Bioluminescence Imaging of Canine Osteosarcoma in an Orthotopic Murine Model

THESIS

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By

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Abstract

Osteosarcoma (OSA) is the most common primary bone neoplasm in dogs and humans, and behaves aggressively in both species. In canine patients, lung micrometastases are often present at the time of diagnosis. Mouse models have been enormously valuable in aiding our understanding of the biology of OSA growth and metastasis, but it is often difficult to detect micrometastasis in the live animal. Non-invasive imaging techniques such as computed tomography (CT), positron-emission tomography (PET) and bioluminescence imaging (BLI) are useful tools for detecting and monitoring the growth of primary and metastatic tumors, as well as tumor response to chemotherapy. Oftentimes these modalities are combined to provide a more complete assessment of the question at hand. In previous work, it has been demonstrated that PET imaging lacks the sensitivity to track tumor metastasis in a mouse model of canine OSA. In this study, we evaluated the utility of BLI in quantifying tumor growth and metastasis, and determined the feasibility of developing a viable mouse model with a reliable luciferase reporter in a canine osteosarcoma cell line. The findings from this study will allow us to further refine our mouse model for future use in OSA therapy trials by ensuring a highly successful metastatic rate at the start of the experiment. Ultimately, an improvement in recognition
of metastases may provide a guide for individualized chemotherapy in humans as well as companion animals.
Acknowledgments

I would like to express my sincere gratitude to Feng Xu for her invaluable assistance in this project and to Krista LaPerle, D.V.M., Ph. D., D.A.C.V.P., for her assistance in reviewing Aperio and ImageScope with me. I would like to thank my research advisor, Matthew J. Allen, Vet. M.B., Ph. D., for his guidance and support and my clinical advisors and committee members Wm Tod Drost, D.V.M., D.A.C.V.R. and Amy Habing, D.V.M., D.A.C.V.R. for their guidance and support throughout my residency. Finally, I would like to thank my predecessor, Alexis McMurray, D.V.M., M.S., D.A.C.V.R., for her guidance as my resident mate and for her previous work involving image metastatic osteosarcoma.
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1998.................................................................B.A. Biology,
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Chapter 1: Introduction

Osteosarcoma is biologically aggressive, with a strong predilection for forming distant metastases, most commonly within the pulmonary parenchyma. In the canine patient, the disease is most prevalent in large and giant breeds, with approximately 75% of tumors affecting the appendicular skeleton.\textsuperscript{22} Largely because of this breed association, a hereditary basis for the development of osteosarcoma is suspected and there is evidence to support this theory, particularly involving the well-documented $p53$ and $RB$ genes.\textsuperscript{3} In the dog, tumors typically affect the metaphyseal regions of long bones, with the distal radius and the proximal humerus as the two most common sites.\textsuperscript{3} Osteosarcoma also has a bimodal distribution in the dog with the first peak incidence at 18-24 months of age, and a second peak occurring around 7 years of age.\textsuperscript{3} Once there is radiographic evidence of metastasis, most dogs develop clinical signs such as malaise within one month. For staging purposes, thoracic radiographs are performed, with less than 10% of dogs demonstrating radiographic evidence of metastatic disease at the time of primary tumor diagnosis.\textsuperscript{3} In humans, 30-40% of patients with osteosarcoma will die from metastases, despite aggressive medical therapy.\textsuperscript{4} It is a clinician’s goal to detect metastatic disease as early as possible, as its presence affects patient prognosis as well as therapy. Improvements in the ability to detect, treat and ideally prevent metastasis have tremendous potential to significantly impact clinical outcomes for patients with this
devastating disease. Given the critical importance of metastasis in the biology of osteosarcoma, it should come as no surprise to find that a significant amount of research effort is being made to develop and characterize laboratory models for studying osteosarcoma growth and metastasis. Much of this work involves mouse models in which xenograft human or canine osteosarcoma cells are implanted into either heterotopic (e.g. subcutaneous) or orthotopic (bone) sites. Within our research group, we have elected to focus on mouse models of canine osteosarcoma with the expectation that the preclinical data from these models will be readily transferable into both the veterinary and the human clinic. We have worked with both standard intra-osseous injection models, as well as with a newer tumor implantation model in which small fragments of OSA tumor are implanted directly into bone. The primary advantage of the solid tumor fragment model is that it is associated with a significantly reduced risk of embolic spread of tumor to the lung, making the model potentially more relevant as a surrogate for the natural disease in both dogs and humans.

