circles in Figure 4C).

- Inject the prostate cancer cell suspension into isolated vossicles. It is important to place the needle tip in the vossicle.
 - In general, $0.5\sim 1 \times 10^4$ cells in 3–5 μ l volume. However, the optimal number of cells should be determined beforehand (Koh et al. 2005; Liao et al. 2008; Pettway and McCauley 2008). A varying number of tumor cells can be tested for bone formation or destruction at a desired time point.
 - Priming the hypodermic needle with regular 1ml syringe before attaching to Hamilton Syringe will help remove trapped air bubbles.
 - Place one vossicle in each quadrant of the animal's flank (Figure 4C).
 - Mice are placed in a lateral position during transplantation.
 - Four implantations per mouse can be used for different endpoint histological analysis (e.g. immunostaining, histomorphometric analysis, *etc.*).
 - Close wounds with surgical wound clips.
4. Bring animals to clean cages, and monitor until the animals are completely awake.
 5. Monitor tumor growth
 1. Wound clips should be removed after two weeks.
 2. If tumor cells are labeled with luciferase, *in vivo* bioluminescence (as described in Basic Protocol 2. Step #7).
 3. If tumor cells are not labeled, determine the endpoint by harvesting and analyzing tumors serially in a preliminary experiment.
 6. Necropsy and pathologic examination
 1. Animals should be euthanized by an IACUC-approved method (e.g. CO₂ asphyxiation, anesthetic drug overdosing, *etc.*)
 2. Dissect vossicle implantation.
 3. If desired, take plain radiographic images (e.g. FaxitronR) and/or photographs of vossicles.
 4. Fix tissues in 10% buffered formaldehyde (Fisher brand 10% Formalin from Fisher Scientific) for 24 hours, followed by decalcification (e.g. in 10% EDTA solution for 14 days with occasional buffer change). Adequate fixation and decalcification techniques must be used according to antigen retrieval method of desired immunohistochemical staining.
 5. Embed in paraffin and section for histology.

COMMENTARY

Background Information

Prostate cancer is a major health problem in the United States, and is second only to lung cancer as a cause of cancer-related death in American men (Jemal et al. 2008). Similar to many other types of solid tumors, distant metastasis is the major cause of mortality and morbidity of prostate cancer patients. Cancer metastasis is a very selective process, determined not by anatomic vascular draining pathways, but by highly specific interactions

between disseminating tumor cells ('seed') and the microenvironment of target organ ('soil') (Paget 1889; Fidler 2003). As a result, tumor metastasis develops in a predictable manner, and prostate cancer has a very specific propensity to metastasize to bones and lymph nodes, and less frequently to lungs, liver, pleura and adrenal glands. Approximately 90% of advanced stage prostate cancer patients develop bone lesions, resulting in morbidities as severe bone pain, immobility, hematopoietic complications and spinal cord compression (Bubendorf et al. 2000; Rubin et al. 2000). Bone metastasis involves most frequently the pelvis, vertebrae and femurs. Currently, there is no effective cure for bone metastatic prostate cancer, and thus considerable further investigation is urgently required to understand the biology of metastasis and to develop novel therapeutics for afflicted patients. Many such investigations employ mouse models of prostate cancer.

The major impediment of mouse models to study prostate cancer growth and metastasis is experimental reconstitution of the tumor microenvironment. The prostate gland is comprised of three major types of cells (secretory luminal cells, basal cells and neuroendocrine cells). In addition to the parenchymal prostate tissue, increasing evidence now supports that fibromuscular tissue, supporting connective tissue, and endothelium all together significantly contribute to prostate cancer growth and metastasis (Cunha et al. 2003; Micke and Ostman 2004; Park et al. 2007; Paland et al. 2009), and it is practically impossible to experimentally reproduce the organ microenvironment *in vitro*. Moreover, bone, the most favored site of prostate cancer metastasis, is a very unique microenvironment, not only because of the matrix calcification but also because of multiple types of constituting cells, including bone cells (osteocytes, osteoclasts and osteoblasts), hematopoietic cells, immune cells, stromal cells, adipocytes and endothelial cells. Particularly, it is now firmly believed that hematopoietic stem/progenitor cells and hematopoietic stem cell niche are critical regulators of bone metastasis (Sun et al. 2005; Shiozawa et al. 2008), but this process remains poorly explored, partly due to the complexity of experimental models. Consequently, *in vivo* tumor models are fundamental and indispensable tools to study the biology of prostate cancer metastasis.

