Preclinical Modeling of Musculoskeletal Cancer

Dissertation

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By

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Abstract

Preclinical animal models serve as invaluable tools in the investigation of disease and the development of novel therapeutic strategies to prevent and treat disease. Osteosarcoma is the most common primary bone tumor in humans and dogs, and is associated with a high rate of lung metastasis. In order to successfully develop new strategies to improve outcomes for OSA patients, we sought to develop a clinically relevant mouse model with spontaneous metastasis. We successfully developed a model that mimicked the biologic behavior seen in dogs and in people with osteosarcoma. This included development of a primary bone tumor that was surgically amputated followed by development of lung metastasis. Lung metastases were quantified using stereological principles with an average of 33% of lung affected by metastasis in the model. We then used this model to investigate changes in gene expression that occur between primary and metastatic OSA that might be critical to the metastatic cascade and therefore be viable targets for therapy. Eighty genes were identified as differentially expressed between primary and metastatic tumors. Identified genes ranged from well-known genes involved in cancer to others that are yet unnamed. The model was also used with primary canine-derived patient OSA tissue. These tissues will be used to further investigate selected genes identified by microarray.
Radiation therapy is an important modality in the treatment of many types of cancer; however there are important adverse effects to bone that must be considered. Following exposure to external beam radiation, there is loss of trabecular bone with an associated decrease in bone strength and increase in brittleness. This leads to a markedly increased rate of fracture in irradiated bones. We utilized a previously developed mouse model of radiation induced bone damage and examined changes in mechanical properties as well as changes in osteoclasts. Changes in mechanical properties mirrored those previously reported for the model. We also discovered that osteoclasts increase initially, followed by a decrease at 12 weeks post irradiation. To further investigate the effects of radiation on osteoclasts, we exposed osteoclast precursors and mature cells to radiation and quantified cell viability. As expected, mature osteoclasts were more radioresistant than osteoclast precursors. Multiple factors are likely involved in radiation-induced changes in bone, including changes in osteoclast number and function.
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Chapter 1: Background on Osteosarcoma

Introduction

Osteosarcoma (OSA) is the most common primary malignancy of bone in humans, affecting approximately 500-1000 children and adolescents in the United States each year, and is the second most frequent cause of cancer-related death in adolescents.\textsuperscript{1-3} The tumor is composed of a neoplastic mesenchymal population that produces osteoid and/or immature bone, and is thought to arise from mesenchymal stem cells of the osteoblast lineage.\textsuperscript{4} Osteosarcoma mortality is most often attributed to hematogenous spread leading to pulmonary metastasis, which occur in approximately 80\% of cases if surgical resection of the primary bone tumor is the only treatment pursued.\textsuperscript{2} In humans, the peak incidence occurs in the second decade of life and is thought to be related to the rapid bone growth and turnover associated with adolescence.\textsuperscript{5} Many other mammals are affected by osteosarcoma, in particular, domestic dogs. The incidence of osteosarcoma in pet dogs in the United States is estimated to be over ten times that in humans, reaching over 8,000 cases per year in the United States. In addition, canine osteosarcoma shares many characteristics with human osteosarcoma with regard to disease biology and therefore serves as a valuable model for the condition in people.\textsuperscript{6}
Osteosarcoma in Humans

Epidemiology

All bone and joint cancers together make up approximately 6% of cancers diagnosed in the United States.\(^7\) OSA is the most common primary malignancy of bone followed by chondrosarcoma and Ewing sarcoma, and has a bimodal age distribution with the largest group affected being children and adolescents in the second decade of life. The second smaller peak occurs in people over 65 years of age.\(^8\) The incidence of osteosarcoma in the United States is approximately 4-5 per million adolescents with between 500-800 new cases each year.\(^2, 7, 9\) The propensity for osteosarcoma to occur in the 10-14 age range\(^2\) corresponds with the period of rapid skeletal growth and bone turnover. Although osteosarcoma can occur in any bone, the most common locations are in the long bone metaphysis of the appendicular skeleton.\(^10\) In addition, there is a higher incidence of osteosarcoma in males\(^11\) and children diagnosed with OSA are taller than their peers and have increased birth weight.\(^12, 13\) Hispanics and African Americans are at higher risk of developing OSA than their white counterparts.\(^7\) The second peak in OSA incidence that occurs in people over 65 years of age is more likely to occur as a secondary condition. Pre-existing conditions include Paget’s disease, previously irradiated bone, bone infarcts, chondrosarcoma, and other bone related pathologies.\(^14\) These patients generally have a poorer prognosis due in part to the increased likelihood of an axial location, and also due to a reduced tolerance to chemotherapy.\(^15, 16\)
Etiology

The vast majority of cases of OSA have an unidentified etiology; however, there are known genetic, environmental and concurrent bone diseases that are identified in some cases and may shed light on the pathogenesis of the development of OSA. Osteosarcomas have complex karyotypes and are typically aneuploid; therefore common chromosomal alterations have not been clearly identified. With regards to specific genetic etiologies, there is a well-documented increased risk associated with hereditary retinoblastoma, Li-Fraumeni syndrome, Rothmund-Thompson syndrome and Bloom and Werner syndromes.

People with hereditary retinoblastoma are born with a germline mutation in the RB1 gene, located at chromosome 13q14. RB1 is a tumor suppressor gene which negatively regulates cell cycle progression at the G1 to S phase. In normal, non-dividing cells, the retinoblastoma protein binds and suppresses E2F transcription factors. When the cell is stimulated by growth factors to replicate, cyclin dependent kinases, including CDK4 and CDK6, hypophosphorylate the Rb protein. This leads to release of E2F and subsequent transcription of genes involved in the progression of the cell cycle. Mice that lack RB1 do not survive past embryonic day 15 and demonstrate defects in multiple organ systems. Mice that are heterozygous for RB1 develop pituitary tumors at 6 months of age, but interestingly do not develop retinoblastomas or osteosarcomas. This may be due in part to other members of the Rb family, including p107 and p130, or yet undiscovered functions of the Rb protein. Patients with RB1 mutations are at a greatly increased risk of developing OSA, with an incidence 500 times greater than the normal
population.\textsuperscript{24} RB1 is also found to be mutated in 30-75\% of spontaneous OSAs, in addition to being altered in many other types of cancer.\textsuperscript{25}

People with Li-Fraumeni syndrome have an autosomal dominant mutation in the p53 gene and are at a high risk of developing OSA among many other cancers. p53 is a tumor suppressor gene that can detect DNA damage and then halt the cell cycle to allow for DNA repair via activation of p21WAF1 or cause apoptosis in part through activation of the pro-apoptotic protein Bax.\textsuperscript{26} Loss of functional p53 has been implicated in many different cancers, including brain, breast, adrenocortical tumors, leukemias and soft tissue sarcomas.\textsuperscript{27} Importantly, OSA is the second most common cancer reported in patients with Li-Fraumeni syndrome.\textsuperscript{28} Mechanisms leading to loss of p53 include allelic loss, point mutations and gene rearrangements.\textsuperscript{29} In addition to direct inactivation of p53, there are many regulators of p53 that many be altered in osteosarcoma. MDM2 protein inactivates p53 and can block transcriptional activity and transport from the cytoplasm into the nucleus. MDM2 also targets p53 for proteosomal degradation via its ubiquitin ligase activity.\textsuperscript{30} Amplification of the MDM2 gene has been associated with metastatic OSA in humans.\textsuperscript{31}

A subset of patients with Rothmund-Thompson syndrome have a mutation in the RECQL4 gene, which encodes a DNA helicase. When cells lack functional RECQ proteins, there is an increased rate of mitotic recombination, chromosome mis-segregation, defects in meiosis and increased sensitivity to DNA-damaging agents.\textsuperscript{32} These patients are at a greatly increased risk of developing OSA.\textsuperscript{33} Additionally, they are at increased risk for toxicities associated with chemotherapy. In contrast to RB1 and p53,
RECQL4 has not been identified as a mutation in spontaneously occurring OSA. Bloom and Werner syndrome patients also have mutations in RECQ-helicases and have increased risk of multiple types of cancer including OSA.

One of the most well documented environmental risk factors for osteosarcoma is exposure to ionizing radiation and it is implicated in 3% of OSA cases. The median time period between radiation and the diagnosis of an OSA is 12-16 years and the mean age at presentation is 45.6 years. Both therapeutic and accidental exposure to radiation has been documented to contribute to OSA and pre-natal exposure to x-rays increases the risk of developing OSA. Despite historical assumptions that radiation-induced OSA had a worse prognosis, recent studies have shown that radiation-induced OSA has a similar prognosis to sporadic OSA. Research has shown that alkylating agents also contribute to an increased risk of osteosarcoma, particularly evidenced in Ewing sarcoma patients that undergo high-dose radiation and alkylating agent chemotherapy.

Paget’s disease is a chronic skeletal disorder characterized by rapid bone remodeling with resultant abnormal bone formation. Paget’s disease occurs in the over 40-year-old population and contributes significantly to OSA risk, particularly the widespread form, with approximately 1% of patients developing OSA. This represents a several thousand fold increased risk. When patients with Paget’s disease develop OSA, they have a poorer than average prognosis, particularly if the OSA occurs in the skull or pelvis. Other pre-existing conditions of bone such as infarcts, enchondromatosis (Ollier’s disease), fibrous dysplasia, and chronic osteomyelitis have also been associated with an increased risk of developing OSA.
Diagnosis and Staging

Patients with OSA most often present with non-specific symptoms of pain and/or swelling in a bone or joint of several months duration that may have been misdiagnosed as a sports-related injury.5, 46 Five to ten percent of patients present with a pathologic fracture and no previous diagnosis of OSA.47 The sites most commonly affected are the metaphyseal region of long bones, more specifically the distal femur, proximal tibia and proximal humerus. Plain film biplanar radiography is the first step in diagnosis and typically demonstrates an aggressive lytic bone lesion, sometimes accompanied by osteoblastic features and/or a soft tissue mass. The most common radiographic pattern of osteosarcoma includes both lytic and proliferative areas with a wide zone of transition between neoplastic and normal bone.8 A triangular area of new subperiosteal bone that lifts the periosteum off of the bone, often referred to as “Codman’s triangle”, alludes to the aggressive nature of the lesion in many cases. Exuberant osteoblastic lesions may exhibit a “sunburst” appearance. Pathologic fracture is present at diagnosis or occurs pre-operatively in approximately 10% of cases of high grade conventional OSA,48 and can lead to an increased risk for local recurrence.47 However, pathologic fracture does not appear to affect the prognosis as long as surgical margins can be achieved with complete removal of the tumor at the primary site in bone.49 Skip metastases are defined as bone metastases that occur in the same bone as the primary tumor at a more proximal location and are best detected by magnetic resonance imaging (MRI).50 Despite improved detection of skip metastasis with current imaging modalities, prognosis is guarded in
these patients. Radionuclide bone scanning with technetium can also be used to detect primary or secondary sites in bone and is fairly sensitive; however, its lack of specificity limits its utility. Bone cysts and reparative processes will both cause uptake of the radiotracer.

Following a radiographic suspicion of osteosarcoma, a bone biopsy is performed, of which there are two techniques for the procedure: open and closed biopsy. Open biopsies are performed under general anesthesia and allow for collection of multiple specimens. Closed biopsies are usually performed as an outpatient procedure, but may require multiple needle aspirates to provide adequate tissue for diagnosis. Correlation of the biopsy results with the radiographic findings is important to ensure that the correct tissue was sampled. It is important that the location of the biopsy tract or incision be carefully considered such that it will be removed in the future resection surgery, in order to ensure local control of the tumor.

The lung is by far the most common site of OSA metastasis, followed by bone. Metastasis to the regional lymph node and other organs is much less common. Initial staging for metastasis is done using thoracic computed tomography (CT) to detect lung metastasis. Although CT is the best modality to detect pulmonary metastasis, in 26% of cases the number of metastases might be underestimated, and lesions less than 5 mm can’t be reliably classified. Additionally, CT is unable to distinguish between benign and metastatic masses. Metastatic disease is detected in less than 20% of patients diagnosed with high-grade conventional osteosarcoma. PET imaging with 18F-fluorodeoxyglucose (18F-FDG) can be used to detect metastases, but is not considered
superior to CT. It may however be useful around metallic implants or to monitor the response to chemotherapy that may not be apparent morphologically. Uptake of the 18F-FDG indicates an increased rate of glycolysis and may aid in biopsying the more biologically active and therefore more diagnostic areas of the tumor. Dynamic contrast enhancing MRI is a functional imaging technique that utilizes a bolus of gadolinium and may be the best way to detect an early response to chemotherapy in osteosarcoma by detecting residual viable tumor that is not apparent using other imaging modalities.

The staging systems for sarcomas are different than the typical TNM system common to other cancers because they rarely metastasize to the regional lymph node. Two commonly accepted staging systems for OSA exist: the Musculoskeletal Tumor Society Staging System and the American Joint Committee on Cancer (AJCC) Staging System. The Musculoskeletal Tumor Staging Society system is based on the histopathological grade of the tumor (low or high), whether the tumor is intra- or extra-compartmental, and the presence of metastasis. The AJCC staging system is based on tumor size (T), regional lymph node (N), distant metastasis (M), and histopathological grade (G).

Classification

A diagnosis of osteosarcoma is made based on the production of osteoid and/or bone by neoplastic mesenchymal cells. The most common variant of osteosarcoma is primary conventional high grade and arises from the intramedullary canal. Conventional
OSA may be further classified based on the predominant matrix produced by the neoplastic cells. Those producing only bone and osteoid are termed osteoblastic, while those producing cartilage as the primary product are classified as chondroblastic and lastly, those producing mostly fibrous connective tissue are termed fibroblastic.\textsuperscript{61} Variants other than conventional OSA can be classified according to morphology, location of origin on the bone surface, or clinical presentation.

