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Brian T. Grisez
West Virginia University

Justin J. Ray
West Virginia University

Phillip A. Bostian
West Virginia University

Justin E. Markel
West Virginia University

Brock A. Lindsey
West Virginia University

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Highly Metastatic K7M2 Cell Line: A Novel Murine Model Capable of *In Vivo* Imaging via Luciferase Vector Transfection

Brian T. Grisez, **Justin J. Ray**, **Phillip A. Bostian**, **Justin E. Markel**, and **Brock A. Lindsey**
Department of Orthopaedics, West Virginia University, PO Box 9196, Morgantown, WV 26506-9196

Abstract

Osteosarcoma is rare and little improvement in survival rates has occurred in the last 25 years despite modern chemotherapeutic treatment. Bioluminescent cell lines for the modeling of osteosarcoma have shown success in tracking metastases *in vivo*, but commonly use adenoviral vectors to transfect the native cell line with bioluminescent reporters. The purpose of this study was to develop an orthotopic model for metastatic osteosarcoma capable of *in vivo* monitoring of metastatic and primary tumor burden in an immunocompetent mouse and compare that model to its wild type pathogenesis. K7M2 cells were transfected using a plasmid vector and were stable after 12 weeks. Thirty-four female BALB/c mice aged four to five weeks underwent orthotopic implantation of either wild type (*n=12*) or transfected (*n=22*) K7M2 cells in the proximal tibia. Mice were monitored for tumor growth and weekly In Vivo Imaging System (*IVIS*) imaging was performed to monitor for pulmonary metastasis. Although tumors developed sooner in the wild type group, no significant differences were seen compared to Transfected Group 1 in rate of inoculation, growth rates after first detection, metastatic rate, and time between inoculation and death. This study establishes a new murine model for metastatic osteosarcoma using the K7M2-wt cell line transfected with a non-viral plasmid luciferase vector. The benefits of this preclinical model include an intact immune system and orthotopically driven metastatic disease; this model appears comparable to its wild type counterpart. In the future, the model may be used to examine promising immunomodulatory therapies using bioluminescence *in vivo*.

Keywords

Animal cancer models; imaging of tumor progression and metastasis; immunotherapy; sarcoma; tumor microenvironment

Corresponding Author: Brian T. Grisez, MD, West Virginia University, PO Box 9196, Morgantown, WV 26506-9196, 304.293.1168, 304.293.0231, bgrisez@hsc.wvu.edu.

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Introduction

Primary osteosarcoma is rare and has an incidence of 800 new cases diagnosed yearly, with half of cases diagnosed in children.[1] Five-year survival rate for localized disease is 60–80% and a dismal 15% with a metastatic presentation.[2] Complete surgical resection with reconstruction in combination with neo-adjuvant and adjuvant chemotherapy is the mainstay of treatment. However, with the addition of modern chemotherapeutic agents, little progress has been made in patient survival rates in the past 25 years.

Multiple factors contribute to the difficulty of developing new therapies, including a low incidence of disease and rapid clinical course with the onset of metastasis. However, because of the poor survival outcomes, new treatment modalities are crucial. Immunotherapy is an innovative approach to treating osteosarcoma. Spurred by the observation that patients who develop post-operative infections have increased survivorship, the goal of immunotherapy is to harness the beneficial effects of a pro-inflammatory response while limiting destructive side effects.[3–5] Most current murine models of osteosarcoma are insufficient to examine immunomodulation because the models use immunodeficient animals.[6–10] In an attempt to study tumor burden and metastatic progression *in vivo*, a model of metastatic osteosarcoma in immunocompromised mice using the luciferase vector and bioluminescent imaging was recently established.[11,12]. However, the use of an orthotopic driven metastatic model in an immunocompetent model is needed.

Metastatic disease is of particular interest in osteosarcoma; the ability to monitor such progression is paramount in determining the efficacy of novel treatments. Methodologies that are currently available to detect disease *in vivo* include the use of micro computed tomography (micro CT) and magnetic resonance imaging (MRI). These modalities have high sensitivity to detect metastasis in murine models, with the lowest detection limits of 600 um in the lungs.[13] However, drawbacks include need for prolonged anesthesia to complete the scan partly because images are obtained between respirations to produce the best resolution. Further, machine capacity often allows for only one animal to be imaged at a time.

In osteosarcoma research, bioluminescent imaging has recently emerged as an important method for tracking both primary tumor burden and metastatic spread. It has the advantage of *in vivo* monitoring in live animals, precluding the need for euthanasia to survey metastasis and tumor burden. Its primary benefit is reduction in the number of animals required for *in vivo* studies.[12,14,15] The *in vivo* imaging system (*IVIS*) quantifies flux produced by a reaction in which the luciferin substrate is oxidized by the luciferase enzyme. The gene for this enzyme can be transfected into the cancerous cell using plasmid vectors or viruses.

The purpose of this study was to develop an orthotopic model for metastatic osteosarcoma capable of *in vivo* monitoring of metastatic and primary tumor burden in an immunocompetent mouse and compare that model to its wild type pathogenesis. Most murine models consist of xenotransplants and have limited utility in examining the interactions among the host, the microenvironment, and the cancerous cells.[16–19] In this syngeneic model, we used the highly metastatic K7M2-wt murine cell line transfected with

the luciferase gene and applied cells orthotopically to the tibiae of BALB/c mice. We followed the progression of the tumor and metastasis using IVIS and performed palliative amputations of the affected extremity; at euthanasia, metastatic tumor size was quantified and cross referenced with IVIS detection. These data were also compared to the wild type model to determine differences in inoculation rates, tumor growth rates, and metastatic rates.