With both injection and implantation models, there is rapid and predictable initial growth of tumor (over 3-4 weeks) followed by spread to the lung. Surgical removal of the tumor, by amputation of the affected leg, makes it possible to maintain the animal for an extended period of time and to explore the pathophysiology and treatment of metastasis. However, at present our ability to leverage the full potential of these mouse models is limited by the lack of a validated, sensitive and inexpensive ante mortem method for detecting and quantifying tumor burden in the lung.
In a recent study in mice, McMurray et al. showed that micro-CT/PET imaging does not accurately quantify canine osteosarcoma metastases, but can increase the efficiency of detecting its presence. As an extension to that study, which made use of relatively sophisticated and expensive imaging modalities, we decided to explore the potential for using bioluminescent imaging (BLI) to track tumor growth in real time. Previous studies have shown that bioluminescent signals are detectable earlier in the disease course, compared to radiography, and that BLI correlates well with microscopic bone marrow metastases, establishing this system as a sensitive in vivo test for studies of metastatic osteosarcoma.

For this Master’s project, we hypothesized that BLI would provide a sensitive, specific and quantifiable marker of tumor metastasis to the lung. We compared the results from BLI analysis to both gross and microscopic pathology in both primary and metastatic OSA.
Chapter 2: Materials and Methods

Cell Lines

The original Abrams canine osteosarcoma cell line was obtained from Dr. Doug Thamm at Colorado State University. The cell line was subsequently transduced with a lentiviral proviral plasmid vector (pLentiloxEV-Luc) containing the firefly luciferase gene under the control of the CMV promoter (Vector Core, University of Michigan Medical School, Ann Arbor, MI). Transduced cells were selected through antibiotic challenge and the stability of the Luc insert verified by in-vitro testing prior to use in mouse work. For routine cell culture, the Abrams-Luc cells were maintained in Dulbecco’s modification of Eagle’s Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine and 1% penicillin-streptomycin solution. To address the issue of relevance to tumors other than osteosarcoma, we also used a commercially available, luciferase-expressing human breast cancer cell line (MDA-MB-231 D3H2; Perkin Elmer Life Sciences, Waltham, MA) that was also maintained in DMEM/10% FCS. Cells were passaged when they were determined to reach ~70% confluency. In preparation for in vivo use, cells were suspended in PBS at a concentration of 1 million cells per milliliter.
Validation of Luciferase Expression

A dose-response curve was performed using both cell lines and a linear relationship was established between cell number and luciferase output (as measured in terms of photon flux).

Experimental Animals

Female athymic nude mice (Fox nu/nu), 4-5 weeks of age, were obtained from Taconic Farms (Germantown, NY). Animals were housed in groups of 5 per cage, fed a commercial mouse chow and provided access to water ad libitum. Mice were provided with appropriate nutritional and environmental enrichment, as mandated by local IACUC guidelines, for the duration of the experimental period.

Orthotopic (Intratibial) Injection of Tumor Cells

The mice were placed in a Plexiglas inhalation chamber and general anesthesia induced with inhaled isoflurane (1-5% in oxygen). Subsequently, the mice were removed from the chamber and placed in dorsal recumbency in preparation for injection. Anesthesia was continued using a face mask to deliver isoflurane in oxygen. Two different sites were injected, depending on the cell line that was being used. For the Abrams-Luc (OSA) cells, the hind limb was scrubbed and draped for aseptic surgery, as per established by prevailing IACUC guidelines for aseptic surgery in rodents. The stifle joint was flexed and 100,000 to 1,000,000 tumor cells (in a volume of 10 or 20 µl) were injected into the proximal tibia using either a glass syringe fitted with a 27-gauge needle, or a standard insulin syringe fitted with a 29-gauge needle. Upon completion of the
injection, the left and/or right ear was notched to provide a unique animal identifier, buprenorphine (0.01 mg/kg SC) was administered to control post-procedural pain relief, and the mice were removed to a heated recovery cage for post-operative monitoring. Mice were examined daily and weighed twice weekly to assess for evidence of ill health. Mice were euthanized if they showed evidence of significant weight loss (20% of baseline body weight), signs of pulmonary compromise (e.g. dyspnea) or overt lameness consistent with pathological fracture.

**Orthotopic (Intratibial) Implantation of Solid Tumor**

In a subset of experiments, solid tumor fragments (rather than cells in suspension) were evaluated in mice. Tumor xenograft tissue was initially generated by injecting Abrams-Luc cells subcutaneously and then harvesting the resulting tumor, which was then minced and frozen in cell culture freezing medium (DMEM containing 10% v/v DMSO and 20% v/v FCS).

For surgical implantation, mice were anesthetized as described previously and the right hind limb was scrubbed with surgical antiseptic and isolated with sterile paper drapes. The medial aspect of the proximal tibia was exposed through a 5-mm skin incision and a small hole was drilled through the medial tibial cortex with a 27-gauge needle. Under direct magnification with a surgical microscope, tumor fragments were inserted into the medullary cavity of the tibia. The skin edges were then apposed with cyanoacrylate surgical adhesive. Post-operatively, mice were injected with buprenorphine (0.01 mg/kg SC twice daily) and then transferred to a warmed cage for recovery.
Radiographic Assessment of Bone Disease Secondary to Tumor Growth

Microradiography was used to monitor the local effects of tumor cells growing within bone. Radiography was performed in digital cabinet microradiography unit (Model LX-60; Faxitron Bioptics LLC, Tucson, AZ) operating at 30kV with an exposure time of 5 seconds while mice were sedated and recovering from anesthesia. Radiographs were generally repeated at 2-week intervals, or more frequently in the event that mice displayed evidence of clinically significant lameness after tumor injection. Radiographs were scored for the presence and severity of tumor-mediated osteolysis, according to a published scoring scheme.