Mice are the most commonly used animal model of cancer research in general. Regarding prostate cancer, the murine prostate has somewhat different anatomical features from the human prostate, yet is similar enough to be considered a valid model for studying human prostate cancer (Abate-Shen and Shen 2002). In contrast to humans, the murine prostate gland consists of four distinctive lobes (the anterior, two dorsolateral and ventral lobes) (Huss et al. 2001). Although there is no definitive anatomic analogy between human and murine prostates, the murine dorsolateral prostate is most similar to the peripheral zone of the human prostate that is most prone to cancerous alterations (Huss et al. 2001). However, despite these anatomical distinctions, there exist striking similarities in the molecular mechanisms underlying disease progression in mice and humans (Huss et al. 2001). For example, human epithelium and murine mesenchyme (and vice-versa) can be recombined to form the prostate gland (Hayward et al. 1997), supporting the validity of murine prostate as a model to study microenvironment.

Critical Parameters and Troubleshooting

The most critical factor in establishing xenograft tumor models is to achieve consistency throughout the implantation of tumor cells. Generally, each experimental or control group contains 5 to 10 mice and tumor size variation should not exceed more than 10% of average tumor size to obtain statistically significant results. For this goal, several factors are important: 1) cells should be transplanted as soon as possible (ideally within one hour) after detachment from the culture, 2) animals should be randomized into experimental groups after tumor cell implantation, 3) injection volume should be consistent, 4) all animals should be injected by the same technique and by one investigator.

Troubleshooting: General

1. Tumor does not develop: Check contamination of cell culture with mycoplasma or murine viruses (VenorGeM Mycoplasma detection kit from Sigma; and commercial diagnostic services such as Bionique Testing Laboratories Inc. or Multiplexion). Optimize injection cell number (e.g. increase injection cell number and repeat the experiment). Check the specific pathogen-free condition of mouse colony (e.g. Research Animal Diagnostic Services from Charles River Laboratories, Inc).
2. Uneven tumor size: Agitate the cell suspension well for each syringe loading.
3. High surgery-related mortality: Reduce anesthetics dosage. Use heating lamp or heating pad to maintain body temperature.

Troubleshooting: Intra-prostatic injection

1. Tumor nodules in mesentery and abdominal cavity: Prevent leakage of tumor cell suspension after injection by holding cotton swab on the injection site for extended time. Confirm bulla formation when injecting.

Troubleshooting: Intra-cardiac injection

1. Loss of blood pulsation into syringe during injection: If correctly positioned, pulsated blood should be observed (in the hub of needle or in the cell suspension) during injection. Improper hand rest and positional change of needle while advancing syringe plunger is a potential problem. Firmly rest one hand on the supporting hand, with the elbow of the injecting arm attached to the torso. Use a syringe needle with clear hub to increase visualization of blood pulsation.
2. No metastasis (after 2–3 weeks follow-up): Confirm tumor cell circulation in the whole body by bioluminescence imaging within 24 hours after injection. Bioluminescence signaling should be detected in the whole body.

Troubleshooting: Intra-tibial injection

1. Ectopic tumor growth in the muscle, with or without tumors growing in the bone: Forceful drilling of injection site perforated through the tibia bone, leading to tumor inoculation in the muscle. Drilling should be performed gently with light force. Use a new needle for each mouse. After drilling, take X-ray to confirm the correct position in the trabecular bone.

Troubleshooting: Vossicle Model

1. This model can be prone to issues of variability. To ameliorate this problem, try to use the same vertebrae for implants (e.g. lumbar vertebrae L1–L3).
2. Variability can also be accounted for by increasing n values.
3. As many as 4 implants can be placed in each donor mouse. This number may increase the n values but statistically implants in the same host mouse cannot be treated as independent samples. Consultation with a statistician is helpful for appropriate experimental design. In addition and for example, one implant can be used for histologic embedding in paraffin; while another is embedded in plastic for a different analysis, and another analyzed biochemically.
4. Numbers of cells to inject for different cell lines needs to be evaluated prior to experimental testing and in order to assure appropriate tumor/bone interaction without allowing the tumor to take over the bone.

5. At times, cartilage will be carried into the implant and can be visualized histologically. Such remnants appear generally inactive and do not seem to alter the tumor growth.