Methods

Growth and preparation of transfected and wild type K7M2 cells

The K7M2 murine osteosarcoma cell line (*ATCC CRL-2836*, *ATCC, Manassas, VA*) was kindly donated by Dr. Kurt Weiss, MD (*University of Pittsburgh Medical Center, Pittsburgh, PA*) in April 2014. Mycoplasma testing is not routinely performed in our lab. The K7M2 cell line is derived by harvesting pulmonary metastases of the K7 murine osteosarcoma cell line, reimplanting the metastatic cells orthotopically and repeating the harvest of a pulmonary metastatic lesion. The cell line is considered highly aggressive with a reported pulmonary metastatic rate of over 90% in mice.

Wild Type (*WT*) K7M2 osteosarcoma cells were cultured in Dulbecco's Modified Eagle's Medium (*DMEM*) containing 10% fetal bovine serum and 1% penicillin and streptomycin (*Life Technologies, Carlsbad, CA*) to obtain 75% confluency. The cells were washed with phosphate buffered saline (*PBS*) without calcium and magnesium (*Corning Inc.*, *Corning, NY*) and harvested with Trypsin (*0.25% Trypsin, 0.1% EDTA in HBSS w/o Calcium, Magnesium and Sodium Bicarbonate, Corning Inc.*). The cell density was adjusted to one million cells (1.0×10^6) in 25 μL of PBS for inoculating mice. Passage one cells were used for orthotopic implantation.

Transfected K7M2 osteosarcoma cells were prepared by adding 1.0 ml (approximately 3.0×10^5) WT cells into each well of a six-well plate containing 1.0 ml of DMEM containing 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were incubated at 37°C for 24 hours to obtain 75% confluency. A 3:1 transfection ratio complex was created using ViaFect Transfection reagent (*Promega, Madison, WI*) and pGL4.51 [luc2/CMV/Neo] plasmid vector (*Promega*) prepared per the manufacturer's specifications and incubated with K7M2 cells for 24 hours. The cells were washed once with PBS, 2.0 ml of DMEM media was added to each well, and the cells were incubated for 48 hours at 37°C. The selective drug Geneticin-418 (*Life Technologies*) was then added to kill non-transfected cells. After further propagation, the cell density was adjusted to one million cells (1.0×10^6) in 25 μL of PBS for inoculating mice. Passage one transfected cells were used for orthotopic implantation.

Stability of transfected cell line

To assess if the luciferase reporter vector was stably passed to future generations, *in vitro* bioluminescence imaging was performed for 12 weeks. For this verification, 4.5×10^5 transfected K7M2 cells were seeded in a 12-well plate, covered with media and incubated for 24 hours which created a final concentration of 5.0 x 105 cells per well. The media was removed just prior to imaging and 1.0 ml of 0.5 mM luciferin was added to each well. The

cells were imaged using the *In Vivo* Imaging System (*IVIS*) Lumina II with Living Image Software (*PerkinElmer, Waltham, MA*). Bioluminescent imaging was set to medium binning with an exposure time of five minutes.

Surgical implantation of K7M2 cells

All animal procedures were conducted in accordance with the Institutional Animal Care and Use Committee (*IACUC*). Thirty-four female BALB/c mice age four to five weeks were purchased from Jackson Laboratories (*Bar Harbor, ME*) and maintained in pathogen free animal housing facilities. A twelve-hour light/dark cycle was used, the animals were fed autoclaved chow, and had access to sterile water ad-libitum.

The Wild Type (WT) group (n = 12) underwent inoculation with wild type K7M2 cells. Transfected Group 1 (n = 10), Transfected Group 2 (n = 10), and Transfected Group 3 (n = 2) were all inoculated with luciferase transfected K7M2 cells. Transfected Group 1 received intraperitoneal injection of luciferin only for tumor detection. Transfected Groups 2 and 3 received both intraperitoneal and intranasal luciferin for tumor detection, along with shaving of the operative limb and chest prior to imaging.

The animal was weighed on a calibrated scale and placed into an anesthesia induction chamber. General anesthesia was induced using isofluorane (*Piramal Enterprises Limited, Andhra Pradesh, India*) with 100% oxygen at a rate of 2.5 liters/min until loss of the righting reflex occurred. The animal was then transferred to a heated operating stage, placed supine, and maintained under general anesthesia using a nasal cone at a rate of 2.5 L/min. Opthalamic lubricant was placed in both eyes and 0.02 ml of 1 mg/ml buprenorphine SR (*Wildlife Pharmaceuticals, Windsor, CO*) was injected subcutaneously using a 25 gauge needle.

The animal's operative hind limb was prepped using betadine solution and sterilely draped. Using sterile technique, a small incision was made over the anterolateral leg under an operating microscope. Dissection was carried sharply to the tibia and directed proximally until the metaphyseal flair was identified. An 18 gauge needle was then used to create a small bore hole in the center of the tibia at the level of the proximal tibial flare. As soon as a small pilot hole was created, the needle was angled approximately 45° in the sagittal plane and advanced until the cortex was penetrated. The needle was twisted gently which caused the anterior flare/cortex to separate from the intact tibia, creating a cortical window. The posterior cortex remained intact to avoid a bicortical fracture. Twenty-five microliters of PBS containing one million K7M2 cells was then injected into the defect. The muscle was pulled back over the bone and the skin was closed in a running fashion using a 4-0 vicryl suture. Animals were awakened and recovered in the usual fashion.

