Video Article

A "Patient-Like" Orthotopic Syngeneic Mouse Model of Hepatocellular Carcinoma Metastasis

Dibash K. Das[1,2], Victoria Durojaiye[1], Adeodat Ilboudo[1], Michelle K. Naidoo[1], Olorunseun Ogunwobi[1,2]

[1]Department of Biological Sciences, Hunter College of the City University of New York

[2]Departments of Biology and Biochemistry, The Graduate Center of the City University of New York

Correspondence to: Olorunseun Ogunwobi at Ogunwobi@genectr.hunter.cuny.edu

URL: https://www.jove.com/video/52858
DOI: doi:10.3791/52858

Keywords: Medicine, Issue 104, Survival surgery, metastasis, hepatocellular carcinoma, circulating tumor cells, orthotopic, syngeneic, solid organ tumors

Date Published: 10/24/2015

Citation: Das, D.K., Durojaiye, V., Ilboudo, A., Naidoo, M.K., Ogunwobi, O. A "Patient-Like" Orthotopic Syngeneic Mouse Model of Hepatocellular Carcinoma Metastasis. *J. Vis. Exp.* (104), e52858, doi:10.3791/52858 (2015).

Abstract

The majority of cancer-related deaths are caused by the metastasis of the cancer rather than the primary tumor itself. Yet, the underlying mechanisms of cancer metastasis are still unclear. Animal models are essential for elucidating the mechanisms and for evaluating novel strategies for the treatment of metastatic cancers. Here, an in-depth description of a “patient-like” orthotopic syngeneic mouse model for exploring the mechanisms of metastasis of solid organ tumors is provided. The survival surgical implantation of BNL 1ME A.7R.1 mouse hepatocellular carcinoma cells directly into the liver (the organ of origin) of the inbred wild-type immune competent laboratory mouse strain, BALB/c is described. The success and reproducibility of this methodology recommends it for widespread use in elucidating the biological mechanisms of solid organ cancer metastasis.

Video Link

The video component of this article can be found at https://www.jove.com/video/52858/

Introduction

Hepatocellular carcinoma (HCC) is one of the most lethal cancers, with poor prognosis and low life expectancy. Nearly all cancer-related deaths are due to the metastatic spread of the disease from the originating organ to additional distant organs[2-6].HCC progression is a complex process. Therefore, being able to model the tumor microenvironment that is naturally found in metastatic HCC in animal models can prove to be a successful and useful way to reveal relevant mechanisms in humans.[7]Unfortunately, the mechanisms of cancer metastasis are still largely unclear. Therefore, there is a need to establish animal models that will enable us to elucidate the underlying molecular mechanisms of metastases of cancers such as HCC[3,5].

Mouse model systems are a very useful approach for delineating mechanisms and evaluating novel strategies for treatment of metastatic human cancers[5,8]. The various mouse model systems that presently exist are a testament to efforts of researchers to correctly depict the complexity of the disease[5,8,9].

Ogunwobi and colleagues recently used a survival surgical approach to demonstrate establishment of a novel orthotopic syngeneic mouse model for the study of metastasis in HCC[3]. Their work established a “patient-like” mouse model that recapitulates features of aggressive and metastatic HCCs[3]. They further demonstrated that this mouse model system can be used to study the biology of circulating tumor cells, and that this holds potential for gaining novel insights into the mechanisms of cancer metastasis[3].

The aim of this paper is to describe in detail the methodology used in establishing this “patient-like” orthotopic syngeneic mouse model of HCC metastasis[3]. The methodology of how to implant BNL 1ME A.7R.1 mouse HCC cells directly into the liver (the organ of origin) of the inbred wild-type immune competent laboratory mouse strain, BALB/c[3] using survival surgery will be described. Unlike other mouse xenograft tumor models where human tumor cells are implanted into immune deficient mice, this system is syngeneic and is, therefore, suitable for studying the role of the immune system in tumor metastasis[3,5,8]. This approach will likely gain widespread use for studying the mechanisms of metastasis in solid organ cancers.

Protocol

Ethics Statement
All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of Hunter College of The City University of New York.