Osteosarcoma variants classified based on morphology include telangiectatic osteosarcoma, low-grade intraosseous osteosarcoma and small cell osteosarcoma. Telangiectatic OSA is characterized by blood filled spaces lined by tumor cells rather than endothelial cells, and sparse amounts of osteoid spicules. It has an aggressive osteolytic appearance and is rapidly expansile. Radiographically it often resembles an aneurysmal bone cyst. Grossly, telangiectatic OSA resembles hemangiosarcoma and cannot be differentiated without histopathology.\textsuperscript{62} In the past telangiectatic OSA has been reported to have a worse prognosis,\textsuperscript{63} however, subsequent studies have refuted this.\textsuperscript{62, 64, 65}

Low-grade intraosseous (central) osteosarcoma is rare and occurs most commonly in the knee of young adults. It typically appears less destructive radiographically and is often misdiagnosed as fibrous dysplasia. Histologically, the tumor is composed of spindle cells displaying minimal cytologic atypia and producing variable amounts of bone and collagen matrix. Surgical resection without chemotherapy is considered curative in these patients.\textsuperscript{66, 67}
Tumors classified as small cell osteosarcoma resemble Ewing’s sarcoma and are composed of small round cells with oval nuclei in a fine eosinophilic matrix. The presence of osteoid confers a diagnosis of small cell osteosarcoma; however, the relationship of these two entities has been questioned.68, 69

Osteosarcoma variants classified based on originating from the bone surface include parosteal OSA, dedifferentiated parosteal OSA, periosteal OSA, and high-grade surface OSA. Parosteal OSA arises from the outer periosteum and is generally a slow growing low-grade tumor. It occurs most commonly in the posterior distal femur of females 20-40 years of age. Radiographically it appears as a densely ossified mass adjacent to the cortex and does not elevate the periosteum, in contrast to periosteal OSA. Unlike conventional high grade OSA, parosteal OSA metastasizes very late in the course of disease and chemotherapy is not indicated if wide resection is achieved.70, 71 In up to 20% of cases of parosteal OSA, a more malignant mesenchymal component of the tumor may develop, termed “dedifferentiated parosteal OSA”. Although the radiographic appearance remains much the same, there are low-grade and high-grade areas present histologically. This form is much more aggressive and likely to metastasize, and therefore chemotherapy is an important component of treatment.72

Periosteal OSA, on the other hand, arises from the inner periosteum causing elevation and thickening of the periosteum, and often has a large chondroblastic component. It most commonly arises from the diaphysis of a long bone and is generally considered an intermediate grade OSA. Although wide surgical resection is a key component of treatment, the need for chemotherapy is debatable.71, 73, 74
High grade surface OSA may resemble periosteal OSA radiographically and arises from the long bone diaphysis. However, histologically it is identical to a conventional high grade (central) OSA. It can be distinguished from dedifferentiated parosteal OSA by its less differentiated matrix. The prognosis for this variant is poorer than for that of other surface osteosarcomas and therapy is similar to standard therapy for high grade conventional OSA.\textsuperscript{75, 76}

Osteosarcoma variants that are best classified based on clinical presentation include gnathic OSA, OSA of the skull, Paget’s-associated OSA, post-radiation OSA, and multicentric OSA (also known as osteosarcomatosis). OSA of the jaw and skull occur more often in older adults than in the adolescent age group. Gnathic osteosarcomas often have a high cartilaginous component and are much less likely to metastasize. They are more prone to occur in the mandible and in men.\textsuperscript{77} OSA of the skull is rare and occurs more frequently in the vault than at the base. Unlike gnathic OSA, skull OSA is more often osteoblastic than chondroblastic. Neoadjuvant chemotherapy may be useful in obtaining good surgical margins, but post-operative chemotherapy is not a mainstay.\textsuperscript{78} Paget’s-associated osteosarcoma and post-radiation osteosarcoma are the same as conventional high grade OSA with regards to histologic appearance and treatment.\textsuperscript{79} Paget’s disease is a disorder of bone remodeling in middle-aged to older adults characterized by vastly increased osteoclastic resorption and bone formation resulting in osteosclerosis. In rare cases (<1%), osteosarcoma can occur in the pagetic bone.\textsuperscript{80} Radiation-induced OSA occurs in irradiated bones often a decade or more after ionizing
radiation exposure occurred. Multicentric OSA is characterized by multiple bone lesions occurring simultaneously or sequentially and carries a poor prognosis.

Prognostic factors

Patients that present with metastatic disease, typically in the lung or bone, represent less than 20% of those presenting with OSA; however, they have a markedly worse prognosis than those with undetectable metastases. The overall 5-year survival for patients with metastatic disease at diagnosis is 20%-50%. Some authors have speculated that the poorer prognosis is attributable to more aggressive intrinsic biologic properties of metastatic OSA versus those that have not metastasized. Conversely, other studies indicate that diagnosis at a later stage of disease and therefore, more advanced spread is the reason for the guarded prognosis. In those patients with metastatic lung disease at diagnosis, disease confined to one side of the lung conveys a better prognosis than bilateral disease. Additionally, a smaller number of metastases impart a better prognosis, although the proposed “cut-off” number varies from study to study.

Tumor site and size have been shown to be prognostic factors, with larger tumors and an axial location being negative prognostic factors. A large tumor was defined as one which extended at least 1/3 of the length of the involved bone; therefore, this parameter can only be used for appendicular OSA. Specific variants of OSA confer an improved prognosis compared with conventional OSA; these include parosteal OSA, periosteal OSA and low-grade central OSA. Age does not appear to be a prognostic factor and
previous reports of a negative prognosis with increasing age may be attributed to an axial
tumor location, which is more common in older patients. Female osteosarcoma patients
seem to fare better according to some studies, but not in others.\textsuperscript{85, 88}

Following surgery to remove macroscopic evidence of tumor, patients who fail to
achieve surgical remission have a poorer outcome.\textsuperscript{85} The most reliable and widely used
prognostic factor for patients without detectable metastatic disease at the time of
diagnosis is based on the response of the primary bone tumor to neoadjuvant
chemotherapy. Following surgery to remove the primary bone tumor, the tumor is
sectioned longitudinally and examined histologically to determine the percentage of
tumor tissue that has undergone necrosis. Tumors which exhibit less than 90\% necrosis
impair a poor prognosis. This represents a response grade of 1-3 on the six-grade Salzer-
Kuntschik scale where grade 1 denotes no viable tumor; grade 2, solitary viable cells or
one focus of less than 0.5 cm; grade 3, less than 10\%; grade 4, 10\% to 50\%; grade 5,
more than 50\% viable tumor; and grade 6, no effect of chemotherapy.\textsuperscript{89} Unfortunately
there are no chemotherapy intensification protocols that have been proven to improve
survival in patients with a poor response to neoadjuvant chemotherapy, although various
agents have been tried including ifosfamide, vincristine, bleomycin, and increased
dosages or intraarterial administration of cisplatin.\textsuperscript{90-92}

Treatment
Specific treatment protocols vary depending on the location, extent, tumor stage, and other patient factors. Even in patients that only present with localized disease, historical data shows that approximately 80% have micrometastatic disease that is undetectable with imaging modalities. The most widely accepted therapeutic plan for patients with conventional high grade osteosarcoma of the appendicular skeleton includes neoadjuvant chemotherapy, surgical resection of the primary bone tumor and lung metastasis followed by post-surgical chemotherapy.

The introduction of chemotherapeutic agents in the treatment of OSA has led to a greatly improved prognosis for patients over the past forty years and is a vital component of current therapy. The most commonly used chemotherapeutic protocols employ the use of doxorubicin, cisplatin, and methotrexate; other agents include ifosfamide, etoposide and cyclophosphamide. Methotrexate, an anti-metabolite that allosterically inhibits dihydrofolate reductase, was one of the first chemotherapeutic agents used widely to treat osteosarcoma in the 1970’s. High dose methotrexate was combined with leucovorin as a rescue agent to prevent myelosuppression and is still a mainstay of therapy. In the late 1970’s and 1980’s, some of the investigators at the Mayo Clinic doubted the efficacy of methotrexate and disputed the validity of using historical controls, arguing that the natural disease was altered and patients were diagnosed earlier, rather than the drug improving survival. Although many investigators disagreed, there was nonetheless enough controversy to incite the prospective “Multi-Institutional Osteosarcoma Study” using control patients that received no chemotherapy. This clinical trial proved that chemotherapy did indeed improve patient survival compared with then present-day
controls. Five year survival rates went from <20% to approximately 65%. A second mainstay chemotherapeutic agent, doxorubicin, which acts as an intercalating agent, was later added in combination with methotrexate and significantly improved the survival rate of patients with OSA. In addition, cisplatin, which crosslinks DNA, is a common agent effective against OSA. Ifosfamide (or cyclophosphamide) and etoposide are also commonly used in certain protocols, but the agents with the most evidence supporting their use are methotrexate, doxorubicin and cisplatin. Intensification protocols that increase the dose or add additional agents have proven largely unsuccessful. Despite the vast improvement in survival since the pre-chemotherapeutic era, the past 25 years have not yielded any great successes with regard to prolonging overall or event free survival in OSA.

As OSA patients began to live longer with the introduction of adjuvant chemotherapy, efforts were made to reduce the number of amputations and increase the number of limb sparing procedures. As a result, the use of preoperative chemotherapy emerged. Originally used as an attempt to treat the tumor while awaiting custom limb spare prostheses, neoadjuvant chemotherapy with a delay in surgical resection was shown to have a positive effect on outcomes by allowing more time for planning and post-operative healing. It also allowed for evaluation of the tumor response to a particular regimen, which was later found to be an important prognostic factor. It was theorized that preoperative chemotherapy may eradicate microscopic foci of metastasis. As a result of these and other studies, neoadjuvant chemotherapy became a mainstay of therapeutic regimens.
One of the most important components of OSA therapy is complete surgical resection of all detectable malignant lesions. Two to three weeks prior to surgery, neoadjuvant chemotherapy is halted to allow for recovery of bone marrow hematopoietic cells. Most surgical resections employ limb salvage techniques, assuming that clean margins can be achieved and reconstruction of the limb is possible. The Musculoskeletal Tumor Society recommends a wide local excision that removes the “primary tumor en bloc along with its reactive zone and a cuff of normal tissue in all planes”. Wide local excision is successful in preventing local recurrence in approximately 95% of patients, according to one study, which is important given the poor prognosis for patients that experience recurrence at the primary site. Imaging technologies today allow for more tissue preservation, facilitating limb-sparing techniques which aim to maintain function and address cosmetic concerns. Tumor removal and skeletal reconstruction are typically combined into a single surgery. There are three general criteria a patient must meet to be a candidate for limb-spare: response of the primary tumor to pre-operative chemotherapy, ability to achieve a clean surgical margin, and the ability to reconstruct the limb so that a reasonable degree of function is maintained. Limb spare surgeries may employ various techniques including prosthesis, autografts from the fibula, allografts, and rotationplasty. Limb-sparing surgery in patients less than 8 years of age (who are still very skeletally immature) is controversial due the need for multiple surgeries over several years to maintain limb length equality.

Radiation therapy, although not a mainstay in the treatment of surgically resectable OSA, has a role in palliation and treatment of unresectable tumors.
general OSA has been considered a relatively radioresistant neoplasm. Additionally, the long term effects of radiation therapy must be considered, particularly in a young patient population with a long lifespan in which to develop late term effects or in older patients that may already be experiencing age-related bone loss. There are some indications that charged particle therapy such as protons may be a more effective form of radiotherapy for OSA. In addition, recent studies indicate that radiation is indeed an effective therapy in craniofacial and gnathic OSA and improved overall and disease free survival at 5 and 10 years following diagnosis. In cases of metastatic OSA, radiation therapy plays an important role in palliation of painful metastasis. One study reports that in 76% of OSA metastasis sites that were irradiated for pain control, patients experienced pain relief. The importance of pain control in these patients with poor prognoses is of utmost concern and is underscored by the young age of many patients. Despite the fact that radiation is not typically indicated for conventional OSA that has not metastasized, its utility in palliation of metastases and in treatment of unresectable primary OSA warrants its use in selected cases.

**Osteosarcoma in Dogs**

**Epidemiology**

Osteosarcoma occurs in many veterinary species, but is particularly common and well-described in domestic dogs. In the United States there are an estimated 8,000 cases per year, which is ten times more than the number seen in humans annually. OSA
tends to occur in middle-aged to older dogs, with a median age of 7 years. Some papers report a bimodal age distribution with a second peak at 2 years of age. Although there has been a reported increased incidence in male dogs compared to female dogs, this finding has been contradicted by other studies. As in humans, a location in the long bone metaphysis of the appendicular skeleton accounts for the majority of cases. The most common locations include distal radius, proximal humerus and proximal tibia. The disease affects large and giant breed dogs most often; within the large dog breeds, Irish setters, St. Bernards, Rottweilers and Doberman pinchers have been reported to have a higher risk of developing OSA.

Etiology

The etiology of canine osteosarcoma is largely unknown. As in humans, there are a number of well-described risk factors. These include ionizing radiation, previous fractures, bone infarcts, metallic bone implants, and genetic abnormalities. One study found that Rottweilers that underwent gonadectomy prior to 1 year of age were at an increased risk of OSA, but these data have not been substantiated by additional studies.

Ionizing radiation, whether therapeutic or experimental, has been a known risk factor for osteosarcoma for many years. When administered in coarse fractions, it has a stronger impact on late responding tissues such as bone, as does orthovoltage radiation, compared with megavoltage radiation. Ionizing radiation likely causes OSA as a result of
mutagenic effects on the tissues. In one study, 3.4% of dogs were reported to develop OSA 1.7 to 5 years after radiation therapy for soft tissue sarcomas ceased.

Many of the same genetic abnormalities found in human OSA are found to be abnormal in dogs with OSA as well. Mutation of the p53 gene that results in loss of function is a frequent occurrence in both species. P53 is an important regulator of cell cycle and is altered in many different types of cancer. p53 alterations may contribute to a poorer prognosis in dogs with OSA. Phosphatase and tensin homolog (PTEN) is another important tumor suppressor that is mutated in some cases of canine OSA. Amplification of the proto-oncogenes c-sis and c-myc have been described in dog OSA tumors and are important in cell cycle and proliferation. Platelet-derived growth factor-B (PDGF-B) is a c-sis gene product and was shown to be increased in both canine and human OSA tumors. Furthermore, PDGF is a known chemotactic agent that attracts osteoblasts and may stimulate their growth and differentiation from precursors. Insulin-like growth factor-1 plays a role in cell proliferation and invasion of OSA cell lines from both species, which isn’t surprising considering its normal affects on osteoblast stimulation. Mesenchymal-epithelial transition factor (MET) is another proto-oncogene that is known to play a role in metastasis and angiogenesis, and is expressed aberrantly in canine OSA. Ezrin, a membrane-cytoskeleton linking protein has been implicated in metastasis and a more malignant phenotype in OSA.

Diagnosis and Staging
Dogs with OSA typically present with lameness due to pain, and swelling of the affected limb. The lameness may appear to have an acute onset in the cases of pathologic fracture, and the owner may report an incident associated with the onset of lameness. Plain film radiography of the affected site reveals changes similar to that seen in humans including a lytic and proliferative bone lesion, often accompanied by soft tissue swelling. A definitive diagnosis is achieved with a bone biopsy, but the signalment, presentation and radiographic findings often impart a high degree of suspicion themselves. Thoracic radiographs are used to assess the dog for pulmonary metastasis, although less than 10% of dogs present with detectable metastasis.\textsuperscript{115,138} Despite this, approximately 90% of dogs have undetectable pulmonary metastasis, based on studies examining dogs that underwent amputation alone.\textsuperscript{116,138} Less common sites of metastasis include other sites in bone and regional lymph nodes. Staging in dogs with OSA is done based on the “TNM” (tumor, node, metastasis) system. Stage I includes low-grade tumors (G1) without evidence of metastasis; stage II includes high-grade tumors (G2) without metastasis; and stage III includes dogs with metastatic disease.