Clinical monitoring of mice

Mice were monitored weekly until a visible tumor was appreciated after which they were measured daily using digital calipers (*greatest width x by greatest length*). Tumor volume was calculated using the formula $V=(length \times width^2)/2$. The mice were graded using our institution's tumor burden scoring system weekly. This scoring system ranges from 0 to 60 (*0 = healthy animal; 60 = requires euthanasia*) and is based on general appearance (fur,

mucus membranes, response to stimuli), body condition (well nourished, thin, cachectic), neurologic status (head tilt, bulging eyes, depression, self-mutilation, limb paralysis, seizures), tumor appearance (ulcerated, limits ability to ambulate, bleeding), and respiratory rate (normal, increased rate/effort, severe distress). After the elevation of any scoring variable, the animals were monitored daily and veterinary staff was consulted to ensure humane treatment and minimization of pain.

IVIS imaging

IVIS imaging uses a charge-coupled device camera that is able to detect light emitted from the luciferin-luciferase reaction within the tumor bed. Once a visibly detectable tumor was identified, animals were imaged weekly using the IVIS Lumina II with Living Image Software to monitor metastatic burden. After isoflurane induction, the animal was injected with luciferin (*150 mg/kg*) in the intraperitoneal cavity and placed onto the imaging platform, dorsal side up. Exposure varied between one and five minutes and bioluminescence binning was set to medium. Images were taken approximately five minutes post luciferin injection. After the first set of images was performed, the lower extremity (*if present, see Tissue harvest below*) was shielded for the second set of images. This method improves sensitivity for small lung tumors that are below the low threshold intensity obtained when a tumor is present in the limb (Figures 1a and 1b).

Intranasal luciferin

Ten BALB/c mice (Transfected Group 2) aged four to five weeks underwent inoculation with one million K7M2 cells according to the same protocol as mentioned above. These mice received 30 microliters of intranasal luciferin (150 mg/kg) in addition to the intraperitoneal luciferin as mentioned above. The operative extremity and chest of these mice were shaved prior to imaging. Otherwise, IVIS imaging settings, exposures, and images were taken in accordance with the protocol mentioned above. The purpose of this transfected group was to show improved IVIS sensitivity and earlier detection of pulmonary metastatic disease using intranasal luciferin.

Tumor luminescence over time

Two BALB/c mice (Transfected Group 3) aged four to five weeks underwent inoculation with one million K7M2 cells according to the same protocol as mentioned above. These mice received both intranasal and intraperitoneal luciferin according to the protocol mentioned above. The operative extremity of these mice was shaved prior to imaging. IVIS imaging settings, exposures, and images were taken in accordance with the above protocol. The purpose of this transfected group was to monitor luminescence and primary tumor size over time. Mice were imaged twice weekly until the tumor score reached 60, at which time the mice were euthanized.

Tissue harvest

Once the tumor reached sufficient volume *(around 1.7 cm^3)*, a palliative amputation of the affected hind limb was performed. The mouse was anesthetized and analgesic was provided as described previously. After prepping with betadine and sterile draping, a longitudinal

incision was made on the medial third of the tumor bed. The tumor was dissected away from the peritoneum, taking care not to violate the peritoneal cavity. The femoral vessels were identified along the medial tumor and traced toward the pelvis. Using 4-0 vicryl suture, the vessels were ligated just distal to the inguinal ligament. The hip was disarticulated from the pelvis by sharply incising the capsule, exposing the femoral head and dislocating it from the acetabulum with the tip of the scalpel. The limb was amputated by circumferentially dividing any remaining soft tissue attachments in line with the original incision. The skin edges were approximated and closed using a 4-0 vicryl suture in a running fashion. The animal was then awakened and recovered in the usual fashion.

Weekly IVIS imaging was continued (*per above*) until the tumor score reached 60 or at 16 weeks post-inoculation. At this time, the mice were euthanized using carbon dioxide asphyxiation. The lungs were harvested *en bloc* and placed into a conical tube containing 10% neutral buffered formalin (*NBF*). The specimens were delivered to the Pathology Laboratory for Translational Medicine, processed, and embedded per their protocol. The paraffin blocks were mounted on a microtome and 5 μm sections were obtained every 25 μm of sectioning until the entire specimen was exhausted. The slides were stained with hematoxylin and eosin. Slides were analyzed by one author (*BG*) as well as a board certified pathologist to confirm the presence of metastatic disease. The number of metastatic lesions was counted as well as the dimensions of the single largest tumor identified in the specimen.

Results

Stability of transfected cell line

There were no significant differences in radiance of the transfected cells at baseline and at 12 weeks ($3.93x10^6$ *p/sec/cm*^{2} *versus* $9.71x10^6$ *p/sec/cm*^{2}). Linear regression modeling did not reveal significant differences in radiance of the transfected cells over time ($R^2=0.019$, $p=0.65$).