Anticipated Results and Time Considerations

Orthotopic intra-prostatic implantation of PC-3MM2GL (5×10^4 cells in 50 μ l), a highly metastatic variant of PC-3 cells, will develop tumors of 1~1.5g (wet weight) in 4 weeks, with approximately 100% lymph node metastases (medial iliac lymph nodes in peri-aortic area). Experienced investigators can palpate tumors with fingers when tumor size is approximately 5–8mm (for PC-3MM2GL, 10–14 days after implantation). PC-3MM2GL is one of the fastest growing prostate cancer cells lines, and less aggressive cell lines (for example, LNCaP or PC-3 parental cells) will produce smaller tumors in the same period. A tumor size of 1~1.5g is generally the maximum (intra-prostatic) tumor burden allowed for mouse models.

The orthotopic injection model is well suited to examine effects of drug treatment on tumor growth and lymph node metastasis. A recent example of use of this model for the study of dasatinib, a Src family/Abl inhibitor now in clinical trial, is described by (Park et al. 2008). However, these models are also useful for examining effects of altering gene expression *ex vivo*, then determining the effects on tumor incidence as well as intra-prostatic growth and metastasis. For example, we examined the effects of decreased c-Met expression on tumor growth by infecting cells with an adenovirus harboring a ribozyme, as described below and detailed previously (Kim et al. 2003). The highly metastatic variant of PC3 cells, (PC-3P-LN4) were infected with replication-defective adenoviral vectors harboring a ribozyme to c-Met (Ad-c-Met) or with a control adenovirus control (Ad-PU-1). Subsequently, to produce orthotopic prostate tumors in nude mice, a single cell suspension of more than 95% viability containing 5×10^5 PC-3P-LN4 cells was injected as described in the above Basic Protocol 1. Mice were necropsied four weeks after implantation and tumor incidence, tumor weight and the incidence of lymph node metastasis were recorded. As shown in Table 1, a single *ex vivo* infection with an adenovirus harboring a c-Met ribozyme was sufficient to affect tumor incidence. As shown in Figure 7, with proper dissection, primary tumor and number of tumor-containing lymph nodes are easily detected.

In an intra-cardiac tumor inoculation model, bioluminescence imaging offers tremendous advantages in monitoring metastatic tumor development. Soon after tumor inoculation (within 24 hours), tumor cells in the general circulation should be confirmed by bioluminescence imaging (Figure 5A and B). When tumor cells are correctly inoculated in the arterial circulation, a bioluminescence signal will be evident throughout the whole body. Mice showing bioluminescence signaling only from the injection site (heart) indicate improper injection technique and thus the mice should be removed from the experiment. Within a week, most tumor cells will die in the circulation, and bioluminescence signaling will be observed in the reticuloendothelial phagocytic organs (i.e. spleen). PC-3 cells (2×10^5 cells in 100 μ l) and its derivatives typically develop metastatic lesions first within 2 weeks in the mandible (Figure 5C). Subsequently, metastatic lesions in the hind limbs can be observed (more than 90%) and other organs including ribs, sternum, kidney and adrenal glands are very common metastatic sites (Figure 5D and E).

The intra-tibial injection model will produce more uniform bone metastatic tumors within 4–6 weeks. Tumor growth can be followed by small animal X-rays (Faxitron X-Ray) or bioluminescence imaging. Depending on the characteristics of cell lines used, different lesions (i.e. osteoblastic, osteolytic or mixed) will be observed on plain X-rays. For example, PC-3 cells typically develop osteolytic lesions (Figure 6A) whereas MDA118b cells develop osteoblastic lesions (Figure 6B). LNCaP cells forms mixed lesions. LNCaP-

LN4 (1×10^6 cells in 20 μ l) or PC-3MM2 (2×10^5 cells in 20 μ l) will result in approximately tumor of 1–2g weight (calculated by subtraction of control leg weight from tumor leg weight) in approximately 6 weeks, and investigational treatment efficacy can be observed by improvement of bone architecture and reduction of tumor size (Figure 6B).

Intra-tibial injection leads to a reproducible, valuable model for drug testing. An example of its use was described by Kim *et al.* (Kim et al. 2006) for the study of imatinib (GleevecR or STI571) with or without paclitaxel for PC-3MM2-MDR cells, a drug-resistant variant of PC-3MM2 cells. To produce multi-drug resistant bone tumors, a single cell suspension of 4×10^5 PC-3MM2-MDR cells was injected into the proximal tibiae of nude mice as described in the Basic Protocol 3 above. Fourteen days later, mice were randomized ($n=10$ each) as follows:

1. Mice were treated with vehicle solution by daily oral administration and weekly i.p. injection,
2. Mice were treated with paclitaxel (8mg/kg, weekly i.p. injection) and vehicle solution (oral administration, daily)
3. Mice were treated with STI571 (50mg/kg, oral administration, daily) and vehicle solution (weekly i.p. injection)
4. Mice were treated with paclitaxel (8mg/kg, intraperitoneal injection, weekly) and STI571 (50mg/kg, oral administration, daily).