Prognostic factors

Although less than 10% of dogs have detectable metastatic disease at the time of diagnosis, it is an important negative prognostic factor in those cases. Standard chemotherapy is generally considered ineffective for these dogs, leaving only palliative therapies.\textsuperscript{115,139} Increased serum alkaline phosphatase (ALP) has also been shown to be a
negative prognostic factor, decreasing survival time by 50%.\textsuperscript{140, 141} Larger dogs, possibly due to a lower overall dose of chemotherapy, may have a worse prognosis according to one study. The same study showed a worse prognosis for dogs with OSA of the humerus, perhaps due to delayed detection in this location.\textsuperscript{142} A histologic grading system described by Kirpensteijn in 2002 is based on cellular pleomorphism, mitotic figures, cellularity, production of tumor matrix, and degree of necrosis. Dogs with tumors that were a grade 3 had significantly shorter survival than those with a grade 1 or 2.\textsuperscript{143} As in humans, parosteal osteosarcoma imparts a better prognosis due to its much lower metastatic rate.\textsuperscript{144} A study by Hammer et al. found that a telangiectatic histologic subtype, location on the rib or scapula, higher body weight, and incomplete excision were negative prognostic factors for dogs with OSA of the axial skeleton. One of the most perplexing prognostic factors is that dogs that undergo limb sparing procedures and experience post-operative infections have longer survival times than those that do not experience infection.\textsuperscript{145} Some investigators theorize this is due to an increased immune response, but others hypothesize that commonly used fluoroquinolone antibiotics may have an anti-tumor effect.\textsuperscript{146}

Treatment

The best practices regarding treatment for dogs with osteosarcoma entails complete surgical resection of the primary bone tumor via amputation or limb-sparing technique, followed by multiple cycles of doxorubicin or cisplatin-based chemotherapy.
The median survival with surgery alone is less than 5 months, while the addition of post-operative chemotherapy increases survival up to 16 months.\textsuperscript{138, 142, 147-150} Once metastatic lesions are detectable, chemotherapy is considered to be ineffective.\textsuperscript{139} Palliative therapies include the use of non-steroidal anti-inflammatories, other analgesics (e.g. opiates) and bisphosphonates. Bisphosphonates may be effective in decreasing bone pain due to osteolysis.\textsuperscript{151, 152} Radiation therapy can be used as a palliative therapy and may not only decrease pain, but improve limb function in up to 75\% of dogs according to one study.\textsuperscript{153, 154}

Molecular Markers

A number of molecular markers that are important in the pathogenesis of metastasis in osteosarcoma have been identified. One of the first steps in the metastatic cascade involves the metastatic cells detaching from the primary tumor mass and migrating through the extracellular matrix. Matrix metalloproteinase 2 and 9 have the ability to degrade components of the ECM, thus facilitating metastasis and both have been implicated in OSA.\textsuperscript{155, 156} In addition, the NOTCH pathway has been identified as an important initiator of invasion and migration.\textsuperscript{157} Numerous studies have shown that Wnt/β-catenin is important in OSA and likely plays a role in migration as well.\textsuperscript{158-161}

For tumors to proceed along the “metastatic cascade”, it is essential that the tumor cells survive in the bloodstream, avoid apoptosis and evade the immune system in order to reach the target organ. Activation of PI3K and Akt, thought to be mediated by Src,
contributes to survival of osteosarcoma cells once they have detached from the other neoplastic cells in the primary tumor.\textsuperscript{162} Downregulation of Fas on the cell surface of OSA cells may be important in evasion of the host immune system, particularly NK cells, and loss of Fas has been shown to be a negative prognostic factor.\textsuperscript{163, 164}

OSA cells have a strong propensity for metastasizing to the lung and although the exact reason for this tropism remains incompletely understood, some studies have shown that OSA interacts with the pulmonary endothelium via CXCR-3 and CXCR-4 and that this likely contributes to targeting the tumor to lung tissue.\textsuperscript{165, 166} Once the OSA tumor cell has arrived at the metastatic site, it must extravasate and re-establish itself in the new microenvironment in order to be successful as a metastasis. Binding of the chemokine and chemokine receptors mentioned above can lead to an increase in growth factors via the MEK/ERK and NF-kappaB pathways in the surrounding tissue, as well as an increase in matrix degradation proteins, such as the metalloproteinases. This facilitates the invasion and establishment of the tumor cells at the new site in the lung.\textsuperscript{167, 168} Ezrin, a protein that links the cell membrane and cytoskeleton together, is thought to help OSA cells attach to the lung microenvironment; it also upregulates pathways associated with proliferation such as Akt.\textsuperscript{136, 137, 169}

Once the OSA cells have attached and established themselves in the lung parenchyma, they must proliferate and initiate angiogenesis in order to thrive. Pathways associated with proliferation in osteosarcoma metastasis include PI3K and ERK1/2.\textsuperscript{168-170} As already mentioned, Wnt is important in osteosarcoma, influencing metastasis in multiple stages. Wnt influences cell cycle regulation by activation of cyclin-D and
contributes to increased proliferation of the OSA cells.\textsuperscript{158, 160, 161} IGF-1 receptor may also play an important role in proliferation of OSA, particularly in young patients.\textsuperscript{134, 171} Src activation can lead to increases in STAT3 and downstream increases in VEGF, FGF and IL-8, which contribute to angiogenesis in the tumor.\textsuperscript{172}

Since metastasis is the major cause of mortality in OSA patients, key molecular pathways that contribute to the pathogenesis of metastasis represent potential therapeutic targets to prevent and treat the development of metastatic disease. They also provide an opportunity for developing laboratory tests to predict or track the development of metastasis in individual patients.

**Mouse Models of Osteosarcoma**

There are a number of different mouse models of OSA reported in the literature with varying utilities. Syngeneic models are those in which the host animal and cell line are genetically similar enough to be immunologically compatible. Two such example of syngeneic mouse models of OSA are the KM7/K7M2 cell lines derived from BALB/c mice\textsuperscript{173} and the Dunn/DLM8 cell lines derived from a C3H mouse.\textsuperscript{174} In both cases the parent cell line (designated first in the pair) was injected into mice and more highly metastatic clones were selected to produce a more reliably metastatic model. The second cell line in the pair represents the derived cell line with higher metastatic potential. Syngeneic models are beneficial in studying the effects that the immune system plays on tumor growth and metastasis. In these models, the host microenvironment, including
immune cells and stromal cells, can interact with the OSA cells in a more biologically relevant manner than in xenogeneic models. The disadvantage of the syngeneic models is that there may be host specific differences in the biology, genetics and pathogenesis of murine OSA as compared to human or canine OSA. For example, spontaneous murine OSA is very rare and as in the models mentioned above, the metastatic potential must be “enhanced” by selection of specific clones in order to make the model more useful.

Genetically engineered mice that lack functional p53 develop osteosarcomas in addition to many other tumor types including lymphoma, fibrosarcoma, rhabdomyosarcoma, neuroendocrine tumors, and hibernomas. In conditional knockouts where p53 loss is restricted to cells of the osteoblast lineage, OSA occurred most commonly in the jaw and skull and had a low rate of metastasis, quite different from the biologic behavior observed in human patients. These genetically engineered models may serve as valuable model to examine the role certain genes play in the development of OSA, but rarely do these models closely mimic the natural disease found in humans and dogs.

Finally, xenogeneic models, such as the model we developed, utilize actual tumor tissue or cell lines from the species of interest and inject or implant these cells into an immunocompromised host, such as nude, SCID or Nod/SCID gamma mice. These models offer the benefit of maintaining the same genetic and cellular characteristics of the parent tumor (for the most part) from which the cells were derived. Depending on the specific model and cell/tissue used, these models may exhibit spontaneous metastasis at a high rate, similar to that seen in patient populations. However, similar to the
syngeneic models, many of the xenogenic models of osteosarcoma utilize cell lines that have undergone transformation or selection to create more a more metastatic phenotype. For example, both the 143B and KRI human osteosarcoma cell lines originated from a poorly metastatic primary osteosarcoma cell line HOS that underwent Ki-ras transformation. The SAOS-2 human osteosarcoma cell line is not transformed, but a more highly metastatic subclone designated SAOS-LM6 is often utilized when studying metastasis. These transformed or selected subclones of cell lines have been artificially manipulated to increase their metastatic potential and may not be as accurate a model for the natural disease. Xenograft models that use an orthotopic location in bone seem to better mimic the biologic behavior of osteosarcoma than those implanted subcutaneously. When interested in metastasis specifically, some models employ intravenous injection of tumor cells, often into the tail vein, however this modeling system bypasses many of the steps of the metastatic cascade. Orthotopic xenogenic models are the only system where we can study the actual tumor cells or tissue from the species of interest in the proper tissue microenvironment. On the other hand, these models are restricted by the lack of an intact host immune response and the species difference between tumor cells and host microenvironment that might limit tumor-host cell interactions.

Conclusions
Despite the differences that exist between OSA in dogs versus humans, there are an overwhelming number of similarities which exemplify the potential utility of dogs as a preclinical model when investigating novel therapeutic protocols. Conversely, advances in human medicine with regards to osteosarcoma will likely “trickle down” and benefit veterinary patients.

Chapter 2 of this dissertation discusses the development of a clinically relevant model of osteosarcoma with spontaneous metastasis. An accurate model of OSA metastasis will be critical as we seek to elucidate the mechanisms of metastasis and develop new therapeutic strategies. The model we have developed has a few distinct advantages over our previously used mouse model of OSA. These include the ability to grow a tumor in its appropriate microenvironment in bone and the development of spontaneous metastasis following removal of the primary tumor, as is the case in the majority of human and canine OSA. Another distinct advantage is the possibility of using primary tumor tissues harvested directly from human or canine patients.

Chapter 3 extends the use of the mouse solid tumor model with patient derived tissue samples, further validating its utility in studying OSA. We also examine gene expression profiles of primary and metastatic osteosarcoma in our model and identify differentially regulated genes that may be important to the metastatic process and that may therefore have utility as diagnostic or therapeutic targets.

Chapter 4 expands the discussion of musculoskeletal cancer away from cancer pathogenesis and into the arena of cancer therapy. Radiation therapy is commonly used to
control tumor growth in the setting of primary and, more commonly, secondary tumors of the musculoskeletal system. In OSA, radiation therapy plays an important role in palliation of unresectable or metastatic bone tumors. Although the benefits of radiation therapy on tumor control and pain relief have been well documented, radiation is known to damage healthy bone, leading to a risk of skeletal complications even after successful cancer therapy. In this Chapter, we demonstrate the utility of the mouse as a model for studying radiation-induced bone disease, and go on to explore possible mechanisms behind this pathology.

Finally, Chapter 5 will bring the different elements of this thesis together, highlighting what has been learned and, in particular, identifying future opportunities for using the laboratory mouse as a test system for improving cancer care in human and veterinary patients.
Chapter 2: A Clinically Relevant Mouse Model of Canine Osteosarcoma with Spontaneous Metastasis

Introduction

Osteosarcoma (OSA) is the most common primary malignancy of bone in humans and the second most frequent cause of cancer-related death in children. In the United States approximately 1000 children are diagnosed with osteosarcoma annually, with the peak incidence occurring in the second decade of life. This period of time corresponds with the rapid skeletal growth of adolescence, and both male gender and increased height are risk factors for the development of OSA in people. OSA is also the most common primary bone tumor in dogs, most often affecting middle-aged to older large breed dogs. OSA in the canine population is 10 times that of humans, with approximately 10,000 new cases per year in the United States. Furthermore, there are many similarities between human and canine OSA including affected sites and gender predilection, association with increased height, and propensity for lung metastasis. As is the case in humans, the etiology is typically unknown; although, a small proportion of cases are seen in sites that have previously undergone radiation therapy and at the site of bone infarction. Most cases of OSA involve the metaphysis of long bones, with the distal femur, proximal tibia and proximal humerus being the most common sites in both species.
At a microscopic level, OSA is characterized by neoplastic cells that form immature bone or osteoid. These cells are thought to originate from mesenchymal stem cells of the osteoblastic lineage.\textsuperscript{4} The vast majority of osteosarcomas are histologically high-grade and have a strong propensity to metastasize. Histomorphologic subtypes include chondroblastic, fibroblastic, osteoblastic, and telangiectatic forms; however, few prognostic differences have been attributed to the different subtypes.\textsuperscript{187}

Despite improvements in the management of the primary bone disease associated with OSA, largely through the development of advanced limb sparing techniques,\textsuperscript{188} many human and veterinary patients will eventually succumb to distant metastasis, most often involving the lungs. Metastasis to the draining lymph nodes or other sites in bone is less common. At the time of diagnosis, less than 20\% of patients have clinically detectable metastasis; however, over 80\% have micrometastasis at the time of diagnosis as shown by distant recurrence of the tumor following resection of the primary mass.\textsuperscript{189}

In order to reduce the morbidity and mortality associated with OSA, it is critical to address the issue of lung metastasis. Metastasis is a complex process with numerous steps involved including migration through the extracellular matrix, intravasation, evasion of the immune system, extravasation at the metastatic site, invasion and proliferation at the distant site, and angiogenesis.\textsuperscript{190} Although a number of canine, human and murine OSA cell lines have been characterized \textit{in vitro} and \textit{in vivo},\textsuperscript{182, 185} a widely accepted orthotopic mouse model of OSA with reliable metastasis has not been established. A relevant model is critical to advancing our understanding of the disease and to improving our ability to evaluate therapeutic agents. In pilot studies using the Abrams canine OSA
cell line, we identified a number of animals that died acutely following intratibial injection of tumor cells. At necropsy, these mice were found to have evidence of tumor micro-emboli within the pulmonary vasculature. In a subsequent series of experiments we were able to significantly reduce the mortality rate by injecting fewer cells, but concern remained that even the lower number of cells that were injected might be sufficient to cause secondary tumors in the absence of overt metastasis from a primary lesion.

If tumor metastasis following intratibial injection is dependent on the development of an established primary OSA in the tibia, early removal of the primary lesion (by amputation) would be expected to significantly decrease the incidence of lung metastasis in mice. One paper in the literature reports reduced metastasis following amputation in an injection model of OSA, but the finding was not statistically significant.191 We therefore undertook an experiment to determine the effects of the timing of amputation on the incidence of secondary OSA tumors in the lung. Since we expected to find that the micro-emboli identified in the pilot animals would go on to form tumors, we hypothesized that the formation of secondary lung OSA in mice injected with Abrams tumor cells would not be sensitive to the timing of amputation. If confirmed, this would significantly limit the clinical relevance of the Abrams cell injection model to our future work on OSA lung metastasis.

Following on from the first experiment, we undertook a second study to characterize an alternate approach to studying the growth and metastasis of canine OSA in mice. We elected to continue to use the Abrams cell line for this work because of its aggressive biologic behavior and clinical relevance in terms of tissue tropism.134, 185 To
avoid potential concerns over embolization of a cell suspension, solid fragments of Abram’s tumor were transplanted orthotopically into the medullary canal of the proximal tibia. The temporal patterns of tumor growth at the primary site were evaluated by radiography and histology, and the incidence and severity of subsequent secondary OSA development in the lungs was quantified as a relative percentage of lung volume affected using histological sections.

**Materials and Methods**

*Experimental Design Overview*

In the first experiment, the emphasis was on determining whether the timing of removal of the primary OSA lesion (by amputation) had an impact on the incidence of lung metastasis following intratibial injection of a cell suspension. The primary outcomes for this experiment were (a) survival of the animal and (b) the incidence/severity of secondary OSA lesions in the lung. In the second experiment, which was designed as a proof of concept study, the emphasis was on determining the time course of primary and secondary tumor development in the solid fragment implantation model. The primary outcomes were radiographic and histologic assessment of tumor growth within the tibia, and stereologic analysis of tumor growth within the lung.