Surgical Amputation of the Hind Limb

For hind limb amputation, mice were anesthetized with isoflurane (induced in an anesthesia box and maintained via facemask) and the hind limb was aseptically prepped and draped for surgery. A circumferential, elliptical incision around the mid-thigh was made and the skin was reflected. Two to three stainless steel ligating clips (Ethicon Endo-surgery, Blue Ash, OH) were used to ligate the femoral artery. The thigh muscles were transected between the clips and reflected off of the proximal aspect of the femur. The coxofemoral joint capsule was transected to expose the femoral head. The cut end of the sciatic nerve was anesthetized with topical lidocaine (2% v/v) and the muscles of the hip region were sutured over the acetabulum using 5/0 polydioxanone (PDS II; Ethicon, a Johnson and Johnson Company, Somerville, NJ). The skin incision was closed with intradermal sutures of the same material, reinforced with cyanoacrylate tissue adhesive.
Postoperatively, mice were treated with buprenorphine (0.01 mg/kg SC twice daily) and meloxicam (1 mg/kg SC once daily) for three days.

**In Vivo Bioluminescent Imaging**

For in-vivo imaging, we used an IVIS® system that was initially developed by Xenogen corporation. The system is comprised of a cooled, charge-coupled device (CCD) camera, which sits above a light-tight, dark colored imaging chamber into which animals are positioned. To increase sensitivity to very low levels of light, the camera is super-cooled to approximately -90°C, effectively reducing thermal noise. Two different imaging systems (IVIS 100 and IVIS Spectrum), at two different locations, were used during the course of this study. Different experiments were always initiated and completed on the same instrument to avoid any concerns about variations in signal detection between the two instruments.

In preparation for *in vivo* imaging, mice received an intraperitoneal injection of D-luciferin (PerkinElmer, Waltham, MA) at a standardized dose of 150 mg/kg in a 0.15 mL volume. After approximately 10 minutes, mice were placed in a chamber containing isoflurane and oxygen, for induction of anesthesia, then transferred to the IVIS® imaging chamber where mask inhalation anesthesia was maintained with isoflurane. Images were acquired for periods of between 10 and 60 seconds. Regions of interest (ROI) were drawn on saved images to include color pixels corresponding to emitted photons, and the photon flux from each ROI was calculated using the software supplied with the IVIS unit (Living Image; PerkinElmer, Waltham, MA).
**Histology**

The amputated limbs were fixed in 10% formalin for at least two weeks, decalcified in 10% (w/v) ethylenediaminetetraacetic acid (EDTA) and submitted for routine paraffin processing and thin-section histology. Histological sections (4 microns in thickness) were stained with hematoxylin and eosin (H&E), prior to visual examination and scanning (see below).

**Aperio Scanning**

Histology slides were submitted to a pathology laboratory for digital scanning using the Aperio ScanScope XT (Leica Biosystems, Buffalo Grove, IL), producing a virtual slide in a single-file, pyramidal tiled TIFF format. Resultant images were reviewed using the recommended ImageScope software (Leica Biosystems, Buffalo Grove, IL). The reviewer had received an introduction to the Aperio system using ImageScope from a board-certified veterinary pathologist. Regions of neoplastic cells were identified according to the presence of cellular atypia and/or basophilic staining. A region of interest was accordingly encircled and the total area calculated directly from the ImageScope software.

**Data Handling and Statistical Analysis**

Since the primary goal of this study was to determine whether IVIS imaging provides a robust method for identifying and monitoring tumor metastasis to the lung, data analysis focused on determining the sensitivity, specificity, positive and negative predictive values for IVIS. Our initial plan was to use gross autopsy findings as the gold standard for
determining the presence or absence of metastasis. We were also interested in seeing how well the results from quantitative IVIS scanning compared to the morphometric data derived from measuring tumor area on histological sections; for this, we undertook correlation analysis between IVIS signal (measured at the terminal scan) and histological measures of tumor burden. A significance level of $p<0.05$ was used throughout.
Chapter 3: Results

As anticipated from previous work with the original, non-transduced Abrams cell line, the Luc transduced Abrams cell line grew well after subcutaneous injection, intratibial injection and intratibial implantation. Subcutaneous tumors typically reached a size of approximately 10 mm in maximum dimension within 4-6 weeks, at which point they were harvested for subsequent use in the implantation experiments.