Note: Eight BALB/c mice were used in this experimental procedure. Five BALB/c mice were implanted with 5 X 10^6 BNL 1ME A.7R.1 mouse hepatocellular carcinoma cells to produce the primary tumors. Three BALB/c mice with no implantations were used as controls. BNL 1ME A.7R.1 is a murine hepatocellular carcinoma cell line that was derived by chemical transformation from the murine non-tumorigenic hepatocyte cell line BNL CL.2. BNL 1ME A.7R.1 was obtained from the American Type Culture Collection (ATCC).

1. Pre-surgery

1. Ensure that mice for all survival surgeries are healthy. To ensure that mice are healthy, inspect signs of dehydration by tenting of the skin. Do not use mice with any evidence of disease or distress in these experiments.
2. Weigh mice to ensure they are not underweight. Ensure that the weight is at least 16-20 grams.
3. Prepare 5×10^6 BNL 1ME A.7R.1 mouse HCC cells for implantation per mouse:
    1. Aspirate media from a 10 ml 60% - 70% confluent cell culture dish of BNL 1ME A.7R.1 mouse HCC cells. Wash twice with 5 ml PBS. Add 2 ml of trypsin to the dish and incubate at 37 °C in a humidified incubator with air and 5% CO_2 for 5 min.
    2. Subsequently, pipette 10 µl trypsinized cells into a manual hemocytometer for cell counting under a microscope at magnification of 10X. Count the total number of cells found in the four large corner squares and multiply the dilution factor by the total number of cells. Then, divide by the number of squares counted and multiply by 10^6 for the cell concentration (cells per ml).
    3. Using the calculation, collect 5 million cells per mouse in sterile 1.5 ml microcentrifuge tubes. Label 1.5 ml microcentrifuge tubes.
4. Resuspend the 5×10^6 BNL 1ME A.7R.1 mouse HCC cells in 100 µl of phosphate buffered saline (PBS). Keep these cells on ice till implantation in surgery.
5. Perform activities required for preparation of mice for surgery such as anesthesia induction and hair clipping away from sterile equipment and materials to avoid contamination.
6. Induce general anesthesia in mice by inhalation of isoflurane using an isoflurane vaporizer along with oxygen at a constant concentration flow rate of 1 L per min (approx. 2.1%). Note: The system is self-reliant, and includes a precision vaporizer, an oxygen supply and a charcoal canister that is linked to a breathing circuit needed for gas/anesthetic scavenging.
7. Ensure that anesthesia is adequate to prevent the animal from undergoing pain during the surgery. Perform toe-pinch and observe animal for non-responsiveness to confirm anesthesia.
8. At that time, switch the valve line, seal to the box and open to the nose cone.
9. As soon as the mouse is anesthetized, apply a tiny amount of an ophthalmic ointment to the corneas as a preventive measure from drying and damage.
10. Subsequently, move the animal to the preparation area, with the nose cone properly placed and connected (for maintenance anesthesia). Prepare the site of the surgical incision for surgery by depilation at this preparation area.
11. Ensure that the area of surgery is at a location free of all other activity.
12. Ensure that the work surface is sturdy and appropriately sanitized, unsoiled and clutter-free before the surgery. Place a sterile piece of linen or surgical tray over it.
13. Ensure that the operative field is available to only persons carrying out the surgery. Ensure that the surgical room is cleaned and swept with an appropriate cleaner and sprayed with an appropriate disinfectant.
14. Ensure that it is also positioned away from doors and windows as air currents can spread dust leading to contamination of the site.
15. Perform the survival surgery with sterile instruments. Either soak instruments in a disinfectant according to manufacturer's recommendation or autoclave. Use basic sterile supplies that are ready for use.
16. Separate instruments according to function to help prevent contamination of sterile areas.
17. Shave approximately 1 inch around the site of incision (midline incision) to access the liver of the animal. Remove the hair from the animal away from the site of surgery.
18. After shaving, scrub the area with a soapy disinfectant (betadine surgical scrub) and wash the excess disinfectant away with sterile PBS solution. Perform this several times, with the entire scrub process lasting approximately 5 min. Remove and discarded the used gloves.
19. Afterward, change to protective clothing such as surgical gown, scrub top, and face mask. Next, sanitize hands with proper scrub solution before putting on surgical gloves. Note: This is a necessary step to prevent shedding and contaminating the surgical site with potential contaminants.