*Cell culture*
The Abrams canine OSA cell line was kindly provided by Dr. Doug Thamm, (Animal Cancer Center, College of Veterinary Medicine & Biomedical Science, Colorado State University). The Abrams cell line was derived from a 2 year male Rottweiler dog with pulmonary metastasis, and has been shown to have robust and rapid growth in vivo in a mouse model.\(^{185}\) Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT) and 1% penicillin/streptomycin (Gibco). Vented tissue culture flasks (Corning Life Sciences, Corning, NY) were maintained at 37\(^{\circ}\) C in a humidified atmosphere of 5% carbon dioxide in air, and cells were split when they reached approximately 80-90% confluency.

**Animals**

All animal study procedures and protocols were conducted with approval of the Institutional Animal Care and Use Committee. Female, 4-5 week old, BALB/c Foxn1
nu/nu mice (Taconic Farms, Germantown, NY) were maintained in barrier housing in plastic cages with corncob bedding and fed an irradiated cereal-based diet *ad libitum*. Mice were housed 4-5 animals per cage and allowed to acclimate for at least one week prior to any procedures being performed. Animals were exposed to a 12/12 hour light/dark cycle for the duration of the study. All surgical procedures were performed under sterile conditions. Normal body temperature was maintained through the use of a recirculating warm water blanket followed by recovery under an infrared heat lamp.

**Subcutaneous tumor growth**
Abrams cells were harvested and resuspended in sterile phosphate-buffered saline (PBS) at a concentration of $10^7$ cells/ml. Trypan blue staining was used to verify a minimum of 90% cell viability before and after the procedure. Mice were anesthetized with inhaled isoflurane (induced at 5%, maintained at 2%) and the skin over the right thigh was disinfected with 95% isopropyl alcohol. The skin was tented and a 1 ml syringe with a 27-gauge needle was used to inject 0.15 ml of the cell suspension subcutaneously over the right thigh. Mice were weighed weekly and tumor size was monitored by use of manual caliper measurements. After 4 weeks the tumors had reached a size of approximately 10 mm in diameter and the mice were euthanized by cervical dislocation under 5% isoflurane anesthesia. The tumors were harvested aseptically immediately post-mortem. Tumor tissue was minced into multiple small fragments and bathed in warm supplemented DMEM (as described above) until implanted, or cryopreserved in DMEM containing 20% FCS and 10% dimethyl sulfoxide (DMSO). Cryopreserved tissues were frozen under controlled conditions using a commercial cryopreservation system (Nalgene Thermo-Scientific, Rochester, NY) at -80 degrees C and then stored in liquid nitrogen. Additional tumor fragments were snap frozen by immediately immersing them in liquid nitrogen and stored at -80 degrees C.

*Intratibial cell suspension injection technique*

Abrams canine OSA cells were harvested and resuspended in PBS. From pilot work performed prior to initiating this experiment, a cell dose of 20,000 cells in a volume of 20 μl had been found to be effective in supporting robust primary tumor development without a high incidence of mortality at the time of injection. Trypan blue staining was
used to verify a minimum of 90% cell viability before and after the procedure. Mice were anesthetized with inhaled isoflurane (induced at 5%, maintained at 2%) and the right hind limb was disinfected with 4% chlorhexidine scrub followed by 95% isopropyl alcohol. A 100uL Hamilton microsyringe with a 27-G needle was used to draw up 20 μl of the cell suspension. The needle was inserted through the proximal anterior tibial cortex using a rotating motion until the cortex was breached as indicated by a sudden decrease in resistance. The cell suspension was slowly injected over 30 seconds and then the needle was removed.

The growth of the tumor in the proximal tibia was monitored by weekly digital radiography (Model LX-60, Faxitron Corporation, Tucson, Arizona) using an exposure time of 5 seconds at 30kV. In addition mice were weighed weekly and examined for signs of overt limb swelling or lameness. In order to determine the effects of early surgical excision of the primary lesion, mice underwent amputation of the affected limb after 1 day, 1 week, 2 weeks, 3 weeks or 4 weeks. The amputated limbs were fixed in 10% neutral buffered formalin, decalcified in 10% EDTA and then submitted for routine histology. Twelve weeks following intratibial injection of tumor cells, or after a 20% loss in body weight (whichever came first), mice were euthanized via carbon dioxide asphyxiation. A complete necropsy was performed by a veterinary pathologist to look for evidence of metastasis. In order to optimize the quality of the histological sections, lungs were inflated with 10% neutral-buffered formalin prior to removal from the body. The inflated lungs were then immersed in a large volume of formalin for at least 48 hours prior to subsequent routine histology processing to confirm the presence of secondary
OSA growth in the lung. Overall mouse survival data were analyzed using the Kaplan–Meier method including log rank (Mantel-Cox) tests (SPSS v 17.0). A significance level of \( p<0.05 \) was used throughout.

_Surgical implantation of tumor fragments_

Freshly harvested or previously frozen fragments of Abrams tumor tissue (harvested as described earlier and either cryopreserved or snap-frozen, depending on the experiment) were placed in a sterile Petri dish and immersed in warm DMEM. Mice were anesthetized with inhaled isoflurane and the right hind limb scrubbed with surgical antiseptic (4% chlorhexidine) followed by 95% isopropyl alcohol. The skin over the craniomedial aspect of the right tibia was incised to expose the medial tibial cortex, and a 25-gauge needle was used to drill a small hole through the cortex and multiple small fragments of solid tumor tissue (totaling approximately 0.5 mm\(^3\) of tissue) were inserted into the medullary cavity of the tibia (Figure 2.1). The skin edges were then apposed with liquid tissue adhesive (3M, St. Paul, MN).

Tibial tumor growth was monitored by weekly digital microradiographs (Model LX-60; Faxitron) using an exposure time of 5 seconds at 30kV. In addition mice were weighed weekly and examined for signs of ill-thrift, lameness or swelling around the surgical site. All mice underwent amputation of the affected limb after 5 weeks. The amputated limbs were fixed in 10% formalin and underwent a micro-computed tomography scan at approximately 30-\(\mu\)m resolution (Skyscan 1172, Aartselaar, Belgium). The legs were then decalcified in 10% EDTA and submitted for routine
histology. Twelve weeks following intratibial injection of tumor cells or after a 20% loss in body weight (whichever came first) mice were euthanized via carbon dioxide asphyxiation. A complete necropsy was performed by a veterinary pathologist to look for metastasis. The lungs were similarly inflated with formalin, immersion fixed in formalin for at least 48 hours and then processed for histologic analysis (see below).

Hind limb amputation

Mice were anesthetized with inhaled isoflurane (induced at 5%, maintained at 2%) and the right hind limb was disinfected with 4% chlorhexidine scrub followed by 95% isopropyl alcohol. A #15 scalpel blade was used to make a circumferential elliptical incision around the mid-thigh and the skin and subcutaneous fat of the medial thigh were reflected to visualize the femoral artery. A small stainless steel ligating clip (Ethicon Endo-surgery, Blue Ash, OH) was used to ligate the femoral artery just distal to the caudal femoral branch. Another clip was placed 5 mm distal to the first. The thigh muscles were circumferentially transected between the two clips and reflected off of the proximal aspect of the femur. Careful dissection with the tip of the scalpel blade along the medial aspect of the femur revealed the coxofemoral joint capsule, which was then cut to free the femoral head. The femur was dislocated from the pelvis and any additional muscle attachments were severed. The amputated limb was placed in formalin. The cut end of the sciatic nerve was anesthetized with topical 2% injectable lidocaine and the muscles of the hip region were sutured over the acetabulum using resorbable 5-0 monofilament suture (Syneture, Mansfield, MA). The skin incision was repaired with two to four intradermal sutures and the suture line reinforced with tissue adhesive. Post-
operatively, mice were treated with buprenorphine (0.25 μg SC twice daily) and meloxicam (2.5 μg SC once daily) for 3 days for analgesia.

**Tissue collection, processing and analysis**

Formalin-inflated lungs were embedded in a 3% agar solution (Fisher Scientific BP1423, Fair Lawn, NJ) that was allowed to set at 20° C overnight. A tissue-sampling matrix (Zivic Instruments, Pittsburgh, PA) was used to generate random uniform sections at 2-mm increments through the entire volume of the lung tissue. Each slice was then processed into paraffin wax and a standardized sectioning protocol was used for each block. Bioquant Image Analysis Software (Nashville, TN) was used to manually quantify total lung area and lung area effaced by tumor. These numbers were used to calculate the percentage of lung (lung area affected by tumor / total lung area *100) that contained OSA tumor tissue in each mouse. Once all the lungs were analyzed, the mean percent of lung affected and the standard deviation was calculated (IBM SPSS v 17.0).

**Results**

*Intratibial cell suspension injection technique*

Twenty-three out of 25 (92%) mice survived the intratibial injection of 20,000 Abrams cells. Radiographic evidence of osteolysis and neoplastic new bone formation, consistent with successful growth of the tumor, was evident in the injected tibia at 3 weeks post-injection (Figure 2.2). Following amputation, OSA was confirmed by
histology in all of the specimens with radiographic lesions. Beginning at 7 weeks post-injection, many mice reached early removal criteria due to weight loss or general ill-thrift and were euthanized. In every case, necropsy examination revealed gross evidence of lung metastasis, with replacement of a significant amount (>25%) of lung tissue by tumor. The mean survival times for mice amputated one day, one week, two weeks, three weeks, and four weeks were 74.6 days, 68.6 days, 75.6 days, 70.3 days and 76.0 days respectively. There was no significant difference in the incidence of lung metastasis or in survival among mice amputated at different time points (p=0.901; Figure 2.3). Other than the lung and pleura, no other metastases were detected grossly or radiographically.

Surgical implantation technique

A preliminary feasibility study was performed using 5 mice implanted with fragments of fresh Abrams tumor derived from a solid tumor grown subcutaneously in a donor mouse. All 5 recipients developed primary OSA lesions in the tibia. Radiographic changes (bone destruction with some new bone formation) progressed rapidly (Figure 2.4) until they required amputation at 5 weeks post-implantation. Upon histologic evaluation, the primary lesions in the tibia were characterized by large areas of bone destruction associated with an expansile intramedullary mass of neoplastic osteoblasts displaying marked anisocytosis, cellular atypia, a high mitotic index and central necrosis (Figure 2.5). All 5 mice survived to 12 weeks post-implantation, at which point 4 of 5 mice (80%) had gross and microscopic evidence of lung metastasis (Figure 2.6). Lung lesions were characterized by multifocal, variably-sized nodules composed of neoplastic osteoblasts, sometimes producing an osteoid-like matrix and displaying marked
anisocytosis, a high mitotic rate and prominent central necrosis, very similar to the primary bone lesions (Figure 2.7 & 2.8). The remaining mouse did not have any evidence of metastasis at the 12-week end point.

In the main experiment, we sought to characterize the development of the primary bone lesion, quantify metastasis and examine the possibility of utilizing cryopreserved tumor tissue. Fifteen mice were implanted with fresh tumor tissue and 2 mice were euthanized each week (up until amputation at 5 weeks) to examine the time course of primary and metastatic tumor development. At 5 weeks post-implantation the remaining 7 mice underwent right hind limb amputation and were monitored for an additional 12 weeks. In parallel with this, another group of 5 mice were implanted with cryopreserved tumor fragments, amputated at 5 weeks post-implantation and monitored for 12 weeks. Of the 12 mice that were amputated at 5 weeks post-implantation, 6 (including 4 out of 5 implanted with cryopreserved tissue) had confirmed evidence of osteosarcoma in the implanted tibia and all 6 of these developed pulmonary metastasis. Finally, a group of 4 mice were implanted with snap-frozen tumor tissue. None of the mice in this group developed any evidence of primary or metastatic tumors after 12 weeks.

Micro-CT imaging of the tibias at different time points showed progressive tumor growth and destruction of pre-existing tibial cortex with formation of new neoplastic and reactive bone. None of the mice euthanized prior to 3 weeks had any evidence of lung metastasis. One out of two mice euthanized at 3 weeks and at 4 weeks had lung metastasis, but a minimal percentage of the lung volume was affected (0.04% and 0.03% respectively). Of the 6 mice with confirmed primary tumors (including 4 implanted with
cryopreserved tissue), all 6 had metastasis to the lung at the time of euthanasia. Lungs from these mice were analyzed and the mean percent of affected lung was 33.4% +/- 24.3% (Figure 2.9).

**Discussion**

Despite its relative rarity in humans, OSA continues to be a devastating diagnosis due to the young age of the patients and the high rate of metastasis. Over 80% of human patients are believed to have microscopic metastasis at the time of diagnosis.\(^{189}\) The same can be said for metastatic disease in canine patients.\(^{192}\) For these reasons, it is critically important to have a clinically relevant mouse model that can be used to investigate the molecular mechanisms of metastasis and to evaluate novel therapeutic agents, with a focus on preventing or reducing metastatic burden. Given the similarities in the biology of the disease in humans and in dogs, we anticipate that knowledge gained from studies on either species will translate into clinical benefits for both.

Prior to starting the experiments reported in this paper, we had definitive evidence of tumor micro-embolization following intratibial injection of Abrams cells. The goal of the first experiment reported here were therefore to determine whether these micro-emboli had clinical consequence. The results indicate that they do. Acute removal of the primary lesion, by amputation, did not result in a reduction in the incidence or severity of lung pathology, or in a change in overall survival. While the data fall short of proving that embolization is the cause of all of the secondary tumors in the lung, they are
sufficient to support the original hypothesis. As such, they cast doubt on the validity of using this injection route for studying lung metastasis from Abrams cells. It should be emphasized that these findings may be specific to this cell line, and not readily generalizable to other OSA lines. For example, one paper describing the cell suspension injection technique using the OSCA-40 canine OSA cell line showed a trend toward a reduction in metastasis following amputation of the primary bone tumor. Another paper, utilizing the KRIIB human OSA cell line, showed that pulmonary metastasis following intratibial tumor cell injection could be prevented by amputation of the injected limb 2 weeks later. Other proponents of a model using a cell suspension injection and amputation of the tumor-bearing limb, have not studied the effects of amputation of the development of lung metastases. Previous studies with intra-cardiac or intravenous injection of other tumor cell lines have shown that even though there is initial widespread systemic distribution of tumor cells, the vast majority of the tumor cells will die, and only a small percentage go on to develop into secondary tumor deposits. It has been reported that Abrams cells are especially aggressive in vitro and in vivo and it is possible that even a small number of embolic cells will be sufficient to establish a secondary tumor within the lung. It is also important to note that although the secondary deposits of Abrams cells in the lung may not faithfully recapitulate the biology of metastasis, they may still offer a useful model for exploring in-vivo responses of pulmonary OSA lesions to chemotherapy.