Treatment was continued for 10 weeks and mice were evaluated by the digital radiography (FaxitronR) following the last treatment. Mice were necropsied and tumor incidence, tumor weight and the incidence of lymph node metastasis were recorded. The results are shown in Table 2. An example of tumor growth/bone preservation is shown in Figure 8.

The vossicle model is an effective approach for certain applications where tumor/bone factors are under consideration. To investigate the role of genetic alterations of the host microenvironment (e.g. knockout a gene of interest specifically in the bone microenvironment, not in the tumor cells), experimental model can often be complicated since many genes important for bone microenvironment are lethal. To circumvent this possible problem, vertebrae of any given transgenic background can be implanted into athymic mice together with tumor cells. Luciferase-tagged tumor cells can be monitored *in vivo* via bioluminescence to monitor tumor growth over time. Complementary studies using vertebrae from luciferase-tagged mice and non-luciferase cancer cells can be used to monitor the osteoblastic response (i.e. increase in bone) over time. Bones from mice with lethal gene knockouts can be used to evaluate the genetic dependence of osteoblastic factors on tumor growth or *vice versa* and bypass other systemic issues. This model will provide strong tumor-bone interactive lesions within a short time (1–3 weeks) depending on numbers of cells used. The benefit of using neonatal vertebral bone is that it is particularly responsive to growth induction and readily forms woven bone of a similar appearance to that of the human osteoblastic response to prostate cancer. Osteolytic lesions will also present with this model and an aggressive tumor can result in total bone lysis in 4 weeks.

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Figure 1. Intra-prostatic implantation of tumor cells

A. Lower end of abdominal midline incision starts 5–8mm above the external genitalia and the incision is approximately 1.5–2cm long. Urinary bladder locates directly under the incision. Light press on both sides of incision will gently protrude urinary bladder.

B. Seminal vesicles are subsequently exteriorized with cotton swabs. Seminal vesicles are white sac-like organs, located directly adjacent to the bladder.

C. Tumor cells are injected into the dorsal lobe of prostate.

D. Rectum is gently dissected with a cotton swab. Margins of dorsal prostate (solid line) and rectum (dotted line) are marked. Note that urinary bladder is shrunk.

Figure 2. Intra-cardiac inoculation of tumor cells

A. Topographic anatomical points for intra-cardiac injection are depicted on a 10-week old male athymic mouse. Proper immobilization is important to achieve consistency. Upper limbs are stretched and taped, perpendicularly to the midline (dotted lines). Note that cross-abdominal taping (described in the text) is not shown on the picture. Jugular notch is located at the superior end of bony sternum; and xyphoid process is clearly visible. Lower margin of rib cage and midline are illustrated (solid lines). Injection point is 1–2mm left of the midline on the mouse and equally distant from both xyphoid process and jugular notch.

B. A loaded syringe is shown. Air bubble is required to allow blood pulsation. Advancing syringe plunger to 100 μ l graduation will inject equal volume of cell suspension.

Figure 3. Intra-tibial injection

A. Correct positioning and visualization of right tibia of a male athymic mouse are shown. A mouse is placed in a recumbent position, and ankle of right hind limb (medial and lateral malleoli) is tightly held. Combinatorial movements (circular arrow) of plantar flexion, lateral rotation and flexion of knee joint result in clear visualization of anterior crest of tibia (solid lines). Knee joint ligament is also clearly visible (dotted arrow).

B. A new 27½G $\frac{1}{2}$ inch long needle attached to a 1ml syringe is inserted percutaneously to the proximal tuberosity of tibia. Note that needle-syringe shaft is perfectly in line with long axis of tibia (solid line). Proximal tibia is drilled by rotational movement of syringe. Extra care must be paid not to change long axis of rotational movement while drilling.

C. A lateral view of drilling motion is depicted. Syringe axis is aligned with long axis of tibia (solid line). Skin and upper hind limb are extended gently by body weight (solid arrow and dotted line).