**Histopathology as an Indicator of Primary and Metastatic Disease**

Using the presence of macroscopic tumor (grossly visible at autopsy) as the “gold standard,” histology proved to be extremely reliable as a test for determining the presence of primary disease, but less reliable for detecting an absence of disease. For tumor growing within bone, histopathology had a sensitivity of 69%, specificity of 90%, a positive predictive value of 92%, and a negative predictive value of 64% (Table 1). Measures of sensitivity, specificity, positive and negative predictive values were calculated from these primary data, assuming gross autopsy to be the gold standard for determining the presence of primary tumor.
<table>
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</tr>
</tbody>
</table>

**Table 1. Primary tumor pathology: sensitivity and specificity of histopathology as compared with gross necropsy findings**

In the setting of metastasis (i.e. tumors growing in lung), histology had a sensitivity of 47%, specificity of 100%, a positive predictive value of 100% but a negative predictive value of just 10% (Table 2). This data represents a combination of canine osteosarcoma and human breast cancer cell data.

<table>
<thead>
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<tr>
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**Table 2. Comparison between gross post-mortem findings and histopathology for detecting tumor metastasis to the lung.**

**Bioluminescence Imaging as an Indicator of Primary and Metastatic Disease**

Using the presence of gross (macroscopically visible) tumor as the reference, bioluminescent imaging with IVIS was extremely robust as a non-invasive diagnostic test. In the primary setting (i.e. tumors growing within bone), IVIS imaging had a sensitivity of 100%, specificity of 100%, and 100% positive and negative predictive values (Table 3).
In the context of metastatic disease, again with gross necropsy findings as the benchmark, IVIS imaging had a sensitivity of 100%, specificity of 100%, positive predictive value of 100% and negative predictive value of 100% (Table 4).

<table>
<thead>
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</tr>
<tr>
<td>Total</td>
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</table>

Table 3. Primary tumor pathology: sensitivity and specificity of bioluminescent (IVIS) imaging as compared with gross necropsy findings

In order to assess the diagnostic utility of IVIS scanning, we performed a side-by-side comparison of the degree of concordance between IVIS and histopathology. The raw data are presented in Table 5 (primary tumors) and Table 6 (metastatic tumors). Calculations of positive (PPA) and negative percent agreement (NPA) were made according to published guidelines given by Feinstein and Cicchetti. Due to a lack of true negatives in this set of animals, it was only possible to calculate meaningful values for the PPA. For primary tumors, there is 100% agreement between IVIS and histopathology. For metastatic tumors, this falls to 71%.
Histology

<table>
<thead>
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Table 5. Overall agreement between bioluminescent imaging and histopathology for detecting primary tumors

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</table>

Table 6. Overall agreement between bioluminescent imaging and histopathology for detecting secondary (metastatic) tumors

Relationship between Radiographic Scores, Primary Tumor Growth and Subsequent Metastasis

Radiography proved to be highly predictive of bone disease in bone cancer (Table 7). In general, one would anticipate that IVIS signal (measured as photons per second) would increase as the tumor enlarges, and that there would be a positive relationship between IVIS signal and radiographic lysis score. The data from this experiment support the notion that IVIS signals generally increase with lysis score (Figure 8) but this effect was not statistically significant with the numbers of animals in this study. There does appear to be a robust relationship between the intensity of the IVIS signal and the size of the primary bone lesion (Figure 9).
Faxitron images were reviewed for radiographic changes and graded according to the previously reported grading scheme.¹ None of the osteosarcoma mice had a lysis grade of 2, but all other grades were assigned.

<table>
<thead>
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<tbody>
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<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
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</table>

Table 7. Relationship between radiographic findings (presence or absence of bone lysis) and necropsy for primary osteosarcoma lesions in mice
Figure 1. Schematic illustration of the technique for intra-osseous injection of tumor cells
Figure 2. Schematic illustration of the technique for intra-osseous implantation of fragments of xenograft tumor
Figure 3. Scoring of radiographs on the basis of degree of bone lysis. Representative examples of lysis grades 0, 1, 3 and 4 (grade 2 not seen in the osteosarcoma line)
Figure 4. Virtual microscopic image of murine tibia with osteosarcoma and encircled region of interest
Figure 5. Virtual microscopic image of normal murine tibia (unaffected by neoplasia)
Figure 6. Mice positioned within the imaging chamber of the IVIS. No luminescence was seen in these mice.
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Figure 8. Bioluminescent (IVIS) data categorized according to lysis score. Although there was a general trend towards higher luminescent signals from tumors with more lysis, there were no statistically significant differences across the groups.
Figure 9. Relationship between IVIS signal and tumor histology--data reflects results for primary tumors only.
Chapter 4: Discussion

Osteosarcoma is a biologically aggressive cancer, with a strong predilection for forming distant metastases, most commonly within the pulmonary parenchyma.¹ A previous report found that 100% of human patients with stage IIB extremity osteosarcoma developed pulmonary metastases and subsequently died.¹⁰ It also has been shown that an increasing number of pulmonary nodules is associated with a poorer prognosis in humans, and chemotherapy-induced necrosis of pulmonary nodules can be correlated with survival.¹⁰