On the basis of the confirmatory results from the first experiment, we elected to focus on the development of a more clinically relevant model for our subsequent studies.
While the surgical implantation technique proved to be more technically challenging and time consuming than the injection technique, we expected that the use of intact tissue fragments would limit the possibility of tumor embolization following the initial tumor implantation. None of the mice implanted with tumor for less than 3 weeks developed lung metastasis. These results contrast starkly with those from the intratibial injection model, in which there was early development of lung lesions that progressed rapidly, independent of whether the primary lesion was removed. The solid fragment technique proved equally successful with fresh or carefully cryopreserved tissue; as expected, snap frozen tissue did not maintain enough viability to grow once implanted. The growth of the tumor at the primary site in bone, followed by amputation and later, a high rate of lung metastasis, successfully recapitulates the typical clinical disease course in humans and dogs. The reliable occurrence of true spontaneous lung metastasis from a primary bone tumor in this model allows us to investigate individual steps in the metastatic cascade and identify potential therapeutic targets. This model also allows us to evaluate primary canine or human OSA tumor samples by directly implanting them into an orthotopic site in a mouse model while maintaining components of the original tumor microenvironment, without the need for *in-vitro* growth. Multiple studies have shown that protein and gene expression levels can be significantly altered by *in-vitro* versus *in-vivo* growth, not to mention the varied conditions under which cells might be grown *in-vitro*. 197, 198

The tumor fragment model requires only a very small, 0.5 mm$^3$ core of tissue which can be easily obtained at the time of biopsy or resection of the primary bone
lesion. Collection of both canine and human OSA tissue from the primary site in bone with subsequent implantation in mice is currently underway to verify the ability to utilize this model with patient tissues. This ability to grow patient-derived, untreated OSA tissue in its natural microenvironment (in bone) is expected to result in better retention of the molecular and cellular characteristics of the original tumor. Furthermore, as we move towards a more personalized approach to diagnosis and treatment, the model will facilitate comparison of different therapeutic regimens against a patient’s specific tumor and may be predictive of the patient’s response to therapy. From a quantitative perspective, it is clearly extremely hard to ensure consistent numbers of tumor cells in each specimen. With this in mind, we are actively exploring the use of bioluminescent imaging and micro-positron emission tomography (PET) as tools for quantifying and tracking tumor burden with the Abrams cell line and with primary patient specimens respectively.

A systematic and comprehensive comparison of gene expression profiles in primary versus metastatic lesions using a microarray approach is also underway. This will serve to further validate the model and to potentially identify novel markers of metastasis. Although many studies have examined gene expression profiling in canine OSA, none of these studies have looked at paired samples of primary and metastatic canine tumors, likely due in part to the logistical difficulties associated with obtaining tissues at the time of euthanasia/death. With our model it is possible to collect tumor tissue from the primary site in the patient’s bone, and grow the tumor in mice so
that we could also collect cells from lung metastases that have completed all steps of the metastatic cascade in the mouse.

In the specific case of the Abrams OSA line, the surgical model that we have described overcomes potential limitations associated with early micro-embolic spread of tumor to the lung following intratibial injection of a cell suspension. Although we have yet to exploit all the utilities of the surgical implantation model, we believe that this model provides a clinically relevant model of both primary and secondary OSA growth. With this model, we expect to be better equipped to investigate the factors contributing to metastasis and, ultimately, to develop therapeutic strategies that will improve the prognosis for canine and human patients diagnosed with OSA.
Figure 2.1 Tumor implantation technique diagram.
Figure 2.2 Radiograph of an intratibial cell suspension-injected mouse tibia at three weeks post-injection. Note the osteolysis and neoplastic new bone formation.
Figure 2.3 Kaplan-Meier survival curve demonstrating the effects of early removal of the primary tumor (by amputation) following intratibial injection of Abrams OSA cells. There was no difference in survival between groups of mice amputated at 1 day, 1 week, 2 weeks, 3 weeks, or 4 weeks after tumor cell injection (p=0.901).
**Figure 2.4** Sequential radiographs of a mouse surgically implanted with solid osteosarcoma fragments. Radiographs were taken at weekly intervals until amputation at 5 weeks post-op. Note progressive osteolysis and neoplastic new bone formation.
Figure 2.5 Photomicrographs of a tibial osteosarcoma lesion 5 weeks following surgical implantation of solid tumor tissue. A. Note the bone destruction by neoplastic osteoblasts, and central coagulation necrosis (N), 0.5X magnification. B&C. At higher magnification, neoplastic cells display osteoid production (asterisk), marked anisocytosis, cellular atypia, and numerous mitotic figures (arrows), 20X and 40X magnifications respectively. Hematoxylin and eosin.
Figure 2.6 Macroscopic appearance of multiple lung metastases in a mouse 12 weeks after undergoing surgical implantation of solid osteosarcoma tumor fragments.
Figure 2.7 Photomicrographs of the lung metastases in a mouse 12 weeks after undergoing intratibial surgical implantation of solid osteosarcoma tumor fragments. A. Variably-sized nodules composed of neoplastic osteoblasts surrounding a central region of coagulation necrosis (N) with occasional dystrophic mineralization (arrow), 0.5X magnification. B&C. Higher magnification of the coagulation necrosis (N); some neoplastic cells produce eosinophilic, osteoid-like matrix (O), 20X magnification. Hematoxylin and eosin.
Figure 2.8 Photomicrograph of a less affected lung lobe with a single osteosarcoma metastasis nodule (star). Hematoxylin and eosin.
Figure 2.9 Lung metastasis in mice 12 weeks after undergoing surgical implantation of solid osteosarcoma tumor fragments (n=14). Using principles of uniform random sampling and Bioquant Image Analysis software, the percentage of lung volume affected by metastasis was calculated. Mice euthanized at 1 week and 2 weeks post-implantation did not have any metastasis. One mouse at 3 weeks and one mouse at four weeks had 0.04% and 0.03% (respectively) of the lung volume affected by metastasis. Mice that were amputated at 5 weeks post-implantation had a mean percentage of affected lung of 33.4% +/- 24.3% at 12 weeks post-implantation.
Chapter 3: Gene Expression Profiles of Primary and Metastatic Osteosarcoma in a Mouse Model

Introduction

Osteosarcoma (OSA) is a devastating and sometimes fatal disease for the approximately 800 children that are diagnosed annually in the United States. The majority of osteosarcoma patients are between 10 and 15 years of age, which underscores the life altering potential of this disease. Most children with OSA will not have evidence of metastasis at the time of diagnosis; however, over 80% will develop metastatic disease with removal of the primary tumor alone. Metastatic disease most often affects the lung, but can also occur at other sites in bone, lymph node or other organs. In addition, domestic dogs are affected by OSA at an incidence over ten times that seen in humans and suffer the same propensity for lung metastasis. Because dogs and humans with OSA share many of the same clinical signs and biologic behavior, dogs can serve as a useful model for human OSA.
Over the past few decades many improvements to the options available for removal of the primary bone tumor, while retaining use of the limb, have been made. Cosmetic outcomes and functionality of the affected limb improved immensely; however, survival rates have not seen dramatic increases since the 1980’s, when adjuvant chemotherapy became standard protocol. Mortality in OSA in both dogs and people is most often attributed to widespread lung metastasis, usually following excision of the primary bone mass. Even with modern chemotherapy, patients that present with lung metastasis at diagnosis have a poor prognosis. Understanding the pathogenesis of lung metastasis in OSA is critical to improving survival for canine and human patients. In order to better understand genes that may play an important role in the metastatic process, we utilized a model of canine OSA that demonstrates true metastatic disease from a primary bone tumor. We examined differences in gene expression patterns between the primary tumor and the metastatic tumors using a microarray approach. We also utilized the model to implant canine patient tissues directly into the mouse model to examine metastatic potential of OSA seen in pet dogs. We hypothesized that genes critical to the metastatic cascade will be differentially regulated in the primary versus metastatic tumors in this model. We also hypothesized that the model would be a valuable tool for evaluating canine OSA tissue specimens.

Materials and Methods
Cell culture. The Abrams canine OSA cell line was kindly provided by Dr. Doug
Thamm, (College of Veterinary Medicine & Biomedical Science, Colorado State
University). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)
(Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS)
(HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Gibco). Vented tissue
culture flasks (Corning Life Sciences, Corning, NY, USA) were maintained at 37°C in a
humidified atmosphere of 5% carbon dioxide in air, and cells were split when they
reached approximately 80-90% confluency.

Animals. All animal study procedures and protocols were conducted with approval of the
Institutional Animal Care and Use Committee (#2008A0062-R1). Female, 4-5 week old,
BALB/c Foxn1 nu/nu mice (Taconic Farms, Germantown, NY, USA) were housed 4-5
animals per cage. All surgical procedures were performed under sterile conditions.

Subcutaneous tumor growth. Abrams cells were harvested and resuspended in sterile
phosphate-buffered saline (PBS) at a density of 10^7 cells/ml. Trypan blue staining was
used to verify a minimum of 90% cell viability before and after the procedure. Mice were
anesthetized and 0.15 ml of the cell suspension was injected subcutaneously over the
right thigh. Mice were weighed weekly and tumor size was monitored. After four weeks
the mice were euthanized and the tumors were harvested aseptically. Tumor tissue was
minced into multiple small fragments and bathed in warm supplemented DMEM (as
described above) until implanted, or cryopreserved in DMEM containing 20% FCS and
10% dimethyl sulfoxide (DMSO). Cryopreserved tissues were frozen under controlled
conditions using a commercial cryopreservation system (Nalgene Thermo-Scientific,
Rochester, NY, USA) at -80° C and then stored in liquid nitrogen. Additional tumor fragments were snap frozen by immediately immersing them in liquid nitrogen and stored at -80° C.

Canine patient samples. All sample collection was performed in collaboration with The Ohio State University Veterinary Medical Center Biospecimen Repository. OSA tumor samples from primary bone lesions were collected from 5 canine patients that had undergone a limb amputation for a clinically diagnosed OSA. Immediately following removal, the amputated limb was dissected and a sterile core biopsy instrument and forceps were used to collect fragments of tumor tissue from the center of the bone lesion. The fragments were immediately placed in warm supplemented DMEM. Within 30 minutes of collection, the samples were minced into smaller fragments, approximately 10mm³, and cryopreserved in DMEM as described above.

Surgical implantation of tumor fragments. Cryopreserved fragments of Abrams tumor tissue were implanted into the proximal tibia of 3 BALB/c Foxn1 nu/nu mice. Mice were anesthetized with inhaled isoflurane and the right hind limb scrubbed with surgical antiseptic (4% chlorhexidine) followed by 95% isopropyl alcohol. The skin over the craniomedial aspect of the right tibia was incised to expose the medial tibial cortex, and a 25-gauge needle was used to drill a small hole through the cortex and multiple small fragments of solid tumor tissue (totaling approximately 0.5 mm³ of tissue) were inserted into the medullary cavity of the tibia (Figure 3.1). The skin edges were then apposed with liquid tissue adhesive (3M, St. Paul, MN, USA). Tibial tumor growth was monitored by weekly digital microradiographs (Model LX-60; Faxitron) using an exposure time of 5 s
at 30kV. In addition mice were weighed weekly and examined for signs of ill-thrift, lameness or swelling around the surgical site. All mice with Abrams tumors underwent amputation of the affected limb after five weeks. The amputated limbs were dissected and the soft tissues comprising the tumor were embedded in OCT (Anapath Cryostat, Newcomer Supply, Middleton, WI, USA) and snap frozen in liquid nitrogen. Twelve weeks following intratibial injection of tumor cells or after a 20% loss in body weight (whichever came first), mice were euthanized. A complete necropsy was performed by a veterinary pathologist to look for metastases. The lungs were embedded in OCT and snap frozen in liquid nitrogen.

Ten mice for each canine patient were implanted with canine OSA patient tissues fragments and were monitored as described above. The mice were amputated once the bone lesion approached pathologic fracture radiographically or the mouse exhibited signs of lameness. The leg tumor were dissected away from the bone and divided into three fragments: cryopreservation in supplemented DMEM with DMSO, formalin fixation for histopathology, and snap frozen in liquid nitrogen. Following amputation, mice continued to be monitored for weight loss and were euthanized when they reached 20% loss of body weight or at 24 weeks post-implantation. The lungs were snap frozen in liquid nitrogen.

*Hind limb amputation.* Mice were anesthetized and the right hind limb was disinfected. A circumferential elliptical incision around the mid-thigh was made and the skin and were reflected. A stainless steel ligating clip (Ethicon Endo-surgery, Blue Ash, OH, USA) was used to ligate the femoral artery. The thigh muscles were transected between the two clips and reflected off of the proximal aspect of the femur. The coxofemoral joint capsule
was transected to free the femoral head. The cut end of the sciatic nerve was anesthetized with lidocaine and the muscles of the hip region were sutured over the acetabulum. The skin incision was closed with intradermal sutures and reinforced with tissue adhesive. Post-operatively, mice were treated with buprenorphine and meloxicam for three days.

**Laser capture microdissection and RNA isolation.** Laser capture microdissection was performed at the Laser Capture Molecular Core of The Ohio State University Wexner Medical Center. OCT embedded leg tumor and lungs were cryosectioned at 8 μm, sections were placed on polyethylene naphthalate (PEN) membrane covered slides and then stained with hematoxylin QS from Vector Laboratories, Inc. (Burlingame, CA, USA). The PALM Microbeam Module Rel. 4.2 from Carl Zeiss Microscopy, LLC (Thornwood, NY, USA) was used to microdissect and selectively capture tumor tissue from the leg and lung slides. A minimum of 4x10⁶ square microns of tissue were collected from each sample. RNA was isolated from each sample using Arcturus PicoPure RNA Isolation Kit (Life Technologies; Grand Island, NY, USA). The Agilent Bioanalyzer 2100 (Santa Clara, CA, USA) was used to measure the RNA quantity and integrity levels.

**Microarray.** 25 ng of total RNA were amplified using the NuGEN Ovation Pico WTA System V2 (Cat# 3302-12 San Carlos, CA, USA) and labeled with NuGEN’s Encore Biotin module (Cat# 4200-12). The labeled samples were hybridized onto Affymetrix Canine Genome 2.0 arrays (Santa Clara, CA, USA). Samples were hybridized using Herring sperm DNA, Acetylated BSA (50mg/mL) and BioArray Eukaryotic Hybridization Controls (Enzo Life Sciences, Farmingdale, NY, USA) for 16 hours at 45°
at 60rpm. The arrays were washed and stained using the Fluidics 450 EukGE_WS2v5 script as per manufacturer’s instructions.

Statistical analysis. Signal intensities were analyzed by Affymetrix Expression Console software. Background correction and quantile normalization was performed to adjust technical bias, and gene expression levels were summarized by RMA method. A filtering method based on percentage of arrays above noise cutoff was applied to filter out low expression genes. Linear modeling was employed to detect differentially expressed genes. In order to improve the estimates of variability and statistical tests for differential expression, a variance smoothing method with fully moderated t-statistic was employed for this study. The significance level was adjusted by controlling the mean number of false positives. Statistical software SAS 9.2 and R was used for analysis. A p-value of less than 0.01 was considered significant.