Figure 4. Vossicle model

A. A four-day old C57BL/6/J pup is shown. Lumbar vertebral column is marked (dotted box). To prepare vertebrae, make dorsal midline incision and cut the margin of lower rib cage and pelvis (two solid arrows).

B. Lumbar vertebral column is stripped of muscles and depicted (lateral view). Each vertebra is isolated by cutting inter-vertebral discs with a sharp surgical blade (dotted lines)

C. After tumor cells are implanted, vossicles are transplanted subcutaneously on the back (away from midline) of immunocompromised mice (dotted circles).

Figure 5. *In vivo* bioluminescence imaging of intra-cardiac inoculation model

A. A male athymic mouse is *in vivo* bioluminescence imaged 24 hours after luciferase-labeled PC-3 cells (2×10^5 cells) inoculation into the left heart ventricle. Bioluminescence signal emitted from the whole body area indicates correct injection of tumor cells in the systemic circulation.

B. Color representation of photon emission is adjusted on the same image (above **A.**) to show spatial distribution of bioluminescence signals. Although bioluminescence signal is emitted from the whole body, highly vascularized organs (liver, guts and lungs) and reticuloendothelial organs emits increased bioluminescence signals.

C, D and E. A representative mouse is selected to show progressive development of metastasis. Luciferase-labeled PC-3 cells (2×10^5 cells) are inoculated i.c. Mice develop typically mandibular lesions first at week-2 (**C.**) with less frequent hind limb lesions. At week-3 or -4, more than 90% of mice develop hind limb lesions (**D.**), and multiple organ metastasis are frequently observed at week-5 or -6 (**E.**)

Figure 6. Intra-tibial prostate tumor model

A. Plain X-ray images of six-week PC-3 intra-tibial tumor model. Two representative animals are shown. One hundred thousand PC-3 cells are inoculated in the right proximal tibia of male athymic mice. Contra-lateral hind limbs are shown as no-tumor controls. Note that PC-3 cells typically form osteolytic lesions.

B. Plain digital X-ray images of eight-week MDA118b orthotopic bone tumor model. 1.5×10^6 MDA118b cells were implanted in the right proximal tibia of male athymic mice. Animals were treated either with vehicle only or an experimental drug. MDA118b cells are very osteoblastic cells, and an experimental treatment decreased tumor development and improved bone structure on X-rays.

Figure 7. Intra-prostatic tumor model to examine effect of c-Met reduction on tumor incidence, growth and lymph node metastasis

A. Effects of mock infection of PC3-LN4 cells. Note the presence of large primary tumor (T) and two lymph node metastases (LN).

B. Effects of *ex vivo* infection of PC3-LN4 cells with Ad-PU-1 control adenovirus. Note presence of tumor and lymph node metastasis.

C. Effects of *ex vivo* infection of PC3-LN4 cells with Ad-c-Met adenovirus containing a ribozyme inhibiting c-Met expression. Figures are from (Kim et al. 2003)

Figure 8. Digital Radiography of hind limbs of nude mice bearing PC-3MM2-MDR tumors
After 10 weeks of treatment, mice receiving vehicle (control), paclitaxel (8mg/kg of body weight), STI571 (GleevecR or imatinib) (50mg/kg of body weight) or both paclitaxel and STI571 were anesthetized with Nembutal and their hind legs imaged by digital radiography. Integrity of tibias of mice treated with imatinib was improved compared to those treated with vehicle or paclitaxel. The combination of STI571 with paclitaxel provided the best preservation. Figure is from (Kim et al. 2006)

Table 1

Tumorigenicity and development of lymph node metastases induced by PC-3-LN4 cells after *ex vivo* infection of adenovirus or mock infection

Group	Tumor Incidence	Tumor Size (g) Median (Range)	Lymph Node Metastases
Mock	8/8	0.58 (0.31~1.01)	8/8
Ad-Pu-1	9/9	0.47 (0.01~2.45)	9/9
Ad-c-Met	1/10	0.07	0/1

Adapted from (Kim et al. 2003)

Table 2

Treatment of multidrug-resistant human prostate cancer (PC-3MM2-MDR) growing in the tibia of nude mice

Group	Tumor Incidence	Median bone tumor weight (g) and inter- quartile range	Lymph Node Metastases
Control	19/19	1.3 (1.0~1.9)	19/19
Paclitaxel	18/18	1.1 (0.9~1.7)	18/18
STI571	9/18	0.3 (0~1.2)	7/18
STI571+Paclitaxel	4/18	0.1 (0~0.3)	3/18

Adapted from (Kim et al. 2006)