2. Surgery

1. Provide an external heat source for warmth throughout the entire period of anesthesia. The animal may experience some hypothermia because anesthesia causes disturbances of thermoregulation; therefore, maintain the body temperature.
2. Lay the mouse on its back throughout the surgery.
3. Fix the fore limbs, hind limbs and tail of the mouse to the surgery table with pieces of surgical tapes.
4. Make a straight line incision immediately inferior to the xyphisternum.
5. Use a sterile self-retaining surgical retractor to expose the liver.
6. With a syringe having a 20G needle, slowly and carefully inject 100 µl of PBS containing 5×10^6 BNL 1ME A.7R.1 mouse HCC cells into the right lobe of the liver.
7. After removing the needle, apply a sterile cotton swab for at least 1 min to stop any potential bleeding due to high vascularity of the liver.
8. Close the subcutaneous tissue with an absorbable suture followed by skin closure using surgical clips. Subsequently, clean the wound once again with betadine scrub. Note: This will ensure the closure of the surgical wound.
9. Subcutaneously give 100 µl of normal saline.

3. Post-surgery

1. Immediately after surgery, transfer the mouse to a clean cage with a post-surgery identification card to notify caretakers of the mouse condition. Provide food and water. Note: In our experience, a mouse that has just undergone surgery will fare best if housed alone with wet food and water for the initial 24-hr period post-surgery.
2. Keep the animal warm with a heat lamp that generates heat not surpassing 38 ºC. Use a thermometer to measure the temperature until the mouse is fully recovered.
3. House the animal in a cage preferably with paper bedding to ensure comfort.
4. After initial recovery, monitor the animal for signs of discomfort, bleeding, pain and distress. Give buprenorphine (0.05 -0.1 mg/kg) to relieve pain. Closely monitor the activity, behavior and appearance, as well as water and food consumption for approximately 2 hr. If ambulatory, move the animal back to the area for regular normal housing of mice and continue to monitor at 6-12 hr intervals for 24 hr post-surgery. Perform a weight check if desired.
5. However if the animal is having issues recuperating, take appropriate supportive care actions. If mice are unable to recover, humanely euthanize mice via CO_2 asphyxiation and cervical dislocation. Follow approved institutional guidelines for euthanasia.
6. Post-surgery, check for clinical evidence of HCC which is usually observable as significant reduction in body condition score. Note: At this point, a primary tumor has likely developed in the liver and metastatic deposits have likely developed in the lungs.
7. At this point, sacrifice all mice (experimental and control) involved in the experiment by humane euthanasia. Subject the mice to carbon dioxide asphyxiation: very slowly release carbon dioxide into the chamber over a period of 5 -10 min. This will cause respiratory arrest.

Representative Results

The livers of Balb/c mice were implanted with 5×10^6 BNL 1ME A.7R.1 mouse HCC cells. Clinical evidence of HCC development was observable 63 days post-surgery. Consequently, mice were humanely euthanized. Necropsy was performed on the euthanized mice. The lungs and liver were resected and a careful gross examination was performed to identify macroscopic tumors. In one mouse, a superficial tumor was observed on the surface of the liver with some attachment to the abdominal wall (**Figure 1A**). In a second mouse, an abnormally-looking liver was observed. The abnormality very strongly looks like a superficial tumor (**Figure 1B**). As expected, mice in the control group with no implantation of BNL 1ME A.7R.1 HCC cells developed no tumors, as evidenced by a liver (**Figure 1D**) and lungs (**Figure 1E**) from a control mouse. Also, the lungs from the mice with tumors looked somewhat abnormal, displaying areas of hemorrhagic necrosis (**Figure 1C**). Based on previous experience, these lungs may harbor metastatic deposits[3].