Inoculation data of the wild type group

Twelve mice were inoculated (*6 left limbs, 6 right limbs*) with wild type K7M2 cells. The average mouse weight was 18.73 g (*range 17.5 – 21.0*) at time of inoculation. Eleven of twelve mice grew primary tumors at an average of 16.82 days (*range 15 – 26*) after cell implantation. One mouse died 15 days after the inoculation; no tumor growth was noted in this mouse. Eight mice underwent palliative amputation of their limb at an average of 12 days (*range 10 – 16*) after first tumor sighting. Seven of eight survived the palliative amputation; one mouse died due to uncontrollable hemorrhage from the femoral vessels. Three mice did not undergo palliative amputation of their hind limb; these mice were euthanized at the time of primary tumor harvest. Average mouse weight was 23.58 g (*range 21.0 – 26.5*) at time of amputation or early euthanasia and average tumor weight was 2.72 g (*range 1.90 – 4.53*). A primary recurrence of the tumor was noted in six of the eight mice. Five mice were euthanized at an average of 74 days (*range 68 – 89*) after inoculation for tumor scores of 60. All five of these mice had noted recurrent primary disease. Two mice were euthanized at 16 weeks post-inoculation in accordance with the study protocol; neither mouse demonstrated primary recurrence and both had a tumor score of 0 at time of

euthanasia. Three mice were euthanized at an average of 10.3 days (*range 10 – 12*) after first tumor sighting. At the time of euthanasia, lung specimens were collected on 10 mice (*all eight that underwent palliative amputation and two mice euthanized at the time of primary tumor harvest*) and processed for metastatic evaluation *(see below*).

Inoculation data for Transfected Group 1

Ten mice were inoculated *(5 left limbs, 5 right limbs*) with the luciferase transfected K7M2 cells. The average mouse weight was 18.46 g (*range 16.5 – 20.8*) at the time of inoculation. Nine of the ten mice grew a primary tumor at an average of 35 days (*range 27 – 55*) after cell implantation. One mouse failed to develop a tumor; this mouse was euthanized eight weeks after inoculation. Six mice underwent palliative amputation of the affected hind limb an average of 11 days (*range 9 – 18)* after first tumor sighting. All mice survived the palliative amputation. Three mice did not undergo a palliative amputation of their affected limb; one mouse died during IVIS imaging prior to the procedure and two mice were euthanized at the time of primary tumor harvest rather than undergoing an amputation. The average mouse weight at time of amputation or early euthanasia was 25.2 g (*range 23.5 – 26.5*) and the average tumor weight was 3.06 g (*range 2.06 – 4.34*). Four of the six mice that underwent palliative amputation demonstrated a recurrence of tumor growth at the amputation site. Four mice were euthanized at an average of 87 days (*range 77 – 132*) after inoculation due to a tumor score of 60. One mouse was euthanized at 16 weeks post-inoculation in accordance with the study protocol and one mouse died in the IVIS system 91 days after inoculation (*41 days after palliative amputation*). The lungs of seven mice (*all six that underwent palliative amputation and the one mouse that did not develop a primary tumo*r) were harvested at the time of euthanasia for histological analysis (*see below*).

Inoculation data for Transfected Group 2

Ten mice were inoculated *(5 left limbs, 5 right limbs*) with the luciferase transfected K7M2 cells to study IVIS sensitivity and detection of pulmonary metastasis using intranasal luciferin. The average mouse weight was 18.6 g (*range 17.0 – 20.0*) at the time of inoculation. Eight of the ten mice grew a primary tumor at an average of 23 days (*range 21 – 26*) after cell implantation. Two mice failed to develop a tumor; these mice were euthanized sixteen weeks after inoculation. Eight mice underwent palliative amputation of the affected hind limb an average of 12 days (*range 8 – 16)* after first tumor sighting. Seven mice survived the palliative amputation; one mouse died due to uncontrollable hemorrhage from the femoral vessels. The average mouse weight at time of amputation was 23.0 g (*range 20.0 – 24.0*) and the average tumor weight was 2.13 g (*range 1.13 – 3.0*). Seven mice were euthanized at an average of 57 days (*range 48 – 79*) after inoculation due to a tumor score of 60. Two mice were euthanized at 16 weeks post-inoculation in accordance with the study protocol and one mouse died during palliative amputation. The lungs of seven mice (*all seven that survived palliative amputation)* were harvested at the time of euthanasia for histological analysis (*see below*).

Inoculation data of Transfected Group 3

Two mice were inoculated (*1 left limb, 1 right limb*) with the luciferase transfected K7M2 cells to monitor luminescence and primary tumor size over time. The average mouse weight

was 17.0 g at the time of inoculation. Both mice grew a primary tumor at an average of 11 days after cell implantation. These mice were imaged twice weekly until reaching a tumor score of 60, at which time the mice were euthanized. The lungs were not harvested for histological analysis.

Analysis

There were no significant differences between the WT and Transfected Group 1 with regard to inoculation rate, time from tumor detection to removal, tumor weight, mouse weight at death, or time from inoculation to time of death (*any cause*). There was a significant difference between the time of inoculation to time of tumor growth and weight of the mouse at time of primary tumor harvest, however. On average, it took 17.85 days longer for a primary tumor to develop in the transfected mice compared to the wild type strain ($p<0.0001$) and the transfected mice weighed 1.63 g more ($p = 0.0175$) (Table 1).

There were no significant differences between Transfected Group 1 and Transfected Group 2 with regard to inoculation rate, time from tumor detection to removal, time from inoculation to death (days), metastatic rate, or IVIS positivity in histologically-confirmed metastasis. There was a significant difference noted between the time from inoculation to primary tumor detection, mouse weight at time of tumor harvest, and average primary tumor weight. On average, it took 12.2 days longer for a primary tumor to develop in Transfected Group 1 compared to Group 2 ($p = 0.0032$). Tumors for Transfected Group 1 weighed 0.87 g more than tumors for Group 2($p = 0.0269$) (Table 2).

Histology results

Lungs were collected on ten of the twelve WT specimens. Lungs were not collected on one mouse that died prior to developing a primary tumor and one mouse euthanized at the time of primary tumor harvest. Nine of ten specimens showed evidence of metastatic disease when analyzed microscopically. The number and size of metastatic nodules varied per mouse with a range of 1 to >10 lesions measuring 0.1 mm to 5.8 mm.

Lungs were collected on seven of ten Transfected Group 1 mice. Lungs were not collected on one mouse that died during IVIS imaging *(prior to palliative amputation*) and two mice euthanized at the time of primary tumor collection. Six of seven mice showed evidence of metastatic disease by microscopy. Again, size and number of lesions ranged from 1 to >10 and measured 1.3 mm to 20 mm in greatest single dimension. One mouse (*T17*) showed nearly complete obliteration of native lung tissue with tumor; tumor was seen within the lymph nodes as well as invading the diaphragm (Figures 2a–c).

Lungs were collected on seven of ten Transfected Group 2 mice. Lungs were not collected on one mouse that died during palliative amputation and two mice that failed to develop a primary tumor. Six of seven mice showed evidence of metastatic disease by microscopy. Size and number of lesions ranged from 1 to >10 and measured 0.1 mm to 4.0 mm in greatest single dimension. Lungs were not collected for Transfected Group 3 mice.

Histologically, specimens revealed confluent sheets of poorly differentiated cells. There was a spectrum of cells from those with a spindle shaped appearance, prominent chromatin, and

eosinophilic cytoplasm to those with a purely anaplastic appearance. High numbers of mitotic figures were appreciated. There was no statistical difference between the rate of metastasis between the WT and Transfected Group 1 (90% WT versus 86% transfected p=1.0000).

IVIS data

Seven of ten mice in Transfected Group 1 were imaged routinely by IVIS. All primary tumors were confirmed by presence of a bioluminescent signal from the leg region that was inoculated. Three mice showed evidence of a pulmonary metastatic lesion by IVIS. These lesions were seen on average 85.3 days (*range 51 – 128*) after inoculation with tumor cells. The smallest pulmonary lesion that was IVIS positive measured 5.3 mm microscopically. This mouse did not show a pulmonary lesion until 128 days after inoculation and was euthanized four days later (Figures 3a and 3b). The largest non-IVIS positive lesion measured 2.2 mm microscopically.

Seven of ten mice in Transfected Group 2 were imaged routinely by IVIS. These mice received intranasal and intraperitoneal luciferin, along with shaving of the operative limb and chest. All primary tumors were confirmed by presence of a bioluminescent signal from the leg region that was inoculated. Six mice showed evidence of a pulmonary metastatic lesion by IVIS. These lesions were seen at an average of 50 days (*range 44 – 62*) after inoculation with tumor cells. The smallest pulmonary lesion that was IVIS positive measured 1.4 mm microscopically. The largest non-IVIS positive lesion measured 3.8 mm microscopically.

The two mice in Transfected Group 3 were imaged routinely by IVIS to monitor luminescence and primary tumor size over time. Primary tumor was first detected on day 12 via IVIS imaging for both mice. Primary tumor luminescent intensity increased linearly over time as the size of the tumor increased. There was a slight drop in intensity from day 21 to day 33 (Figures 4a–4c).

Discussion

Prior to 1975, osteosarcoma treatment was limited in scope and often ended in amputation. Advances in chemotherapeutic agents led to the widespread use of adjuvant and neo-adjuvant therapy. Concurrent refinement of surgical technique and a paradigm shift from amputation to limb preserving therapy has led to an improved prognosis. Despite these advances, overall prognosis for osteosarcoma remains grave. The most significant challenge surrounding osteosarcoma is its potential for early metastasis and the nature of metastatic lesions that remain elusive to early detection. Indeed, it is estimated that patients presenting with pulmonary metastases have a five-year survival rate of less than 20%. Whereas chemotherapeutic agent expansion in the last 20 years has shown only minimal gains with regard to mortality, new avenues for treatment are actively being sought, including immunotherapy.

Relevant animal models are needed to examine the response of pulmonary metastases to various treatment agents and the primacy of an immunocompetent model cannot be

understated. Further, being orthotopically driven allows cell selection, self-seeding, and other processes to occur *in vivo* that may have an effect on the pathogenesis of this disease and, in turn, on treatment success. The resulting metastatic lesions have a unique subset of cellular characteristics secondary to all of these processes that take part in an orthotopic model. Studies recapitulated in immunocompromised animals,[8–10,21–23] by inducing tumorigenesis via intravenous or subcutaneous cell injection, or use of a transfection method with antigenic vectors do not exploit these essential principles.[24–25]

Comparing the results of the WT cell line to Transfected Group 1, the only clinically significant difference between the groups was the time from inoculation to detection of tumor growth. On average, the transfected tumors took about twice as long to present; thereafter, growth rates were similar. It is not clear why this pattern occurs, but it is consistent with other studies our lab is conducting. We have speculated that it is likely resulting from the additional genetic material that must be transcribed due to insertion of the luciferase vector. *In vitro* analysis of the transfected cell line did not demonstrate a loss of cell viability with propagation. *In vivo*, inoculation rates as well as metastatic rates appear to be similar between the two groups and consistent with previously reported rates for this cell line.[20] The smallest detectable lesion by IVIS measured 5.3 mm, which is larger than the detection limit for MRI or micro CT. However, the specificity of the lesions is 100% in this model because tumors from this cell line will fluoresce, whereas pulmonary nodules could be caused by factors other than cancer. The other advantage is the much shorter length of time under anesthesia as well as the anesthesia being less deep. These issues become much more important for survivability during testing once the animal becomes metastatic. In addition, IVIS is a more cost effective tool and logistically easier to perform compared to MRI.

With the addition of intranasal luciferin and shaving of the chest, the sensitivity of IVIS imaging to detect pulmonary metastasis improved. For Transfected Group 1 that did not receive intranasal luciferin, IVIS imaging detected a signal intensity in three out of six mice with histologically-positive pulmonary metastasis. With the addition of intranasal luciferin to Transfected Group 2, IVIS imaging detected a signal intensity in six out of seven mice with histologically-positive pulmonary metastasis. The sensitivity to detect pulmonary metastasis was 50% in Transfected Group 1 compared to an improved sensitivity of 85.7% in Transfected Group 2 with the addition of intranasal luciferin and shaving of the chest.

Pulmonary metastasis was also detected much earlier on IVIS with the addition of intranasal luciferin and shaving of the chest. On average, pulmonary metastasis was detected on IVIS imaging 50 days after inoculation in Transfected Group 2 compared to 85 days after inoculation in Transfected Group 1 that did not receive intranasal luciferin ($p = 0.0568$). The smallest pulmonary lesion that was IVIS positive measured 5.3 mm microscopically in Transfected Group 1 compared to 1.4 mm microscopically in Transfected Group 2. With the addition of intranasal luciferin and shaving, pulmonary metastasis was detected much earlier and with much smaller histologically sized lesions. The increased sensitivity of intranasal luciferin has been confirmed in previous studies, as one study showed a nearly ten-fold increase in sensitivity on bioluminescent imaging when comparing intranasal to intraperitoneal delivery of luciferin.[26]

The utility of IVIS imaging can also be extended to monitor temporal increases in tumor intensity as the size of the tumor increases. Temporal increases in tumor intensity was demonstrated by the data from Transfected Group 3, which showed that the tumor intensity (photons/second) increased linearly over time as the size of the tumor increased. There was a slight drop in intensity from day 21 to day 33, which likely corresponded to central tumor necrosis or hypoxic areas of the tumor bed as the tumor continued to grow in size. A depiction of potential tumor necrosis or hypoxia is well visualized in Figure 4a for mouse T41 at day 33. Based on these data, it seems reasonable to extend the application of IVIS imaging to monitor metastatic luminescence over time.

Khanna et al. reported the results of orthotopically implanted K7M2 cells in BALB/c mice.[20] They found a tumor take rate of 95% and a pulmonary metastatic rate of 93.3% with an average of 5.4 metastatic lesions per mouse. All mice underwent a palliative amputation of their affected hind limb once tumor volume reached approximately 450 mm^3. Tumor latency (*time from implantation to detection*) was 14 +/− 3.1 days which is consistent with the WT mice in our study; however, in our study, tumors were larger and were removed, on average, ten days sooner. Metastases in the Khanna study were enumerated using India ink via intratracheal injection at the time of euthanasia instead of histology which is the clinical standard. In addition, they did not use a luciferase reporter making it impossible to perform *in vivo* imaging.

Sottnik and colleagues developed a luciferase transfected orthotopic model of mouse osteosarcoma in C3H mice using the DLM8 cell line.[15] A plasmid transfection process was utilized. All mice underwent palliative amputation of their hind limbs at various intervals following implantation. They did find that amputations performed at seven days post implantation did not develop metastases indicating that peripheral organ seeding does not occur at the time of inoculation and metastatic potential requires maturation of the primary tumor bed. All mice amputated after day seven in their study developed metastatic disease by 22 days post-implantation. They did not report the rate of IVIS positive pulmonary lesions and did not have a non-metastatic line for comparison (*the K12 cell line for the K7M2*).

Miretti et al. developed an orthotopic lentiviral luciferase transfected K5 osteosarcoma model in BALB/c mice.[14] They reported all mice had IVIS detectable metastatic lesions by 45 days post-tumor implantation. The wild type and transfected cell lines had similar rates of primary tumor and spontaneous pulmonary disease development. They did not correlate tumor size by histology to the IVIS results. A potential drawback to this study is the use of a lentivirus transfection process which may have an effect on tumor infiltrating lymphocytes moreso than a plasmid vector, which was used in our study.

There are some limitations to our model. First, the K7M2 cell line is murine in origin and not human derived. However, the utility of murine derived tumor models is well established. The use of xenograft models is an alternative; however, it is known that tumor characteristics are influenced by the surrounding microenvironment. Thus, invasion of murine cells may affect the tumorigenicity. The development of metastatic disease does take time following

orthotopic implantation. However, the use of orthotopic models most closely mimics the clinical course of cancer progression in this disease.

This study establishes a new murine model for metastatic osteosarcoma in an immunocompetent mouse using the K7M2-wt highly metastatic cell line transfected with a non-viral plasmid luciferase vector. Bioluminescence and histopathology confirmed the establishment of a primary tumor and metastasis to the lungs. The sensitivity of IVIS imaging was determined by comparison of histopathological analysis of the lungs. To our knowledge, this is the first syngeneic osteosarcoma using the K7M2 cell line that uses bioluminescence to survey and quantify metastatic burden. The primary benefit of the preclinical model is that the immune system is intact, metastatic disease is orthotopically driven, and it appears to have a comparable pathogenesis to its wild type counterpart. The model can be used to examine promising immunomodulatory therapies in the future using bioluminescence *in vivo*.

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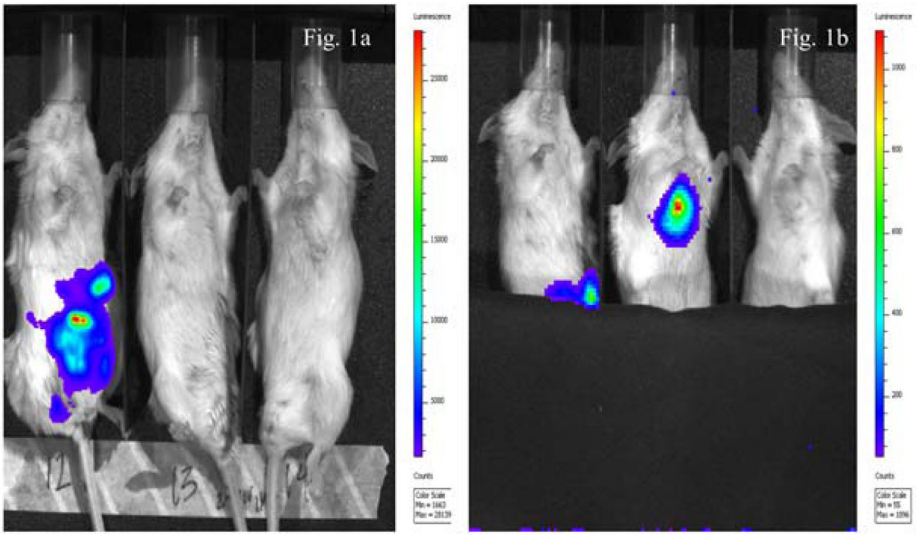

Figure 1.

Figure 1a demonstrates extensive recurrent tumor growth after palliative amputation. No pulmonary lesions appear to be present, although T13 (middle) had evidence of a pulmonary lesion a week prior (not shown). Figure 1b shows bioluminescent signal consistent with pulmonary metastasis after shielding of the lower extremities. The images were taken sequentially the same day.

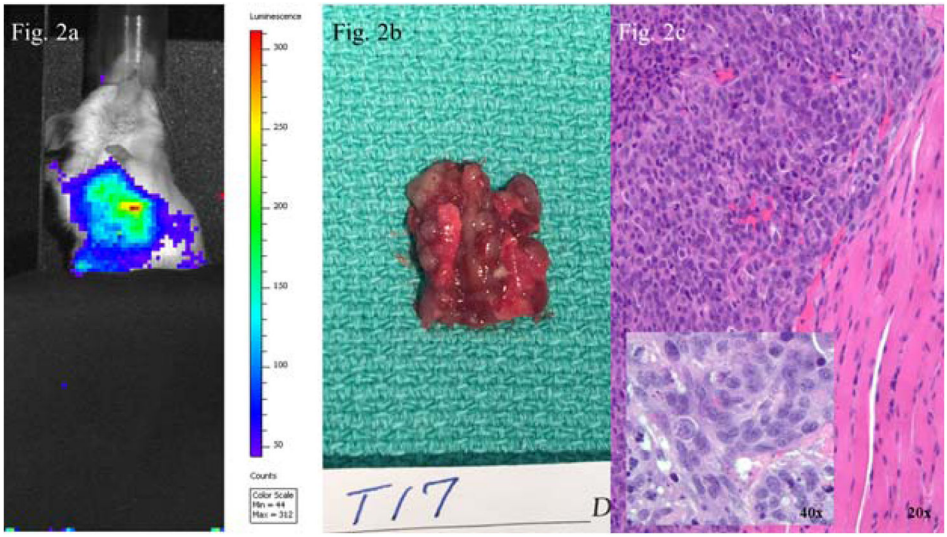

Figure 2.

Figure 2a illustrates extensive pulmonary metastatic disease by IVIS. At necropsy, widespread disease was found with near total obliteration of the chest cavity with tumor (Figure 2b). Histologically, the classic spindle cell morphology is appreciated, with tumor invading the diaphragm (Figure 2c).

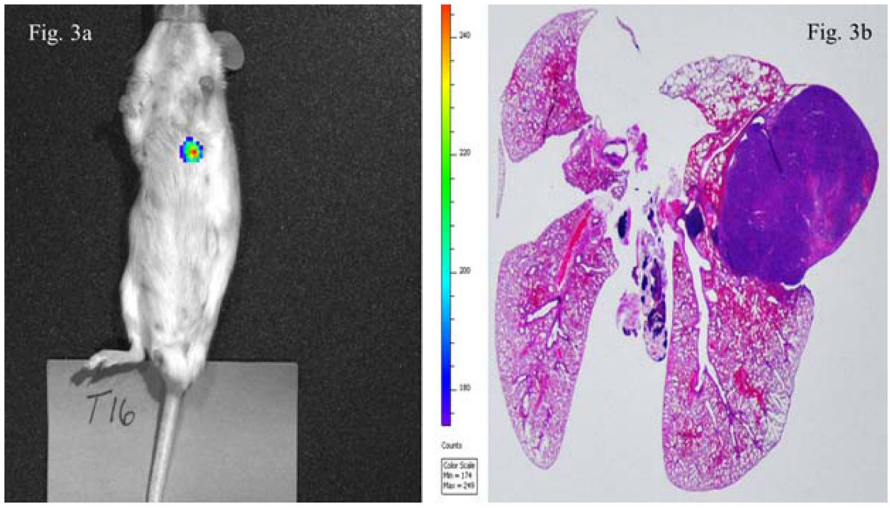

Figure 3.

Figure 3a show that, at128 days post-inoculation, a bioluminescent signal was detected in the pulmonary region of mouse T16. This lesion (Figure 3b) measured 5.3 mm histologically and was the smallest IVIS positive lesion.

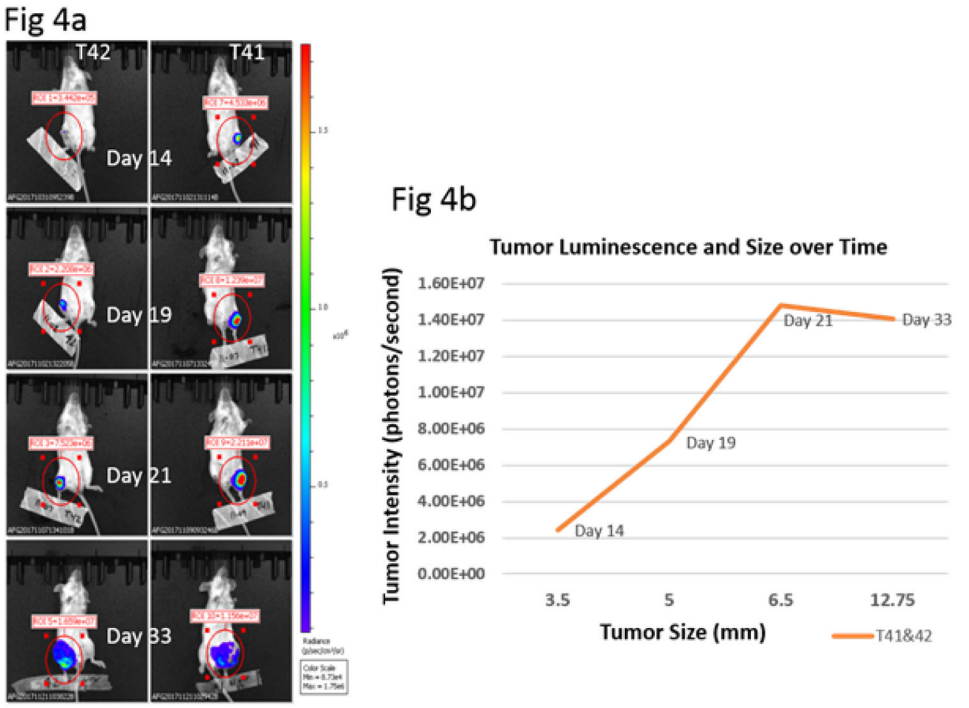

Figure 4.

Figure 4a illustrates temporal changes in luminescent intensity over time in transfected mice T41 and T42. Figure 4b shows a linear representation of the primary tumor luminescence and tumor size over time for mice T41 and T42.

Table 1

Summary of tumor characteristics comparing the wild type and transfected cell lines.

	Wild Type	Transfected	P-Value
Inoculation Rate	11/12	9/10	1.0000
Time from Inoculation to Primary Tumor Detection (Days)	16.8 ± 2.0	34.7 ± 2.2	<0.0001*
Time from Detection of Primary Tumor to Tumor Removal (Days)	11.91 ± 0.8	12.5 ± 1.0	0.6526
Mouse Weight at Time of Tumor Harvest (g)	23.6 ± 0.4	25.2 ± 0.5	0.0175*
Average Primary Tumor Weight (g)	2.7 ± 0.3	3.1 ± 0.3	0.4490
Time from Inoculation to Death (Days)	67.5 ± 10.2	81.33 ± 11.2	0.3748
Average Weight at Death	24.35 ± 0.9	24.19 ± 1.0	0.9053
Metastatic Rate	9/10	6/7	1.0000

Table 2

Summary of tumor characteristics comparing Transfected Groups 1 and 2.

	Transfected Group 1	Transfected Group 2	P-value
Inoculation Rate	9/10	8/10	0.5921
Time from Inoculation to Primary Tumor Detection (Days)	34.7 ± 2.2	22.5 ± 2.6	0.0032*
Time from Detection of Primary Tumor to Tumor Removal (Days)	12.5 ± 1.0	12.1 ± 1.2	0.8299
Mouse Weight at Time of Tumor Harvest (g)	25.2 ± 0.5	22.6 ± 0.5	0.0037*
Average Primary Tumor Weight (g)	3.06 ± 0.26	2.19 ± 0.24	0.0269*
Time from Inoculation to Death (Days)	85.33 ± 12.6	50.17 ± 8.9	0.0568
Metastatic Rate	6/7	6/7	1.0000
IVIS positivity in histologically-confirmed mets	3/6	6/7	0.2127