A syngeneic, orthotopic model of luciferase-transfected, murine osteosarcoma previously has been developed, using immune-competent mice.⁴ Orthotopic models involve implantation of a primary tumor, which subsequently undergoes spontaneous metastasis, indicating the presence of host-tumor interactions.¹¹ The benefit of using this system is that the development of spontaneous metastasis is a good model for the clinical scenario, where metastasis is commonly present at the time of diagnosis; therefore, the process of metastasis and its mechanisms may be more accurately evaluated. To undergo metastasis, a tumor cell must intravasate, circulate, extravasate and survive in distant tissues.⁴,¹¹ Additionally, the interactions between tumor cells, the extracellular environment and host immune cells are more accurately assessed and studied in a
syngeneic model and potential immunotherapeutic agents may be evaluated.\textsuperscript{4,11} The drawback from using such a model is that species-dependent differences in osteosarcoma, i.e., human or canine osteosarcoma, are not evaluated.\textsuperscript{4} Other drawbacks of the orthotopic model, in general, include disruption of tissue stroma and the potential for intravasation at the time of implantation, thereby bypassing natural mechanisms of metastasis.\textsuperscript{11} The use of xenograft tissue (e.g. human or canine osteosarcoma) in an immune competent mouse results in complex interactions between the mouse immune system and the xenograft tissue, limiting its relevance as a model for the natural disease state. However, the use of xenografts in mice are useful for studying agents specifically targeted to the xenograft cells and resultant metastatic lesions.\textsuperscript{4} The microenvironment of tumors has been shown to contain extracellular membrane vesicles, which are derived from both proliferating tumor cells and from infiltrating macrophages and neutrophils, thereby supporting tumor cell behavior.\textsuperscript{13} It is well established that tumor cell-stromal cell interactions are important in the biology of cancer progression and metastasis.\textsuperscript{11,14,15} For example, the use of immune incompetent mice may demonstrate impaired angiogenesis, whereas high natural killer cell activity can lead to elimination of metastatic cells.\textsuperscript{11} Due to these complex interactions, it has been suggested that a combination of xenograft and syngeneic models may be optimal for studying osteosarcoma.\textsuperscript{4,11} One of the great strengths of the xenograft model as a translational research model is that it allows for the assessment of metastasis following surgical resection of the primary tumor. Both the mouse and dog model of appendicular osteosarcoma are useful in this respect because one can amputate the primary tumor to
assess the behavior of metastases without the influence of the primary tumor—if the primary tumor is left in place, it can serve as a source of variability when assessing therapeutic efficacy of agents at hand.\(^4\)

An \textit{in vivo} imaging system is useful for evaluating tumor biology in real time. A major benefit of such a system is the potential for the reduction in numbers of animals required to evaluate potential therapeutics.\(^{13}\) A bioluminescent, \textit{in vivo} imaging system has been established for the study of cell-cell interactions such as occurs in neoplastic processes. Luciferase from the firefly, \textit{Photinus pyralis}, is a common source of luminescence for detection in bioluminescent imaging, though other reporters are also available.\(^6\) We chose luciferase due to its wide use and availability, and because it is the most widely studied of the currently available reporter genes.\(^6\)

The luminescence of luciferase-expressing cells exposed to luciferin occurs as a continuous wave at a constant intensity for a relatively short period of time.\(^{16}\) The ideal time point for detection of luminescence has been established and we used a time point of 10 minutes, which is well within this range. Previous studies also have demonstrated the stability of \textit{Luc} genes, supporting these models as useful for long-term studies.\(^6\)

There is relatively low tissue absorption of photons greater than 600 nm, and hemoglobin is the primary absorber of light \textit{in vivo}.\(^6\) For wavelengths between 600 and 1000 nm, scattering predominates over absorption, hence, the ideal wavelength of light for \textit{in vivo} imaging of tissue greater than 1 cm thick is 600-950 nm.\(^{17}\) The optical properties of tissue affect the detected signal, which depends on photon flux per cell, number of cells and the migration of photons through tissue.
Photon attenuation is strongly nonlinear, and it is a function of depth and tissue optical heterogeneity. Photons emitted from the tissue surface are referred to as surface radiance. At a fixed depth, with other terms held constant, the surface radiance is proportional to cell count. The IVIS imaging system can detect wavelengths in the range of 400-950 nm, and the quantum efficiency of the charge coupled device is high for this range, further establishing the system as highly sensitive. Therefore, tissue optics are the primary limitation of sensitivity of detection of bioluminescent reporters, and only 400 cells are required to get signal above auto-luminescence in bioluminescent imaging.