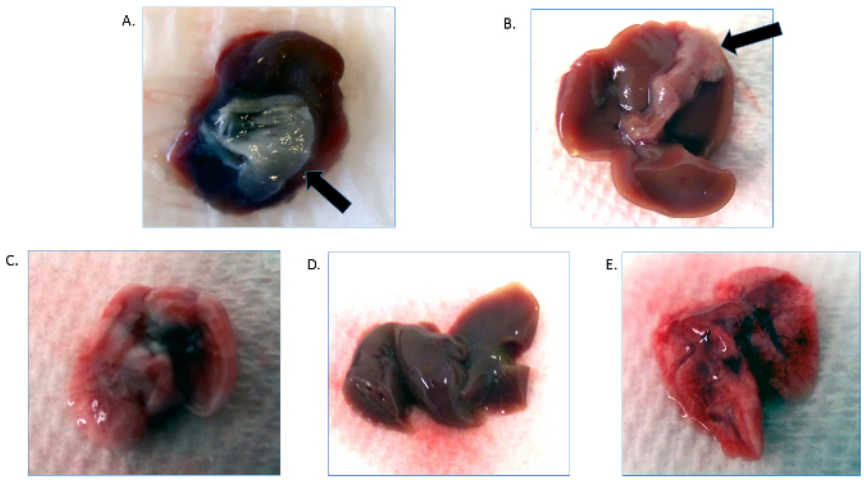

Figure 1. Gross examination of tumor and metastasis. Balb/c mice were implanted with 5 X 10^6 BNL 1ME A.7R.1 tumor cells and euthanized 63 days later. Representative liver tumors observed were photographed and shown here. **(A and B)** both show superficial liver tumors. **(C)** is lungs from tumor cells implanted mice. **(D)** displays liver of a control mouse. **(E)** indicates lungs of a control mouse. Please click here to view a larger version of this figure.

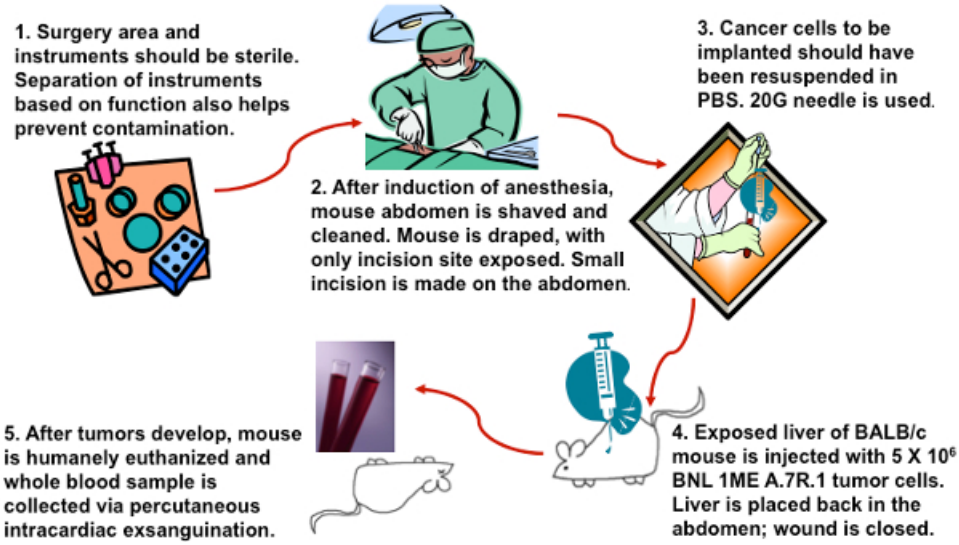

Figure 2. Schematic of the methodology of a "patient-like" orthotopic syngeneic mouse model of hepatocellular carcinoma metastasis. Once anesthetized, mouse liver is implanted with 5 X 106 BNL 1ME A.7R.1 mouse hepatocellular carcinoma (HCC) cells. When clinical evidence of HCC is observable, animal is sacrificed by humane euthanasia (subjected to carbon dioxide asphyxiation, followed by percutaneous intracardiac exsanguination and cervical dislocation). Please click here to view a larger version of this figure.

Discussion

In this article an in-depth description of the method is given that was recently reported by Ogunwobi and colleagues of successful establishment of an orthotopic syngeneic mouse model of HCC metastasis (**Figure 2**)[3]. The take rate of tumors for this procedure is generally high. We have previously observed a tumor take rate of 100%[3]. However, the take rate can be variable depending on the competence of the investigator. In a recent experiment performed by a new graduate student under training, the tumor take rate was as low as 40%. Although most experiments are terminated at 4 - 6 weeks due to decline in body condition score, we believe from experience with subcutaneous implantation of the same cells into the same mice that primary tumors may be established by 2 - 3 weeks. We usually expect to see one tumor nodule from a successful experiment. And although we expect lung metastasis to develop between 4 - 6 weeks, it is conceivable that tumor in the liver may take place much earlier.