Pathway analysis. To identify the statistically significant biological functions and signaling pathways affected by the genes differentially expressed in our comparisons, we performed Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Redwood, CA). The IPA program is based on the Ingenuity Knowledge Base, which consists of millions of relationships between genes, protein complexes and networks extracted from peer reviewed scientific literature. IPA is the largest curated database and analysis system for understanding the signaling and metabolic pathways, molecular networks, and biological processes that are most significantly changed in a dataset of interest. (www.ingenuity.com)
Results

Microarray. Six gene expression profiles were generated from the RNA from laser capture microdissection tissue isolated from the three primary (leg) OSA lesions and three secondary (lung) OSA deposits in mice implanted with the Abrams canine OSA. Expression analysis yielded a total of eighty genes that were differentially expressed between the primary tumors in the leg and the corresponding metastatic tumors from the same mice (Figure 3.2) ranging from a 2.2- to a 15.8-fold difference. The genes with the greatest fold change that were downregulated in the metastatic tumors compared with the primary tumors included NDUFAF4, DHDH, DDHD1, and ANK3. The genes with the greatest fold change that were upregulated included UBE2J1, GTPBP4, and WIF1. Hierarchical clustering of these expression data resulted in separation of the primary leg tumors from the metastatic lung tumors. (Figure 3.3) Additional RNA from the same specimens was saved for confirmation of selected genes by rtPCR. In particular, eight genes were identified as being differentially expressed in the current data set as well as a previously performed microarray (unpublished data). (Figure 3.4) The previous unpublished microarrays utilized pooled samples from the primary leg tumor and metastatic lung tumor. Due to the pooled sampling, statistical analysis was limited and the study was redone using separate gene chips as described above.

Pathway Analysis. Using IPA analysis, we explored the most significant biological network functions and pathways in the differentially expressed genes identified above.
The top network identified was “Digestive system development, embryonic development, organ development” and included 15 of the 80 genes of interest. (Figure 3.5) Two additional networks identified by IPA were “Developmental disorder, hereditary disorder, metabolic disease” and “Hereditary disorder, metabolic disease, cardiovascular disease”. These networks included 14 and 13 of the 80 genes identified, respectively. (Figure 3.6 and 3.7) The top molecular and cellular bio-functions identified were Cell-to-cell signaling and interaction (7 genes), Protein trafficking (2 genes), Carbohydrate metabolism (2 genes), and Cell morphology (7 genes). The top upstream regulators for the differentially expressed genes were APOE, Marlin1, SPTBN4 and POLA2.

*Canine patient samples.* Five canine patients that underwent limb amputation for the management of OSA at The Ohio State Veterinary Medical Center had tissues collected from the tumor site in the amputated limb and cryopreserved. The dogs ranged in age from three to eight years old and were a mix of spayed females and castrated males. Breeds included Greyhound, Golden Retriever, Rottweiler and a Mastiff, which all represent commonly affected large breed dogs. (Figure 3.8) All tumors were located in the radius or tibia. All five dogs received a histopathologic diagnosis based on the amputated surgical specimen of a well- or moderately- differentiated osteoblastic OSA. (Figure 3.8) None of the dogs had radiographic evidence of pulmonary metastasis at the time of surgery to resect the primary leg tumor. Regional lymph nodes from canine samples 1, 2, 3, and 4 were evaluated microscopically and none were found to contain neoplastic cells. The regional lymph node from the fifth dog was not evaluated. Each specimen was implanted into ten nude mice each. All the different tissue specimens
successfully grew in at least one of the ten implanted mice and developed radiographic evidence of OS. (Figure 3.9) The timing of bone OSA development varied and amputation occurred between 7 weeks and 18 weeks post-implantation. Furthermore, histopathology on portions of the amputated leg tumor confirmed the presence of OSA. Mice implanted with three of the five canine specimens also developed metastatic lung OSA seen at necropsy. (Figure 3.10) These mice were euthanized when they developed clinical signs of 20% weight loss or lethargy, or at 24 weeks post-implantation, whichever occurred first. Portions of the primary OSA in the leg and the metastatic OSA in the lung were snap frozen for RNA isolation and future gene expression investigations.

**Discussion**

Metastasis is a multi-step process that likely involves important changes in gene expression in the cells with metastatic capability. We have demonstrated a difference in gene expression profiles between the same tumor at the primary site in bone and that of the metastatic tumor growing in the lung. By using laser capture microdissection, we selectively chose tumor cells and therefore avoided issues with cross-hybridization of mouse host tissues to the canine gene chip.

Within the 80 genes that were differentially expressed, eight were found to be in common with preliminary gene expression microarrays done utilizing pooled samples in an injection model of Abrams canine OSA. These eight genes are listed in Figure 3.4. The first gene identified, MGP (matrix Gla protein), is an important inhibitor of vascular
calcification and regulator of BMP-2 (bone morphogenetic protein-2). Its effects on BMP-2 are not clear, but it seems to be an inhibitor overall, which might promote a less differentiated neoplastic osteoblast. In addition, overexpression of MGP has been linked to multiple types of cancer including gastric cancer, glioblastoma multiforme and breast cancer.

The second gene identified, ANK3 (ankyrin 3) was also one of the genes with highest fold-change difference. ANK3 is involved with neuronal signaling and is implicated in multiple psychiatric disorders. Elevated ANK3 has also been reported in neural and neuroendocrine tumors.

SNX24 (sorting nexin 24) is related to a large group of intracytoplasmic proteins that can bind membranes and may be involved in protein trafficking; however, little is known about this specific protein.

RCOR3 (REST corepressor 3) is part of a family that regulates neuronal gene expression and stem cells, but its function in cancer is unknown. Although little is known about the LOC4766 gene, a similar gene that codes for ribosomal protein L18a has been overexpressed in murine squamous cell carcinoma.

GRIA2 (glutamate receptor, ionotrophic, AMPA2) is expressed mostly in the brain and serves as an excitatory neurotransmitter receptor. There is some evidence that GRIA2 may be a positive prognostic marker in ovarian adenocarcinoma.

The gene WIF1 (WNT inhibitory factor 1) is a well known tumor suppressor gene that binds and prevents Wnt signaling and is altered in many types of cancer.
including osteosarcoma. The Wnt-beta-catenin pathway transmits signals from extracellular ligands that bind cell surface receptors to the cell nucleus, where gene transcription is altered. Without the binding of an extracellular ligand, the signaling molecule, beta-catenin, is proteolytically degraded and signaling ceases. In normal osteoblasts, Wnt signaling contributes to maturation of osteoblast precursors and osteoblast mediated bone formation. Beta-catenin also plays an important role in osteoclast maturation and function via modulation of RANKL and OPG. In osteosarcoma on the other hand, increased expression of beta-catenin in the cell cytoplasm has been associated with metastasis and has been used a biological marker to predict the risk for lung metastasis. Beta-catenin is also implicated in OSA cell motility and invasion. Wnt pathway blockade results in reduced tumorigenicity and decreased metastasis. There is also evidence that the Wnt-beta-catenin pathway is involved in resistance to chemotherapy, including the three most commonly used agents, doxorubicin, cisplatin and methotrexate. Loss of Wif-1 specifically has been shown to increase the susceptibility of mice to radiation induce OSA. Despite this evidence, there is still some controversy over the exact role of Wnt, with some studies showing that stimulation of the Wnt pathway results in decreased OSA cell proliferation. Clearly the Wnt signaling pathway is important in the pathogenesis of OSA and warrants further investigation.

The last of the eight genes is MITD1 (microtubule interacting and transport, domain containing 1), which codes for a protein that interacts with endosomal sorting
complexes required for transport (ESCRT) proteins. The ESCRTs are important in cell division, in particular the final separation of daughter cells.\textsuperscript{233}

The significance of the eight genes that have been identified through this microarray experiment remains unknown but is an area of active investigation at the current time. We are in the process of designing primers for real-time PCR analysis, first to confirm the validity of the microarray data from the Abrams cell line, but also to probe RNA collected from the five primary canine OSA tumors that have been implanted in mice. While we are confident that the microarray results are valid, the use of species-specific primers will help to address lingering potential concerns regarding the possibility of unanticipated cross-reactivity between sequences in the canine gene chip and DNA from normal mouse cells that were unavoidably captured during the laser capture process.

The eight differentially expressed genes from the current and previous microarrays differ from those reported in studies comparing cell lines with different metastatic potentials.\textsuperscript{173, 234-236} The differences identified between cell lines with varying metastatic potential may well differ from those identified between primary and metastatic tumors from the same cell line or patient. Comparing different cell lines could lead to identification of inherent differences in the primary tumor that increase the likelihood of metastasis, whereas comparing the primary and metastatic tissue from a single tumor would identify genes that change as a cause or effect of metastasis.

IPA pathway analysis identified the top networks that the 80 differentially expressed genes we identified are associated with. Although some of the network
functions do not have a clear link to cancer or metastasis, embryonic development and hereditary disorders are often attributed to genes that may play a role in cancer development.\textsuperscript{237-239} Interestingly, the ubiquitin C gene (UBC) was at the center of all three networks. The ubiquitin system is most well-known for its intracellular proteolysis function, but it is also involved in signal transduction, transcription, endocytosis, protein trafficking, DNA repair, cell cycle regulation and cell survival.\textsuperscript{240} Many of these functions are key elements in the development and metastasis of OSA. In fact, a polyubiquitin chain complex has recently been shown to activate NF-kappaB and contribute to lung metastasis in OSA.\textsuperscript{241}

IPA also identified the top molecular and cellular bio-functions associated with differentially expressed genes in our model of OSA metastasis. Seven molecules were identified as being involved in cell-to-cell signaling and interaction. Cell signaling is a critical component that determines the propensity for metastasis to particular organs.\textsuperscript{242} None of the other molecular and cellular bio-functions identified involved more than 7 of the differentially expressed genes, and the bio-functions are common to cell function in general.

Identification of the top upstream regulators of the 80 differentially expressed genes revealed 4 molecules. The first upstream regulator, the APOE gene, encodes the protein apolipoprotein E and is a critical component of the catabolism of triglyceride-rich lipoproteins.\textsuperscript{243} Interestingly, polymorphisms in the APOE gene have recently been associated with aggressive biologic behavior in prostate cancer.\textsuperscript{244} The second upstream regulator, marlin-1, is an RNA binding protein that also binds the GABA receptor in the
brain. It has only recently been described, and its relationship to cancer or metastasis is uncertain. The SPTBN4 gene was identified as the next upstream regulator of interest and encodes the protein spectrin, beta, non-erythrocytic 4. Spectrin links the cell membrane to the cytoskeleton via actin cross-linking, therefore affecting organelle organization within the cytoplasm, protein sorting, cell migration and cell shape and stability. Alterations in spectrin family molecules have been associated with worse prognosis in pancreatic cancer and transgenic mice with reduced beta-spectrin develop hepatocellular carcinomas thought to be modulated via activation of cyclin D1. Lastly, the POLA2 gene encodes the enzyme DNA polymerase alpha subunit B, which is a critical player in DNA replication. It is the only enzyme capable of starting DNA chains de novo. Although the specific role that alterations to the POLA2 pathway may play in carcinogenesis is not known, clearly DNA replication is essential to the development of cancer. Despite the lack of a known metastasis-associated upstream regulator or network in those identified by IPA, the detection of these regulators and pathways provides relevant information to guide future investigations.

The cell line used in our investigation, the Abrams canine OSA cell line, is reported to be a highly tumorigenic and metastatic cell line and this has matched our experience with the cells. In spite of this, many of the genes that have been previously identified as altered in OSA, including VEGF, ezrin and survivin, did not show differential expression in our experiments. A comparison between the Abrams cells and normal canine osteoblasts may shed further light on which genes are likely to be involved in tumorigenesis. Furthermore, studies comparing primary and metastatic OSA
tissue in humans have identified differentially expressed genes that differ from those we
identified. Even within studies comparing similar groups, there is a lack of complete
agreement on a “metastasis signature”. Osteosarcomas display complex genomic
changes that create difficulty identifying important molecular changes and the exact
types that lead to metastasis may differ with each individual tumor. Although all
these methods may be useful in identifying genes that play a role in metastasis, they all
require further investigation to define what role the changes in gene expression play in
OSA.

The information gleaned from the Abrams solid tumor implantation model of
OSA will only be useful if similar changes are present in other patient tumors. This work
will be facilitated by the use of the model to grow patient tissues. We recently reported
on the intratibial surgical implantation of solid Abrams OSA tumor fragments as a
valuable mouse model of OSA with spontaneous metastasis. Due to the nature of this
model and the small amounts of tissue required, patient-derived primary OSA tissues can
successfully be implanted, leading to primary tumor development in the bone followed
by spontaneous metastasis. This ability to maintain tumor within a bone
microenvironment may help retain tumor characteristics that would otherwise be lost or
altered with in-vitro or even subcutaneous growth. This is an important characteristic
of a model that will be used to evaluate therapeutic efficacy or to examine molecular
alterations that contribute to metastasis.

In the current set of experiment, canine patient OSA tissue samples were
successfully used in the model with all samples growing a primary tumor in at least one
mouse, and three of the canine tumors leading to metastasis in the mouse model. Interestingly, the OSA specimen that led to the greatest number of mice with pulmonary metastasis was from a relatively young (3 year old) greyhound. Younger dogs diagnosed with OSA are reported to have shorter survival times with a more aggressive biologic behavior. Furthermore, in a study examining greyhound dogs, OSA was one of the leading causes of death. The fact that the mouse model might have predicted a more aggressive phenotype may lend credence to its use as a predictive prognostic tool. The first canine OSA specimen implanted (sample #1) was one of the specimens that led to metastasis in the mouse model. Correspondingly, this dog was reported to have a very short clinical course and was euthanized due to metastatic disease within 6 months of the diagnosis. At publication, clinical follow-up was not available for the remaining dogs, but future investigations may provide support for the relevancy of the model. Although all the canine specimens in this group had a similar histologic diagnosis of osteoblastic OSA, future studies might examine differences between different OSA subtypes in the mouse model and whether these differences reflect known differences in biologic behavior in the dog.

Conversely, differences in the “take rate” of the implanted tumor tissue in the mouse tibias may reflect the heterogeneous composition of the implanted fragment. With some of the collected specimens, small amounts of tissue remaining after implantation of 10 mice was processed and evaluated histologically; however, whether the tissue examined was representative was questionable, and the apparent viability of these small fragments did not seem to correlate with the propensity to grow in the mice. In most
cases, the tissue was composed of necrotic debris and fibrous connective tissue. Only 3 of the specimens had enough remaining tissue to examine in this manner, so any conclusions drawn are only speculative. In future studies, larger quantities of tissue will be collected to allow for more thorough evaluation, although it will always be limited by the fact that you can’t directly examine the tissue fragments implanted. A second limitation of the model is the difficulty in quantifying the exact amount of tissue implanted. The volume is so small and the exact amount to be implanted is often determined intra-operatively (as much as will “fit” in the tibia), so that a reliable method of quantification using patient derived tissues seems unlikely. On the other hand, when using the Abrams cell line to grow solid tumor fragments, quantification may be achieved using bioluminescence technology. This possibility is currently being explored by the laboratory.

Although canine patient tissues were used for ease of collection, rather than human samples, the model is also relevant for human biopsy samples that could be collected at the time of diagnostic biopsy of the suspected OSA. The model also allows for collection of primary and metastatic tumor tissue form the same patient specimen, which is often difficult or impossible to obtain from the patients themselves. Despite the limitations of the intra-tibial solid tumor implantation model, the ability to take a specimen directly from a patient and grow it in a biologically relevant micro-environment, with a disease course that matches that seen in nature, makes this model an invaluable tool in the ongoing study of OSA.
Figure 3.1 Tumor implantation technique diagram.
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**Figure 3.2** Differentially expressed genes between primary OSA and metastatic OSA
**Figure 3.2 continued**

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Figure 3.3 Heat map and hierarchical clustering of the 80 differentially expressed genes.
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**Figure 3.4** Differentially expressed genes between primary and metastatic tumor that overlap with previous dataset.
Figure 3.5  Major signaling network “Digestive system development, embryonic development, organ development” associated with the genes differentially expressed (shaded green and red) in metastatic OSA tumor compared with primary OSA tumor tissue identified by IPA. The network consists of 35 nodes and includes 15 of the 80 differentially expressed genes. Green shading indicates genes that are upregulated in the metastatic tumor and red shading indicates genes that are downregulated in the metastatic tumor.
Figure 3.6  Major signaling network “Developmental disorder, hereditary disorder, metabolic disease” associated with the genes differentially expressed (shaded green and red) in metastatic OSA tumor compared with primary OSA tumor tissue identified by IPA. The network consists of 35 nodes and includes 14 of the 80 differentially expressed genes. Green shading indicates genes that are upregulated in the metastatic tumor and red shading indicates genes that are downregulated in the metastatic tumor.
Figure 3.7 Major signaling network “Hereditary disorder, metabolic disease, cardiovascular disease” associated with the genes differentially expressed (shaded green and red) in metastatic OSA tumor compared with primary OSA tumor tissue identified by IPA. The network consists of 35 nodes and includes 13 of the 80 differentially expressed genes. Green shading indicates genes that are upregulated in the metastatic tumor and red shading indicates genes that are downregulated in the metastatic tumor.
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**Figure 3.8** Canine patient data. All dogs were diagnosed with osteoblastic OSA with no evidence of distant metastasis.
Figure 3.9  Mouse implanted with canine specimen #1 at 12 weeks post-implantation.
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**Figure 3.10** The ratio of mice with implanted canine OSA tissue that developed primary and metastatic OSA.
Chapter 4: The Pathophysiology of Radiation Injury to Bone: Role of the Osteoclast

Introduction

Ionizing external beam radiation therapy has been shown to be an effective modality for the treatment and/or palliation of a variety of cancers; however, the detrimental long-term effects of radiation therapy are also well-known. One of the important sequelae to radiation is the effect on the growing and mature skeleton. Soft tissue sarcomas, genitourinary cancers, lung and gastrointestinal cancers are all potential candidates for radiation therapy that could include bone in the field of radiation.\textsuperscript{107, 109, 255-258} In addition, bone metastases from a variety of primary cancers including breast and prostate cancer can be treated with radiation therapy.\textsuperscript{259} In pediatric patients, one of the most significant effects of bone irradiation is closure of the growth plate.\textsuperscript{260} In adult patients, some of effects of radiotherapy on bone include osteonecrosis and a large increase in the risk of bone fracture due to a decrease in bone strength.\textsuperscript{109, 261, 262} Fractures that occur following radiation often heal poorly, leading to non-unions in many cases, making treatment more difficult.\textsuperscript{263-265} Another long-term side effect of radiation therapy is the development of neoplasms, including osteosarcoma at the site of irradiated bone.\textsuperscript{266}
There is often a significant lag time between the radiation therapy ending and the appearance of adverse effects on bone, with a reported median time interval of 3.5 years in humans.\textsuperscript{263} As people survive cancer and live longer past their treatment periods, it becomes increasingly important to consider these potential adverse effects.\textsuperscript{110} Although it has been shown that radiation decreases the strength of bone and increases its brittleness, the exact mechanism of radiation-induced bone damage is not fully understood.\textsuperscript{267} Despite the beneficial effects of radiation therapy, the long-term adverse effects must be considered when recommending a patient undergo radiation therapy. Understanding the pathogenesis behind the increased risk of fracture is critical for prevention. Possible pharmacotherapeutic agents or changes to administration protocols could then be developed to abrogate the negative effects of radiation on bone while retaining the positive cancer killing ability.

Previous studies conducted using a mouse model of focal 20 Gy radiation-induced bone damage have shown that there is a reduction in the trabecular bone fraction associated with a decrease in bone strength and a decrease in bone mineral density.\textsuperscript{268} Additionally the bone marrow is lost and replaced by adipocytes. This mouse model that replicates the effects seen in the adult skeleton following irradiation provides an opportunity to investigate the pathogenesis of trabecular bone loss and weakness that occurs following external beam radiotherapy to the skeleton. We utilized this mouse model and hypothesized that an increase in osteoclastic bone resorption was responsible for the loss of bone and resultant weakness. We also conducted \textit{in-vitro} experiments on
osteoclasts and osteoclast precursors to determine their relative sensitivity to external beam radiation.

**Material and Methods**

*Generation of Murine Osteoclast Precursors from Bone Marrow*

Adult Swiss Webster mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. Within 15 minutes of euthanasia the hind limbs were washed with 95% ethanol and removed from the carcass. The skin was removed from the limb, the tibia and femur separated, and the muscles removed. Next, the individual bones were placed in a sterile petri dish containing warm alpha-MEM supplemented with 10% (v/v) fetal calf serum and 1% (v/v) antibiotics and L-glutamine (“complete medium”). A sterile syringe and 25-gauge needle was used to flush the marrow cavity and the resulting bone marrow cell suspension was collected into a sterile 50-ml centrifuge tube. Pooled marrow from all of the bones was then centrifuged at 400g for 5 minutes to pellet the cells. The supernatant was removed and re-spun in another vial. The cell pellets were combined, washed with 20 ml of sterile medium and then resuspended at a final concentration of 5 million cells per ml in complete alpha-MEM containing 100ng/ml recombinant human (rh) M-CSF. The cells were placed in a culture flask in an incubator at 37°C and % CO2.

After 72 hours in culture, non-adherent cells were aspirated and centrifuged for 10 minutes at 400 x g. The pellet was resuspended in alpha-MEM containing 25 ng/ml rhM-
CSF and 30 ng/ml recombinant murine RANKL. The cells were seeded into 24-well plates at a density of 1 million cells per well and the medium changed every 2-3 days.

*Generation of Mature Mouse Osteoclasts from Bone Marrow*

The procedure for isolating bone marrow-derived mononuclear cells has been described above. In order to generate mature osteoclasts, the culture process was modified slightly based on recommendations from Dr. Beth Lee in the Department of Physiology & Cell Biology, The Ohio State University (personal communication). In brief, mononuclear cells flushed from bone marrow were seeded initially into 100-mm Petri plates and cultured in complete medium supplemented with 100 ng/ml M-CSF. After 24 hours, the medium was removed and replaced with fresh complete medium supplemented with 25 mg/ml M-CSF and 30 ng/ml RANKL. Cells were allowed to differentiate for 5-6 days until cell fusion was evidenced by the formation of multinucleate cells. At this point the cell monolayer was scraped and the cells from one 100-mm Petri transferred into 6 wells of a 24-well plate.

*Irradiation Protocol*

Osteoclast precursors or mature osteoclasts were irradiated 24 hours after being seeded. External beam irradiation was performed using a Siemens clinical linear accelerator in The Ohio State University Veterinary Medical Center using a 20 cm x 20 cm field size. Culture plates were covered with a 1 cm thick Superflab tissue-equivalent bolus (Mick Radio-nuclear Instruments, Inc., Mount Vernon, NY, USA) with a specific gravity of 1.02. The cultures were subjected to 6 megavolt photon irradiation to a total
dose of 0 Gy (non-irradiated controls), 1 Gy (98 monitor units, 0.45 minutes) or 5 Gy (490 monitor units, 2.16 minutes). Immediately following irradiation, cells were returned to the incubator and maintained at 37ºC in a humidified atmosphere containing 5% carbon dioxide.

**Quantification of Cell Survival**

The effects of acute irradiation on the viability of immature osteoclast precursors were determined indirectly, through the use of the MTT assay. The yellow, water-soluble MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to an insoluble purple formazan crystal within the mitochondria of living cells. Thus, the assay is technically a measure of the metabolic activity of the cell and therefore is an indirect measure of viability. The amount of formazan product within a cell monolayer is proportional to the total number of metabolically active (viable) cells. Treatment of the monolayer with dimethyl sulfoxide (DMSO) results in lysis of the cell membranes and dissolution of the formazan product. The concentration of formazan is then quantified by measuring optical absorbance at a wavelength of 562 nm.

**Quantification of Tartrate-Resistant Acid Phosphatase (TRAP) Activity**

The effects of acute irradiation on the generation of TRAP-positive mono- and multinucleate cells was determined by staining irradiated and control wells with TRAP reagent (Acid Phosphatase, Leukocyte (TRAP) Kit; Catalog #386A, Sigma Aldrich. St. Louis, MO). Total numbers of mono- and multinucleate TRAP-positive cells were enumerated within 10 randomly selected fields of view (100x total magnification, each
field of view representing 3.14 mm$^2$). One investigator performed all of the analyses in order to reduce inter-observer variability in cell counting.

*Effects of External Beam Irradiation on Mouse Bone In Vivo*

All animal study procedures and protocols were conducted with approval of the Institutional Animal Care and Use Committee (#2008A0062-R1). Female, ~12-week-old BALB/c mice (Taconic Farms, Germantown, NY, USA) were housed 4-5 animals per cage. Mice were anesthetized using injectable xylazine/ketamine combination and placed on a Plexiglas jig with the left hind leg extended and taped into place. Lead shielding was molded over the mouse’s body and over the foot, leaving only the left femur and tibia exposed. The mouse’s leg was irradiated with a dose of either 5 Gy or 20 Gy using a commercial cabinet-style irradiator (Model RS 2000; Radsource Technologies Inc., Suwanee, GA).

At 2, 6, and 12 weeks post-irradiation groups of 6 mice were euthanized and the hind limbs were dissected. Non-irradiated mice served as controls. The limbs were then radiographed, disarticulated and placed in formalin (tibias) or frozen at -20°C (femurs).

After a minimum of 48 hours of formalin fixation, the tibias were decalcified in 10% EDTA for 7-10 days and then underwent routine paraffin embedding. Sections were stained with hematoxylin and eosin for descriptive review, as well as with TRAP reagent to allow for morphometric evaluation of osteoclast number using a dedicated semi-automated image analysis package (Osteo II; Bioquant Imaging Corporation, Nashville, TN). A region in the proximal tibial metaphysis, excluding the primary spongiosa, was
outlined and the numbers of osteoclasts and the percentage of trabecular bone surface area that was positive with TRAP staining were quantified. The area and length of trabecular surface measured varied depending on the section and the amount of trabecular bone present, therefore all measurement and comparisons were relative to the trabecular bone surface measured.

The femurs were wrapped in saline-soaked gauze, frozen at -20°C and sent to Dr. Kenneth Mann at SUNY Upstate Medical University in Syracuse, NY for mechanical testing. The strength of the distal femur was determined by axial compression testing, following an established procedure. Axial compression tests of the distal femur were performed to failure in displacement control (0.5 mm/min) at room temperature on all specimens using a mechanical test frame (Q-Test, MTS Corporation, Eden Prairie, MN). This test configuration was chosen as it loads all elements of the distal femur (epiphyseal, metaphyseal, and diaphyseal bone) with the same magnitude of load. Applied load (Newtons) and displacement (mm) were recorded continuously and used to calculate initial stiffness (N/mm), energy to peak load (Nmm) and energy to failure (Nmm).

Statistical Analysis

For the in-vitro experiments, comparisons between treatment groups (radiation dose) were made using one-way analysis of variance (ANOVA; IBM SPSS v 19.0). For the morphometric analysis of irradiated tibiae, comparisons across time and dose were performed using two-way ANOVA (IBM SPSS v 19.0). Mechanical test data were
analyzed using analysis of covariance, with time post-irradiation as the covariate. A significance level of p<0.05 was considered statistically significant.

Results

Irradiation of primary osteoclast precursors resulted in a dose-dependent reduction in viable cell number (Figure 4.1). Morphologically, cultures that had been irradiated were clearly distinguishable from non-irradiated control wells because of the presence of floating, highly refractive cells within the medium. Similar changes were seen at both 4 days after irradiation (Figure 4.1A) and at 12 days post-radiation (Figure 4.1B), although the magnitude of the irradiation effect was more at 12 days post-irradiation.

When more mature osteoclast precursors (as evidenced by multinucleation) were irradiated, there was a similar dose-dependent effect on overall cell numbers (Figure 4.2). By breaking the total number of cells down into mono- versus multinucleate cells, we were able to document that the total number of multinucleated cells was relatively preserved and was not statistically different from controls. In contrast, the number of TRAP-positive mononuclear cell decreased dramatically over the course of the experiment. This decrease was statistically significant (p<0.05) at the 2 Gy and 5 Gy irradiation levels.

Mice femurs that underwent 20-Gy irradiation showed changes in mechanical properties that followed a similar pattern to that reported previously, with a decrease in
energy to peak load and energy to failure, accompanied by an increase in stiffness (Figures 4.3 to 4.6). Although the temporal pattern of degradation of mechanical properties was not found to be statistically significant, irradiated bones had inferior mechanical properties to normal bones at all time points tested (data not shown).

Histologically, there was marked loss of bone marrow elements in all the irradiated mice and a time-dependent replacement by adipocytes that occurred at the 12-week post-irradiation time point (Figure 4.7). Using TRAP staining to identify areas of osteoclast activity on the surface of bone (Figure 4.8), there was increase in osteoclast number per trabecular bone surface area and an increase in TRAP-positive surface 2 weeks following irradiation of the tibia (Figure 4.9). This change was statistically significant in the 20 Gy mice, but not in the 5 Gy group, despite a trend toward a similar finding. At 6 weeks post-irradiation, osteoclast numbers and TRAP surface area was similar to that seen in the controls. At 12 weeks post-irradiation, mice in the 20 Gy group had a statistically significant decrease in osteoclast number and TRAP-positive surface area compared with controls. The 5 Gy group had similar trends, but was not statistically different. (Figure 4.9)

Discussion

The deleterious effects of radiation on bone are an important consideration when treating patients with radiation for malignancies. Young people may be seriously affected because of the many years they will live in which side effects may materialize. On the
other hand, older people affected by malignancies, particularly women, may already be experiencing decreased bone mineral content and weakening due to the hormonal effects of menopause.\textsuperscript{109} All of these patient subsets would greatly benefit from a greater understanding of the effects of radiation on bone. The pathogenesis of radiation-induced bone damage is undoubtedly complex and the effects are likely mediated by multiple factors and cell types.\textsuperscript{269, 270}

Previous work in our laboratory has shown that mature osteoblasts are relatively resistant to doses of radiation up to 5-10 Gy, and that there is little to no inhibition of mineralization by osteoblasts. (Allen, et al. unpublished data) Our current study supports the hypothesis that osteoclasts play an important role in radiation-induced bone damage. Despite the radiosensitivity of immature osteoclast precursors, more mature multinucleated osteoclasts were relatively resistant to the effects of radiation. This, along with our data showing an increase in osteoclastic activity on the trabecular bone surface following irradiation, support the role of osteoclasts in the process of bone loss and changes in mechanical properties. However, conflicting studies regarding the pathogenesis of radiation-induced bone damage exists. For example, in contrast to our current and previous work, a rat model of focal bone irradiation attributed bone loss to a decrease in osteoblast number and mineralization activity, with few changes to osteoclasts.\textsuperscript{271} Another group reported decreased matrix production by radiation damaged osteoblasts.\textsuperscript{272} Furthermore, another study using whole body irradiation of C3H/HeN mice as a model did not demonstrate changes in osteoclast activity; however this study used relatively low doses of radiation (2 Gy) and did not demonstrate changes in the
mechanical properties of bone despite a loss of trabecular bone volume.\textsuperscript{273} There is likely a minimum threshold of radiation damage that must occur before changes in osteoclast populations or mechanical properties can be detected.\textsuperscript{274} More recently, alterations to the bone material matrix has been implicated as a factor that contributes to loss of bone volume and strength.\textsuperscript{275} A reduction in vascular supply to the bone following radiation therapy has been known for some time and has been described as “obliterative endarteritis”.\textsuperscript{276} More recent investigations support the theory that vascular changes are significant contributors to radiation-induced bone damage.\textsuperscript{277}

Based on our results, osteoclasts likely contribute to the physical and mechanical changes that occur in bone following radiation therapy and may serve as a useful therapeutic target. As we hypothesized, mature osteoclasts were more radioresistant that osteoclast precursors, and similar numbers of TRAP-positive multinucleate cells were seen in irradiated and non-irradiated (control) cultures. At first glance these findings seem to contradict those from the histomorphometry, which showed an early increase in osteoclasts followed by a later decrease. However, we hypothesize that the initial tissue insult resulting from radiation therapy may cause an influx of osteoclast precursors from other areas of the body. It is widely recognized that tissue trauma induces preferential homing of bone marrow-derived stromal cells through up-regulation of chemokine pathways such as the SDF-1/ CXCR-4 axis,\textsuperscript{278} and we anticipate that a similar mechanism occurs following radiation injury. Although marrow hematopoietic tissue is obliterated by the radiation, stromal cells remain and may be sources of pro-migratory and pro-osteoclastogenic factors such as RANKL and M-CSF to support osteoclast
maturation and activation. Furthermore, early increases in osteoclast numbers in C57/BL6 mice following whole body radiation have been described by other authors.\textsuperscript{279} Given the finite lifespan of osteoclasts, the osteoclast numbers will subsequently decrease as the acute stimulus for recruiting remote marrow precursors decays. Ultimately, the chronic reduction in locally available osteoclast precursors is likely the cause of the decrease in osteoclast numbers seen 12 weeks after radiation therapy.