As established by Sottnik, et al., luciferase labeling allows serial monitoring of murine osteosarcoma tumor progression and metastases. Comstock, et al., established that a bioluminescent signal at least strongly correlated with caliper measurement in primary tumors, but this correlation decreased as time elapsed and tumor size increased. Another study has established the increased sensitivity of bioluminescent imaging in detecting bone marrow metastasis of breast cancer, in the absence of radiographic osteolysis. We also demonstrated a general trend of increasing bioluminescent signal in primary tumors with a higher tibial lysis score, but this was not statistically significant. It is likely that low numbers within our study contributed to this finding.

Our study demonstrated the high sensitivity, specificity, positive and negative predictive values for bioluminescent imaging of canine osteosarcoma in an orthotopic murine model in gross and histological assessment of primary tumors, but not for metastatic disease. However, our tissue samples did not include volumetric measurement and the entire
tissue block was not reviewed histologically. As is common practice in research laboratories, only one tissue section was processed per gross tissue sample. Increasing our tissue samples would likely increase the likelihood of detecting pulmonary metastases. A recent study by McMurray et al., determined that micro-CT/PET imaging does not accurately quantify canine osteosarcoma metastases in a mouse model, but can increase the efficiency of detecting its presence. The same study also noted that the expense of such imaging procedures can be a deterrent to its use. Previous work also has established the utility of stereology for assessing tumor volume within lungs, and while this method is somewhat laborious, the use of stereology should be considered for future studies.

In our study, complications with one of the cell lines confounded the data relating bioluminescent signal and primary tumor. Results from our study demonstrated a strong, statistically significant correlation between IVIS signal and 2-D tumor area; \( r = 0.887, p<0.01 \) but this result was likely confounded by the presence of a high number of false negative samples (type ii error: signal-positive, histology negative). Again, this likely relates to the inadequate, single section tissue sampling method, and therefore we do not recommend that method for future studies.

Our cells were maintained in tissue culture, and required passage and selection for use during the study. Having been translated from an artificial, two-dimensional environment to an \textit{in vivo}, three-dimensional environment is likely to have affected the behavior of our tumors \textit{in vivo}. Therefore the mechanisms and pathways by which metastasizing tumors progress are likely to have artificial components. \textit{In vitro} treatment
and maintenance of tumor cells also may impact a cell’s ability to interact with a biologic system. Thus, a potential explanation for the difficulties in developing a successful luciferase transfected canine osteosarcoma cell line may be inherent in the system. The primary source of background noise in this study is auto-luminescence from the live animals, the mechanism of which is poorly understood. This phenomenon occurs in a non-uniform, whole-body distribution, and it is greater in female mice as compared to male mice. In luciferin-injected animals, the auto-luminescent level is 20% higher than in non-injected animals, however the level is still quite low and there are no other sources of background luminescence in the IVIS instrument. In our study, we used a consistent source, strain and sex of mice (female), housed and fed in like manner, to minimize inter-experimental variation. Additionally, all animals imaged received D-luciferin injections and regions of interest were drawn against the whole body as a background. Therefore, the presence of background auto-luminescence is unlikely to have significantly affected the level of radiance determined in our regions of interest.

Osteosarcoma typically originates within the metaphyseal, intramedullary cavity of bone and eccentrically invades into cortical bone and periosteum. It may also directly extend into the adjacent soft tissues. While these changes may be seen radiographically, the absence of radiographic findings does not exclude neoplasia as a diagnosis. Radiography can detect osteolysis and associated soft tissue masses, as well as macroscopic metastases; however, micrometastases are undetectable. In our study we performed radiography as a means of assessing tumor growth and to compare radiographic findings to histologic findings. While none of the mice showed
radiographic changes without histologic evidence of neoplasia, 6/25 (24%) of mice did have histologic evidence without radiographic changes. This number may have been affected by time between imaging and necropsy. Nevertheless, because micrometastasis is common in osteosarcoma, and because these regions serve as potential therapeutic targets, a system to detect micrometastases is desirable. Bioluminescent signal is detectable earlier in the disease course, compared to radiography, and it is detectable within microscopic bone marrow metastases, establishing this system as a sensitive \textit{in vivo} test for studies of metastatic osteosarcoma.\textsuperscript{6}

In one of the groups (5-ALUC), primary tumors were grossly visible but these tumors were not submitted for histopathology. Given the extremely high probability that the tumors were indeed osteosarcoma, these results were included in the Fisher’s exact test which evaluated signal versus gross necropsy and radiographic changes versus necropsy. In this same group, five of the mice had gross and histological data from the lungs, therefore these 5 mice were included in the data analysis comparing necropsy to histology and IVIS to histology. In the 2-HBC group, one of the mice did not have pulmonary histology results and another mouse did not have IVIS images available for review, so these mice were not included in analyses of the corresponding questions. In the 2-BCA group, only one of the mice had lungs submitted for histology so these mice were not included in metastatic disease analyses, but were included in primary tumor analyses. Additionally, 3 of these mice did not have IVIS images available for review so these were not included in IVIS imaging statistics.
The 4 ALUC group was radiographed at 13 weeks; though no IVIS signal was detected in this group, radiographic changes were evident and primary tumors were grossly evident. Because this cell line was not validated immediately prior to injection, we suspect there was either unanticipated insert instability, or selection of a non-transduced cell from what is a heterozygous loss of the insert contamination or recombination of the cells, leading to absence of luciferase expression, or hindering its ability to lyse luciferin. The reason for the absence of signal in the 4-ALUC group is unknown; however, luciferase may alter the behavior of transfected cells in an unknown way. Previous literature has suggested that GFP and luciferase transfected cells may alter the behavior of pulmonary metastases.\textsuperscript{18,4}