Good surgical techniques and aseptic conditions are essential to the success of this survival surgery method. Mice used for survival surgery should be healthy. Weighing pre and post-surgery is a good practice. The average pre-surgery weights of the mice used in or most recent experiment was 18 grams, and at the experimental end-point (63 days post surgical implantation) their average weight was only 22 grams. A gain of only 4 grams of weight during that time period is likely because there was some decline in weight gain due to systemic illness from the tumor. We would have had a more objective assessment of this had we measured mouse weight once or twice weekly throughout the experiment. This is recommended best practice.

As summarized in **Figure 2**, mice should be adequately anesthetized before the surgery and throughout the period of surgery to avoid pain and distress. The area should be free of all clutter, and access should only be available to those performing the surgery. Both the surgery area as well as the instruments used should all be sterile.

A vital step for the success of implantation is the preparation of the BNL 1ME A.7R.1 mouse HCC cells to be implanted. During the preparation, it is crucial to avoid contamination of the collection of the cells from confluent cell cultures dishes. These steps should be performed in a cell culture hood under sterile conditions. A cell count should be also done to ensure 5×10^6 BNL 1ME A.7R.1 mouse HCC cells have been collected in a sterile 1.5 ml microcentrifuge tube per mouse that is resuspended in 100 µl phosphate buffered saline (PBS). Trypan blue staining can also be optionally performed to confirm the viability of the cells. These cells should be kept on ice no longer than 2 hr before implantation in livers of mice.

The implantation of the cancer cells should be handled with care due to the delicate nature of the liver (**Figure 2**). During injection of the cancer cells, avoid too deep a puncture that may result in the needle going through the entire liver. It is essential to inject the cancer cells slowly and carefully. On the other hand, too shallow a puncture with the needle prior to injection will likely cause spillage of the cancer cells injected out into the peritoneal cavity. Also, after removing the needle, there is always a potential of the organ bleeding out due to its high vascularity. Therefore, applying a sterile cotton swab for at least 1 min is recommended. Care and attention will ensure the highest rate of implantation success. The animal should also be provided an external source of heat throughout the procedure as hypothermia is a major concern causing disturbances of thermoregulation during anesthesia. Post-surgery, the animal should be monitored for any signs of discomfort or pain at which point appropriate care should be administered. If the mouse is unable to recuperate from the surgery, it should be humanely euthanized.

As cancer metastasis is the primary cause of fatality from cancer, it is crucial to delineate the mechanisms of hematogenous dissemination[2-4]. Novel circulating tumor cells were established from this orthotopic syngeneic mouse model of HCC metastasis[3]. Moreover, the CTCs derived from this model consistently show increased tumor initiation and metastatic capability[3]. The circulating tumor cells isolated using this method may prove to be very important as they help to unravel specific molecular factors, markers and possible therapeutic targets of cancer metastasis[2-4]. This methodology has already identified that there is increased upregulation of hepatocyte growth factor (HGF) and its receptor,

c-Met in circulating tumor cells as compared to the primary tumor cells of HCC[3]. These results build on previous work demonstrating that an important aspect of tumor metastasis is the cellular process of epithelial-mesenchymal transition[2-4,10]. Further characterizing circulating tumor cells using this novel model will increase our understanding of the intricate mechanisms of factors that promote the process thus leading to the development of more effective management of HCC[1-3,10].

Currently, the most widely used animal tumor models are mouse xenograft tumor models wherein human tumor cells are implanted into immune deficient mice. The model described in this article has important advantages because it is syngeneic and is, therefore, suitable for studying the role of the immune system in tumor metastasis[3]. This model has proven to be highly reproducible for isolating, culturing, and studying of viable circulating tumor cells[3]. It, therefore, has tremendous potential for evaluating clinical applications like screening, diagnosis, prognostication, therapy monitoring and discovery of novel therapeutic strategies. A limitation of the methodology described above is that a significant portion of BNL 1ME A.7R.1 mouse HCC cells implanted into the mice will be incapable of surviving and thus only a small portion of the cells will be viable and adept at introducing tumors at secondary sites[3]. We account for this limitation by initial implantation of 5×10^6 BNL 1ME A.7R.1 mouse HCC cells per mouse.

In conclusion, this orthotopic syngeneic mouse model of metastatic solid organ cancer provides a good platform for elucidating the biological mechanisms underlying the metastatic capability of circulating tumor cells in the blood of cancer patients[3]. This in turn will likely shed novel insights into the mechanisms of cancer metastasis because cancer metastasis occurs primarily via hematogenous spread[11-13]. Novel insights thus gained into the mechanisms of cancer metastasis will be critical to the development of novel beneficial clinical applications[3,14,15].