Although the differential sensitivity of osteoclastic elements is likely to explain at least part of the change in bone mass seen after radiation, much remains to be learned. From a practical perspective, treatment of patients with drugs that inhibit osteoclastic activity (e.g. bisphosphonates or perhaps Denosumab) represents a logical approach to controlling the decrease in bone mass following radiation. Subsequent to our performing these experiments, Dr. Kenneth Mann’s laboratory at SUNY Upstate Medical University produced preclinical evidence that bisphosphonate treatment is effective in reducing bone loss after radiation.\textsuperscript{280} This data is in agreement with another study that showed risedronate to be an effective therapy for radiation-induced osteoporosis.\textsuperscript{281} However, rather intriguingly, bisphosphonates were not effective in restoring the mechanical properties of irradiated bone.\textsuperscript{280} Studies in rats have shown PTH to be a potential therapeutic agent that may prevent bone loss and it is one of the few agents that can not only reduce bone loss, but also increase formation.\textsuperscript{271} Other agents reported to be beneficial in the prevention of radiation-induced bone damage include N-acetylcysteine\textsuperscript{282} and GS-nitroxide.\textsuperscript{283} Additional work is now needed to more completely
explain the interactions between the cellular, vascular and matrix components of bone in the face of radiation therapy.

One major limitation with the current study relates to the use of a single bolus of radiation, rather than a more clinically applicable fractionated radiation protocol. Fractionated radiation protocols allow for a degree of tissue recovery between radiation doses that would not be seen with a single bolus and lead to a reduction in some of the radiation-induced side effects. Additionally, there is a need to repeat the in-vitro studies in order to confirm our findings and reduce the variance in the data set. Furthermore, it would be helpful to expand the cell culture studies to include functional evaluation of osteoclastic activity. This would most easily be accomplished by measuring pit formation on dentine slices cultured for varying periods post-radiation of the osteoclasts.

Despite the limitations of the current study, we have produced significant evidence for the important role osteoclasts play in the physical and mechanical changes that occur following irradiation. Highlighted by the conflicting data in the literature regarding the pathogenesis of radiation-induced bone damage, there is a significant need for further investigation into the negative effects on bone following therapeutic radiation.
**Figure 4.1** Effects of external beam radiation on the viability of osteoclast precursors at 4 days (A) and 12 days (B) post-irradiation. There was a reduction in cell viability in both the 1 Gy and 5 Gy irradiated cells at 4 days and 12 days post-irradiation compared with non-irradiated controls. Lower case letters a and b denote statistically significant
(p<0.05) differences between the 0 Gy and the 1 Gy or 5 Gy treatment groups respectively.
**Figure 4.2** Effects of external beam radiation on tartrate-resistant acid phosphatase activity in committed osteoclast precursors. Bars represent relative numbers of TRAP-positive mononuclear cells (blue) and multinuclear cells (red) in treated wells, expressed as a percentage of numbers in control wells. Data represent mean and standard deviation from N=3 wells per treatment. Asterisks denote a statistically significant (p<0.05) difference in the mononuclear cells irradiated with 2 Gy and 5 Gy as compared with the untreated 0Gy control.
Figure 4.3 Changes in energy to peak load (Nmm) over time following irradiation of mouse femurs to a total dose of 20 Gy. There is a trend toward a decrease in energy to peak over time, following 20 Gy irradiation. (not statistically significant)
**Figure 4.4** Changes in energy to failure (Nmm) over time following irradiation of mouse femurs to a total dose of 20 Gy. There is a trend toward a decrease in energy to fail over time, following 20 Gy irradiation. (not statistically significant)
Figure 4.5 Changes in stiffness over time following irradiation of mouse femurs to a total dose of 20 Gy. There is a trend toward an increase in stiffness over time, following 20 Gy irradiation. (not statistically significant)
Figure 4.6  Histopathology of the proximal tibia of a control mouse tibia (A) and of 20 Gy irradiated mouse tibias at 2 weeks (B) and 12 weeks (C) post-irradiation. There is loss of hematopoietic elements and eventual replacement by adipocytes at 12 weeks post-irradiation. There is also an apparent decrease in trabecular bone as well as disruption of the growth plate at 12 weeks post-irradiation. Hematoxylin and eosin. 100x magnification.
Figure 4.7  Histopathology of the proximal tibia of a control mouse tibia (A) and of 20 Gy irradiated mouse tibias at 2 weeks (B) and 12 weeks (C) post-irradiation. There is an initial increase in TRAP positive trabecular surface at 2 weeks post-irradiation, followed by a decrease in trabecular bone and TRAP staining at 12 weeks. Tartrate-resistant acid phosphatase. 100x magnification.
Figure 4.8 TRAP positive osteoclast surface area as a percentage of trabecular bone area in the 20 Gy irradiated mouse tibias over time. There is a statistically significant (p<0.01) increase (a) in TRAP positive surface area at 2 weeks post-irradiation compared with non-irradiated controls and a decrease (b) in TRAP positive surface area at 12 weeks post-irradiation when compared with non-irradiated control mice.
Figure 4.9 Number of osteoclasts per surface area in tibiae irradiated at 0, 5 and 20 Gy over time. Trends are similar to data reported in Figure 4.8 with osteoclast number initially increase in the 20 Gy irradiated tibias at 2 weeks compared with controls and later decreasing at 12 weeks post-irradiation. The difference in osteoclast number at 2 weeks between 20 Gy irradiated tibias and non-irradiated controls is statistically significant (p<0.01), denoted by an asterisk (*).
Chapter 5: Future directions

Osteosarcoma

Osteosarcoma (OSA) affects hundreds of people and thousands of dogs in the United States each year. Despite improvements in the treatments available for the primary bone tumor, lung metastasis remains a grave and all too common occurrence. In order to make significant strides in improving outcomes for people and dogs with osteosarcoma we must develop therapies that address OSA lung metastasis. To facilitate the development of novel therapeutic strategies, it is imperative to have a variety of model systems to investigate the pathogenesis of metastasis and to evaluate these therapies in a pre-clinical setting. No one laboratory model can address all aspects of a disease.

Although a number of OSA models exist, we were unable to find one to address our questions regarding which genes were important for metastasis of OSA. We initially attempted to utilize a cell suspension injection model of osteosarcoma to answer questions about gene expression, but experienced problems with seeding the lung with tumor emboli from the onset. As a result of this, we sought to develop a model where we could continue utilizing the Abrams canine osteosarcoma cell line, but with only
spontaneous metastasis from a primary bone tumor. We successfully developed and characterized this orthotopic surgical implantation model and went on to utilize the model to examine gene expression profiles in the metastatic tumor compared with the primary tumors. Eighty genes were identified as being differentially expressed and future work will center on selecting a subset of genes to analyze in additional canine OSA tissue samples that have been grown in the model we developed. Some of the genes identified, such as WIF-1 are already known to be involved in OSA and metastasis. Others have not yet been reported to play a role in metastasis or even cancer. Sorting through the information generated from the expression arrays will be an important component of future work in the laboratory. Our first step will be to use canine (i.e. species-specific) primers to confirm that the changes in gene expression that were identified on microarray truly represent changes in gene expression within the tumor itself, and not the surrounding mouse stroma. Once this has been completed, we would move on to quantifying the expression of these 8 candidate genes (and other genes that have previously been implicated in canine OSA, such as ezrin, survivin and Wnt) in primary and secondary tumors from the canine clinical cases that have been evaluated to date. Assuming that we are able to identify one or more genes that exhibit(s) a consistent pattern of differential regulation, the clinical significance of these changes will be definitively explored through a series of up/down-regulation experiments involving selective targeting of the gene of interest. A similar approach has been used in other tumors, including breast cancer, to try to identify a series of pro-metastatic genes that could be used as a “metastasis signature” for the purposes of both diagnosis and therapy.
One of the benefits of using a solid tissue implantation model was the possibility of using patient-derived tissues and implanting them directing into the mouse model, thus generating primary and metastatic tissues that would be difficult or impossible to collect from the patient themselves. We explored this possibility by collecting tumor tissue from dogs undergoing amputations for OSA at the Ohio State Veterinary Medical Center. We then implanted this tissue into the model and monitored mice for the development of primary and metastatic OSA. We were successful in achieving primary tumor growth with all specimens and many of those went on to develop lung metastasis. As the dogs continue with their post-operative therapeutic plan, it will be possible in the future to compare their outcomes with outcomes in the mice implanted with the corresponding tissue to validate the utility of the model as a prognostic predictor. Although we had initially planned to collect human OSA tissue samples at the time of biopsy, the low caseload during our collection period precluded our plan. We were able to collect and implant one human specimen, which unfortunately did not grow. One possibility is that the fragments collected contained necrotic tumor or non-neoplastic surrounding stroma.

This highlights one of the drawbacks to this model; it is impossible to evaluate exactly what the fragments are composed of or accurately quantify the amount of tumor implanted. Given the clinical interest in using advanced imaging modalities such as computed tomography (CT) and positron emission tomography (PET) in identifying and quantifying tumor metastasis, we made a number of attempts to quantify lung metastasis using uCT and combined uCT/PET. Although our data showed that micro-CT/PET was highly sensitive, it was not very specific (67%), and this significantly limits its utility as a
diagnostic test for this disease in mice. Of even greater concern, PET signal from the lung field did not correlate with histologic tumor burden analyzed by stereological methods. We have subsequently been able to transduce Abrams cells with firefly luciferase and we will be using these luciferase-labeled cells in future studies; preliminary data indicate that the Abrams-Luc cells output photos in a linear fashion from ~100 cells to over 50,000 cells, so we expect to be able to use luciferase imaging to track primary tumor growth, the effects of adjuvant chemotherapy, and the development of metastasis.

In addition to enhancing our ability to track tumor metastasis, the ability to quantify metastasis in-vivo would also help overcome one of the other major limitations of any xenograft tumor study, namely the variation in primary and metastatic tumor burden between animals. The utility of the model to evaluate therapeutic agents would be greatly enhanced if metastasis could be quantified in-vivo. With the Abrams model, we can use luciferase imaging to track tumor burden. For other tumor samples, including those derived from canine and human tumors, an alternate approach is needed and we are currently exploring the possibility of using PET tracers to allow for detection of Cerenkov radiation.287

In conclusion, the development, characterization and gene expression profiling of this OSA model helps further our understanding of the biologic behavior and factors involved in metastasis of OSA and gives us a valuable tool to continue our studies on both pathophysiology and therapeutics.
**Radiation therapy and bone**

Radiation is a beneficial therapy for many types of cancer, but its effects on bone can often be detrimental and lead to increased morbidity.\(^{267}\) In particular we were focused on the changes that occur in bone with regards to loss of strength and increases in brittleness that lead to a greatly increased incidence of fractures following radiation therapy. We demonstrated that osteoclasts likely play an important role in this process by increases their number and activity at the site of bone irradiation. Further investigations into the specific signaling molecules that contribute to the pathogenesis are necessary to fully understand what occurs and why osteoclasts are increased. In addition, agents that decrease osteoclast activity such as the bisphosphonates will be examined as a possible strategy to prevent pathologic fractures following radiation. Preliminary evidence reported from Dr. Allen’s previous institution suggest that targeted therapy with bisphosphonates can be partially effective in preventing radiation-induced bone disease, but much remains to be explored. In particular, no one has demonstrated in a small animal model that clinically relevant (fractionated) radiation therapy protocols result in long-term bone damage that serves to delay or even prevent normal healing after pathologic fracture. This is another area of active interest in our lab, along with tissue engineering/regenerative medicine approaches to enhancing and repairing bone healing after fracture.

The role of vascular injury secondary to radiation therapy has not been completely defined, although endothelial cell death is recognized as a potential long-term complication of radiation. Although outside the scope of my thesis work, we have been
actively studying this question through the use of fluorescent microspheres to quantify bone blood flow in normal and irradiated bone.\textsuperscript{288}

In conclusion, this work has increased our knowledge about the role of osteoclasts in radiation-mediated bone damage, but much remains to be learned. It is likely that the chronic damage done to bone represents a complicated mixture of damage to cellular, vascular and matrix (organic/inorganic) components of the bone milieu. Effective therapies, ideally to prevent the initial damage, or at least to treat the established damage, can only be developed after we have a more complete picture of the relative contributions of the different mechanisms of injury.

**Final Thoughts**

Preclinical animal models provide an unparalleled opportunity to explore clinically relevant questions. As a scientist and a veterinarian, I remain committed to pursuing the high quality science in an ethical manner. Much of my thesis work has focused on the development and validation of a mouse model that recapitulates the biology of OSA. I was able to refine the model through the introduction of objective measures such as lung histomorphometry and we looked at the potential for using noninvasive imaging to track tumor metastasis. Five years later, I feel that the solid tumor model is ready to be deployed as both a screening model for new therapeutic agents and as a test system for comparing the biology of primary tumors harvested from patients. We initially conceived of using the mouse model as a rapid screening system for evaluating
response to front-line chemotherapy in OSA, but it is more likely that we will instead use
the mouse as a test model for exploring the relationship between tumor genotype,
response to therapy and clinical outcomes. Much remains to be done with this disease,
but it has been interesting and rewarding to be able to make this contribution.
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