While the level of gene expression of a cell transfected with luciferase is largely dependent upon the promoter chosen in the vector, the introduction of cells within a biologic system introduces inherent, uncontrollable factors. The level of expression determines the extent to which the cell can be detected in vivo.\textsuperscript{6} To determine average cell expression, the photon emission can be determined in tissue culture prior to in vivo use.\textsuperscript{6} We constructed a dose-response curve using transfected Abrams-luciferase osteosarcoma cells to validate the expression of the gene prior to injection, after re-deriving the cell line. This curve demonstrated a linear response of the system. It is known that the attenuation of photons in tissue occurs in a non-linear fashion;\textsuperscript{16} therefore, the primary function of the dose-response curve was to establish the efficiency of transduction and the reliability of signal, rather than to serve as a predictive factor for in vivo performance. The majority of false negative samples were in a group of mice, 4-ALUC, for which the luciferase expression was not validated prior to imaging, stressing
the importance of this test. For the 4-ALUC experiment, it is highly possible that in-vitro growth of the cell line resulting in preferential growth of a subpopulation of cells with low (or no) luciferase expression. This experience highlights the importance of assessing *Luc* expression immediately before the start of each experiment, rather than simply at the start of a series of experiments. Additionally, this cell line was re-derived and used for a subsequent group, 5-ALUC, which reliably produced signal, and this contributes to the explanation of the absence of signal in the 4-ALUC group.

There were additional, unpredictable complications with several of the groups, likely unrelated to the luciferase line itself. In some instances, tibial fractures were diagnosed based on radiographs and mice were subsequently euthanized using a protocol as established by IACUC. One of the mice in the 1A-OSA group died after luciferin injection one week after subcutaneous tibial injection. One of the mice in the MB231-LUC line did not awake from anesthesia after tibial injection, and a second mouse in this group was euthanized during week four, due to hind limb paralysis.

Mouse 12 in the 5-ALUC group had progressively diminishing signal based on IVIS imaging. This mouse also had radiographic changes in the proximal tibia which had largely resolved by week 11. The mechanism for this particular mouse’s response is not understood. Athymic nude mice lack T cell development but retain an innate immune system including natural killer cell activity, as well as a humoral adaptive immune response. The immune system is known to exhibit anti-neoplastic effects, including specific studies regarding natural killer cells.\(^{20}\) It is reasonable to suggest that this particular mouse may have developed NK activity against its tumor; however, this
explanation raises the question of why other mice, either within the same group or within other groups did not demonstrate a similar response. All mice should have received approximately the same tumor cell dose, discounting low tumor cell burden as an explanation. Perhaps increasing numbers of animals used within the study would reveal a similar response by some mice, which would imply simply the low likelihood of mounting an effective immune response against the tumor such that our study numbers simply were not large enough to capture this response rate.

While the OSA-LUC cell line demonstrated a linear response, this was not demonstrated once the cells were used in vivo, as some mice expressed fluctuating levels of luciferase at sequential imaging time points. This was not entirely unexpected, given the known behavior of tumor cell growth and metastasis. Furthermore, as mentioned previously, the attenuation of photons in tissue occurs in a non-linear fashion.\textsuperscript{15} Introducing cells into a biologic system carries numerous inherent, confounding factors as the cells interact with extracellular matrix and stroma.\textsuperscript{21} This is considered one of the advantages of using an orthotopic mouse model to study metastasis: by allowing the tumor cells to respond to a biologic system, the researcher establishes a model whereby the interactions of the neoplastic cells are considered more similar to a naturally occurring neoplasia, enhancing the quality of information to be gained from its study. An additional explanation for the disparate response in vivo compared to in vitro is that the tumors may have developed in an inhomogeneous distribution. In all instances, a single region of interest was drawn for the gross primary tumors, as this incorporated the luminescent regions. While the CCD camera does exhibit excellent spatial resolution, this does not necessarily translate
to the histologic level, and a single region of interest also cannot be drawn to the histologic level in a gross specimen. Additionally, foci of necrosis as evidenced histologically on the slides, were not appreciated grossly, such as absence of signal. A study evaluating infectivity of adenovirus transduction noted that in vivo luciferase signal intensities varied from mouse to mouse, suggesting that the tumors and mice may respond differently to injection with the same vector, even within immune deficient mice.\textsuperscript{22} In that study, spontaneous tumors were injected with the vector containing luciferase. In our study, IVIS regions of interest were drawn to include photons emitted within a focal region on a 2 dimensional image, though the animal and the tumor are both 3 dimensional objects. A region of interest with planar IVIS imaging therefore includes by necessity some non-luminating anatomy. Bioluminescence tomography, as opposed to this study’s planar imaging techniques, can more accurately delineate a region of luminescence by reducing effects of photon scatter and tissue absorption.\textsuperscript{14} The development of a 3-D tumor model for chemotherapeutic trials, as suggested by the literature, likely would be more sensitive, and therefore more ideal.\textsuperscript{24} Another possible explanation for disparate in vivo response is that as the tumor responded to its biologic environment it recombined or even down-regulated the vector containing luciferase. Therefore, such regions would be unable to respond to luciferin by emitting photons of light. Additionally, the length of time over which the dose response curve was generated was dramatically different from the length of time over which the tumors were allowed to grow in vivo. While response to luciferin is acute (10 minutes post-injection), short-lived\textsuperscript{17} and regenerated at each time point, the mouse model time
points were generated at 2 week intervals, while the curve time points were generated at minute intervals, and only on a single day. Therefore, the behavior of the vector in the cell line over a long period of time was not evaluated ex vivo as compared to in vivo.