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

We would like to thank Pascal DuBois for proof-reading the article. The authors would also like to thank the personnel of the Animal Facility at Hunter College of The City University of New York. This work was supported by a NIH RCMI grant to Hunter College.

References

1. Sheng, W. *et al.* Capture, release and culture of circulating tumor cells from pancreatic cancer patients using an enhanced mixing chip. *Lab on a Chip*. **14**, 89-98, doi:10.1039/c3lc51017d (2014).
2. Ogunwobi, O. O., & Liu, C. Therapeutic and prognostic importance of epithelial-mesenchymal transition in liver cancers: insights from experimental models. *Critical Reviews in Oncology/Hematology.* **83**, 319-328, doi:10.1016/j.critrevonc.2011.11.007 (2012).
3. Ogunwobi, O. O., Puszyk, W., Dong, H. J., & Liu, C. Epigenetic upregulation of HGF and c-Met drives metastasis in hepatocellular carcinoma. *PloS One*. **8**, e63765, doi:10.1371/journal.pone.0063765 (2013).
4. Ogunwobi, O. O., & Liu, C. Hepatocyte growth factor upregulation promotes carcinogenesis and epithelial-mesenchymal transition in hepatocellular carcinoma via Akt and COX-2 pathways. *Clinical & Experimental Metastasis.* **28**, 721-731, doi:10.1007/s10585-011-9404-x (2011).
5. Fujiwara, S. *et al.* A novel animal model for *in vivo* study of liver cancer metastasis. *World Journal of Gastroenterology : WJG.* **18**, 3875-3882, doi:10.3748/wjg.v18.i29.3875 (2012).
6. Wirtz, D., Konstantopoulos, K., & Searson, P. C. The physics of cancer: the role of physical interactions and mechanical forces in metastasis. *Nature Reviews. Cancer.* **11**, 512-522, doi:10.1038/nrc3080 (2011).
7. Heindryckx, F., Colle, I., & Van Vlierberghe, H. Experimental mouse models for hepatocellular carcinoma research. *International Journal of Experimental Pathology.* **90**, 367-386, doi:10.1111/j.1365-2613.2009.00656.x (2009).
8. Zhang, L. *et al.* An *in vivo* mouse model of metastatic human thyroid cancer. *Thyroid : Official Journal of the American Thyroid Association.* **24**, 695-704, doi:10.1089/thy.2013.0149 (2014).
9. Menezes, M. E. *et al.* Genetically engineered mice as experimental tools to dissect the critical events in breast cancer. *Advances in Cancer Research*. **121**, 331-382, doi:10.1016/B978-0-12-800249-0.00008-1 (2014).
10. Ogunwobi, O. O., Wang, T., Zhang, L., & Liu, C. Cyclooxygenase-2 and Akt mediate multiple growth-factor-induced epithelial-mesenchymal transition in human hepatocellular carcinoma. *Journal of Gastroenterology and Hepatology.* **27**, 566-578, doi:10.1111/j.1440-1746.2011.06980.x (2012).
11. Hanahan, D., & Weinberg, R. A. The hallmarks of cancer. *Cell.* **100**, 57-70, doi:10.1016/S0092-8674(00)81683-9 (2000).
12. Chambers, A. F., Groom, A. C., & MacDonald, I. C. Dissemination and growth of cancer cells in metastatic sites. *Nature Reviews. Cancer.* **2**, 563-572, doi:10.1038/nrc865 (2002).
13. Kim, M. Y. *et al.* Tumor self-seeding by circulating cancer cells. *Cell.* **139**, 1315-1326, doi:10.1016/j.cell.2009.11.025 (2009).
14. Stolpe, A., Pantel, K., Sleijfer, S., Terstappen, L. W., & den Toonder, J. M. Circulating tumor cell isolation and diagnostics: toward routine clinical use. *Cancer Research.* **71**, 5955-5960, doi:10.1158/0008-5472.CAN-11-1254 (2011).
15. Saad, F., & Pantel, K. The current role of circulating tumor cells in the diagnosis and management of bone metastases in advanced prostate cancer. *Future Oncology.* **8**, 321-331, doi:10.2217/fon.12.3 (2012).