During the course of this study, the cooling device for the CCD camera failed, forcing us to use a second IVIS imaging system located at another campus. While there is potential for this disruption to confound the data obtained at the two different locations, the IVIS imaging system is calibrated against a National Institute of Standards and Technology traceable spectral radiance source. Therefore, variation in the signal detection is unlikely to have been affected by the use of a second system.

The relatively new technology of virtual microscopy involves high-resolution digitalization of histology slides. These systems are composed of an optical microscope system, an image acquisition unit (photography), scanning software and viewing software. The camera contains a charge-coupled device to provide conversion of the analog signal into a digital signal. The acquisition of signal occurs in a linear fashion, with each strip acting as a separate file. As a result, the final image of the whole slide is actually a mosaic composed of multiple files. The focus is adjusted from one line to the next, and the focus is always at the optical axis to avoid 2D optical distortion. The images are acquired with some overlap and the common regions are matched by software when forming a final image. Each uncompressed tagged image file format (TIFF) file is 200 mega-pixels in size, and the file size of an average virtual slide is about 300MB.

Lossless compression of the images through the Aperio imaging software eases the difficulties associated with large files (i.e., sharing and storage). The Aperio ScanScope
XT is capable of producing slides with resolution of 0.5 um/pixel at 20 x and 0.25 um/pixel at 40x. The Image Scope software for viewing Aperio images is sourced for free on the internet. A recent study evaluated inter-observer variability in diagnostic agreement when using virtual microscopy compared to light microscopy to assess renal biopsies. This study found that inter-observer lesion scores were more reproducible with virtual microscopy than with light microscopy. A possible drawback of virtual microscopy is that a single focus plane acquisition determines the optimal focus and leaves open the potential for out of focus regions on a slide.

It is well established that the biology of cancer depends on the specific cancer type; therefore, models of specific cancers must be established for thorough study. When choosing animal models of disease, it is ideal to minimize differences between the human and animal models as much as possible. Recent research has demonstrated a greater homology between man and dog than between man and mouse. Currently, the dog is used as a model to determine the safety of novel therapeutic agents in human Phase 1 clinical trials. The dog is often used as a model for human osteosarcoma, due to the strong similarities between osteosarcoma in the two species as well as the relatively recent recognition of the strong similarities between the human and dog genome. These tumor similarities include similar histology of the primary tumor, similar micrometastatic disease with gross metastases to the lung, and similar responsiveness to anthracycline and platinum therapies. Furthermore, dogs develop osteosarcoma spontaneously, adding to its appeal as a candidate for research, whereas, in the mouse, the majority of spontaneously arising tumors either do not metastasize, have a long latency or exhibit
only intravascular metastasis.\textsuperscript{11} For these reasons, we believe the study of canine osteosarcoma metastasis in a mouse model ultimately will provide a strong body of evidence to support therapeutic trials. A model to detect micrometastases is desirable, as such a system could be useful to evaluate the efficacy of novel therapeutic agents and/or protocols.

In conclusion, our study confirmed the sensitivity of appropriately luciferase-labeled canine osteosarcoma cells within a murine model, as luminescing regions reliably indicated regions of neoplasia. We further supported the literature regarding the relative insensitivity of radiography for detecting osteosarcoma, specifically in an orthotopic murine model of canine osteosarcoma. An efficient model of modeling metastatic disease in osteosarcoma remains to be elucidated. Both bioluminescence tomography and stereology may be appropriate methods to more specifically address correlation between tumor size and luminescence. MRI volumetric studies could also be considered for methods of monitoring response to therapeutic trials though its expense is a deterrent. Finally, our work supported the utility of virtual microscopy in ease of use for storing image files and highlighting regions of interest.
References:


