Cancer is essentially a genetic disease of uncontrolled cell survival and proliferation. Medical therapy has traditionally involved chemotherapy and radiation which inhibit the replication of actively dividing cells in a fairly non-selective manner. Research into the molecular pathogenesis of cancer has enabled the recent development of therapy targeted to receptors or signaling pathways crucial to the development and progression of specific cancers and revealed molecular markers that can be used to predict prognosis and treatment response. The work presented in this thesis examines the expression of two potential molecular markers of malignancy, the R1alpha regulatory subunit of cyclic AMP-dependent protein kinase A (PRKAR1A) and insulin-like growth factor 2 (IGF2) retrospectively in a population of canine osteosarcoma patients. Furthermore, it describes the derivation and preliminary characterization of canine osteosarcoma primary cell cultures and the use of these cells to assess the effects of PRKAR1A expression on sensitivity to chemotherapeutic drug treatment \textit{in vitro}.

Post-chemotherapy overall survival was significantly longer in canine osteosarcoma patients with tumours expressing low levels of PRKAR1A, and PRKAR1A expression did not
correlate with mitotic index. IGF2 expression was generally high in the small numbers of cases examined, did not differ between axial and appendicular sites and did not correlate with either mitotic index or survival. PRKAR1A expression varied between cell cultures, but did not appear to be related to expression levels of phosphorylated cAMP response element-binding (phospho-CREB), a downstream target of cyclic AMP (cAMP)-dependent protein kinase A (PKA). In vitro chemosensitivity did not correlate with PRKAR1A expression, but faster growing cells with shorter doubling times and higher rates of BrdU incorporation tended to be more chemosensitive.

In summary, this work identifies an association between low tumour PRKAR1A expression and prolonged post-chemotherapy survival in dogs with osteosarcoma and provides preliminary evidence suggesting that this survival advantage may not be associated with downstream phosphorylation of CREB or sensitivity of the tumour cells to chemotherapeutic agents.
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There are many people to thank for their help in my training, without whom this thesis would not have made it to print. I very much appreciate the patience of my supervisor, Geoff Wood, who helped me to think like a scientist instead of a clinician. His eternally optimistic attitude has been ever encouraging on those days when nothing seemed to be going right. Many thanks also to my other advisory committee members, Drs. Tony Hayes, Rob Foster and Sarah Boston for their constructive criticism and support.

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Returning to school as a mature student and mother after a hiatus in the “real” world has been challenging to say the least. I could not have completed this program without the support of my husband, Rob. He has served as an advisor on scientific matters, but more importantly as an understanding and loving friend. My young son, Ethan, has also been supportive in his own way, continually reminding me to slow down and take time to think and learn.
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<td>3-(4,5 Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
<td>MTT</td>
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<td>analysis of variance</td>
<td>ANOVA</td>
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<tr>
<td>array comparative genomic hybridization</td>
<td>aCGH</td>
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<tr>
<td>arginosuccinate synthetase</td>
<td>ASS</td>
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<td>ATP-binding cassette G2</td>
<td>ABCG2</td>
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<tr>
<td>bromodeoxyuridine</td>
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<tr>
<td>cyclic AMP-dependent protein kinase A</td>
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<tr>
<td>C-X-C chemokine receptor type 4</td>
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<td>disease free interval</td>
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<tr>
<td>diaminobenzadine</td>
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<td>Dulbecco’s modified Eagle’s media</td>
<td>DMEM</td>
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<tr>
<td>fetal bovine serum</td>
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<td>hypoxia inducible factor 1</td>
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<td>IC_{50}</td>
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<td>proliferating cell nuclear antigen</td>
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protein kinase B
protein phosphatase 2
R1alpha regulatory subunit of cyclic AMP-dependent protein kinase A
Ras homolog gene family, member C
receptor activator of nuclear factor kappa-B ligand
regulatory T-cell
retinoblastoma protein
runt-related transcription factor 2
single nucleotide polymorphism
solute carrier family 1, member 3
tumour sclerosis protein 2
tumour suppressor candidate 3
vascular endothelial growth factor
zinc finger CCCTC-binding factor
Statement of Work Done

Chapter 2: All work in this chapter was done by myself.

Chapter 3: Carmen Chui helped with cell culture derivation and clonal isolation. Doubling time and growth in soft agar were both done by Carmen Chui as was the in vitro chemosensitivity testing. I did all of the immunocytochemistry, including the BrdU labeling, and the western blots.
Introduction

With the gradual advancement and sophistication of companion animal medicine, the early diagnosis and treatment of cancer in pets becomes more commonplace. Osteosarcoma is diagnosed in more than 8,000 dogs in the United States alone each year. The prognosis for these dogs is poor even with aggressive surgical resection and chemotherapy. Median survival is less than 6 months with surgical treatment alone and less than a year with standard of care treatment which includes surgery and chemotherapy (1) (2). A better understanding of the molecular pathogenesis of canine osteosarcoma is necessary to discover potential clinically useful prognostic markers and therapeutic targets to aid in patient management.

Cancer in all species is essentially a genetic disease. Recent sequencing of the canine genome has enabled more advanced investigation of the genetic basis of canine cancers including osteosarcoma (3). Results of canine osteosarcoma research may also be applicable to human osteosarcoma patients making it an attractive subject for collaborative and comparative studies (4).

The work described in this thesis examines the expression of two proteins, the R1alpha regulatory subunit of cyclic AMP-dependent protein kinase A (PRKAR1A) and insulin-like growth factor 2 (IGF2), in canine osteosarcoma tissue and primary cell cultures and investigates the effects of PRKAR1A expression on post-chemotherapy patient survival and chemotherapeutic treatment response. The findings prompt further study of PRKAR1A as a potential prognostic marker and therapeutic target in canine osteosarcoma.
Summary of Following Chapters

The first chapter consists of a literature review discussing osteosarcoma in dogs and humans from both a clinical and molecular standpoint with emphasis on the involvement of PRKAR1A in osteosarcoma tumorigenesis. The histologic appearance of osteosarcoma in relation to subtyping and grading schemes is described. The general mechanisms of action of chemotherapeutic drugs commonly used in osteosarcoma treatment are also presented along with an overview of in vitro chemoresistance in osteosarcoma.

Chapter 2 describes a retrospective investigation of PRKAR1A and IGF2 expression in canine osteosarcoma using immunohistochemistry. Correlations between PRKAR1A expression, tumour proliferation and post-chemotherapy survival as well as their potential clinical significance are discussed.

The derivation and preliminary characterization of canine osteosarcoma and osteoblast primary cell cultures is detailed in chapter 3, including doubling times, BrdU labeling, growth in soft agar and immunocytochemistry. The role of PRKAR1A expression in canine osteosarcoma chemotherapeutic treatment response is examined by exploiting the innate variability in PRKAR1A expression in these cell cultures.
Chapter 1: Literature Review

Clinical Aspects of Osteosarcoma

Canine Osteosarcoma

Osteosarcoma is the most common primary bone tumour in dogs and generally carries a poor prognosis. Metastasis occurs early in the course of disease and is mainly hematogenous with lung being the most frequent metastatic site (5). Pulmonary metastases are detectable at the time of diagnosis in approximately ten percent of cases and progressive pulmonary involvement typically culminates in respiratory difficulty and death or euthanasia. Most afflicted dogs are middle aged or older, however, the range of ages at diagnosis is broad and dogs less than two years of age can be affected (6). Large and giant breed dogs are at increased risk of developing osteosarcoma when compared with small breed dogs (7) and males tend to be over-represented, possibly due to their increased size and body weight (6). Rottweilers, Great Danes, Boxers and Greyhounds have also been reported to be at increased risk in comparison with mixed breed dogs (8) (9) (6). Osteosarcoma most frequently affects the metaphyses or epiphyses of the long bones and the proximal humerus, distal radius, distal femur and proximal tibia are the prevailing sites with the forelimb more commonly affected than hindlimb (6). This forelimb predilection may relate to weight burden which is skewed in favor of the forelimbs in dogs.

Approximately one quarter of osteosarcoma cases affect the flat bones of the axial skeleton, and medium and large sized dogs are most commonly affected (10). However, within the canine population, the ratio of appendicular to axial skeletal osteosarcoma decreases with a
decrease in body size such that a greater proportion of osteosarcomas in small breed dogs affect the axial skeleton than in large breed dogs. Eighty percent of all bone tumors in large breed dogs are osteosarcomas and 83% of these tumors are appendicular. In contrast, amongst small breed dogs, osteosarcomas represent only 43% of all bone tumors and affect the appendicular and axial skeleton with equal frequency (11). Cause of death or euthanasia in dogs with osteosarcoma affecting the axial skeleton more frequently results from clinical problems associated with the primary tumor as opposed to metastatic disease (10). This difference may arise due to the relative difficulty in attaining complete surgical excision in axial locations resulting in subsequent tumor recurrence. Rates of pulmonary metastasis appear to vary with axial tumor location. Detectable metastases are more commonly present at the time of diagnosis with rib and cranium primary tumor sites than with maxillary and mandibular primaries (10). Variations in metastatic rate with tumor distribution may reflect variation in tumor biology, however, differences in tumor size and progression at the time of diagnosis as well as choice of pulmonary imaging modality may be confounding factors.

The standard of care for canine osteosarcoma is amputation or surgical resection combined with chemotherapy. Commonly used chemotherapeutic agents include doxorubicin, cisplatin and carboplatin. Dogs treated with amputation plus adjuvant chemotherapy live longer than those treated with amputation alone (12) (13) (2). Alternating post-operative chemotherapy with carboplatin and doxorubicin has not been found to significantly improve disease free interval or survival over single agent chemotherapeutic protocols (14). Radiation treatment can
be beneficial in cases for which surgical resection, including amputation, is not feasible. Radiation treatment protocols vary depending on the goal of treatment, either palliation or cure. Stereotactic radiosurgery or intra-operative extracorporeal irradiation allow for focused delivery of single high dose treatment to the tumour site with minimal damage to surrounding normal tissues. These protocols are commonly employed when cure is the intent of therapy and they appear to result in better control of local tumour growth than multiple doses of fractioned external beam tumour irradiation which are commonly used in palliative care to reduce bone pain and improve quality of life (15). Radiation therapy is especially beneficial for dogs with axial osteosarcoma because surgery alone is often inadequate for local tumour control at axial sites. Aggressive definitive radiation treatment in dogs with axial osteosarcoma has been shown to result in significantly longer median survival than palliative treatment (16). Treatment is rarely pursued in the small proportion of dogs with grossly detectable metastases at the time of clinical presentation due to the grave prognosis. In a retrospective study of dogs with metastatic osteosarcoma whose owners did elect to treat, patients with metastases to bone lived longer than those with pulmonary metastases and combination treatment with palliative radiation and chemotherapy yielded the longest survival times, however, median survival times were very low at only 76 days (13).

Extraskeletal osteosarcoma is a relatively rare mesenchymal tumor of older dogs. Only thirteen percent of canine osteosarcoma cases in one retrospective study involved soft tissues.
The majority of these tumors (64%) were located in the mammary glands. Similar to skeletal osteosarcoma, the disease course for extraskeletal osteosarcomas tends to be very short with a median survival time of only two months. Cause of death is most commonly attributable to pulmonary metastasis for mammary tumours and post-operative local recurrence for other soft tissue sites. Prognosis varies with extraskeletal location; dogs with intra-abdominal tumours have a median survival time of less than two months whereas dogs with cutaneous or subcutaneous tumours have a longer median survival time of 10-16 months. This difference may relate to relative tumour size at diagnosis; intra-abdominal tumours are more likely to go undetected until late in the course of disease progression and are more difficult to surgically excise (17).

Recent work suggests a possible role for the immune system in the regulation and modulation of osteosarcoma progression in dogs. In a prospective clinical trial, the ratio of CD8+ T cells to regulatory T cells in the peripheral circulation was found to be significantly lower in osteosarcoma patients than control dogs and among these patients, a lower ratio was associated with a significantly decreased survival time (18). A second retrospective study found a correlation between higher numbers of circulating monocytes and lymphocytes before treatment and decreased disease free interval (19). The data from these two studies suggests a possible role for monocytes and lymphocytes, specifically regulatory T-cells, in suppressing anti-tumour immunity in canine osteosarcoma. Measurement of blood monocyte and lymphocyte
counts as well as calculation of blood CD8/Treg ratios may provide prognostic information in canine osteosarcoma patients.

There is a general lack of useful and valid prognostic markers to aid in the identification of the small proportion of canine osteosarcoma patients with a longer than average survival time. Several possible molecular prognostic markers were recently identified using gene expression profiling to compare short (< 6 months) and long (> 6 months) surviving dogs with osteosarcoma. These genetic markers were overexpressed in short survivors and may be important in cell proliferation, drug resistance or metastasis (20). Further mechanistic studies are required to investigate the potential roles of these genes in the clinical progression of canine osteosarcoma.

**Human Osteosarcoma**

Recent human medical advances and discoveries in molecular pathology have provided information on human osteosarcoma that may be applicable to the disease in dogs and, in turn, in an age of comparative medicine and pathology, the dog may serve as an important model for human osteosarcoma (1). Osteosarcoma in dogs and humans tends to be very similar from a clinical standpoint; the main differences being higher prevalence and faster progression to metastasis in dogs (21). Similar to canine osteosarcoma, human osteosarcoma affects the appendicular skeleton more commonly than the axial skeleton, with only 11% of cases in a recent retrospective study involving the flat bones (22). Appendicular osteosarcoma in humans tends to be a disease of adolescents and young adults and males appear to be at increased risk.
In contrast, axial osteosarcoma tends to be a disease of middle-age with no apparent sex predilection (22). In the study by Lee et al, metastases at the time of diagnosis tended to be more frequent among cases of flat bone osteosarcoma than those affecting the extremities and a smaller proportion of flat bone tumors were found to respond well to chemotherapy histologically. Five year overall and event-free survival was also found to be shorter for patients with axial tumors than those with appendicular tumors.

Interestingly, considering the increased prevalence of osteosarcoma in large and giant breeds of dog, a human study has shown that patients with osteosarcoma are significantly taller than the general population and that osteosarcoma patients with femoral primaries, the femur being the largest and fastest growing bone in the human body, are significantly taller than those with tumours at other primary sites (24). This data suggests that similar to the disease in dogs, there may be a role for tall stature and rapid growth in the development of osteosarcoma in humans.

Like canine osteosarcoma, surgery combined with chemotherapy is the standard of care for human osteosarcoma patients. Both pre- and post-operative chemotherapy is recommended and typical regimens include both an anthracycline agent such as doxorubicin and a platinum agent such as cisplatin (25). Findings of a systematic review of clinical studies show higher than 20 % clinical response to doxorubicin, cisplatin, methotrexate and ifosfamide when used as single agents and greater clinical efficacy with the use of regimens including three or more drugs (typically doxorubicin, cisplatin and high-dose methotrexate) over those employing two anti-
cancer agents (26). Another very large retrospective international collaborative study (2,680 patients) found a correlation between increased mortality and amputation as compared with limb sparing/wide resection. The addition of radiation therapy to surgery and chemotherapy was also associated with increased mortality as was the sole use of anthracycline without a platinum chemotherapeutic drug (27). Some of the results of this study are likely to be confounded by individual patient clinical presentation; amputation and radiation therapy may have been preferentially selected for those patients with larger and less readily excised primary tumours and those with residual tumour burden after surgical resection. Future prospective clinical studies may allow for a more accurate comparison of prognosis with different treatment strategies.

Several studies have attempted to identify prognostic markers in human osteosarcoma. A negative correlation has been reported between pre-treatment serum levels of Vascular Endothelial Growth Factor (VEGF), a stimulator of angiogenesis, and both rate of metastasis and survival (28). Increased pre-treatment serum Alkaline Phosphatase, a byproduct of bone formation by osteoblasts, has been significantly correlated with poor prognosis including the presence of metastases at presentation, an increased risk of relapse and a decreased disease free survival time (29). Percentage tumor necrosis and reduction in tumor volume after preoperative chemotherapy have been shown to be independently predictive of increased disease free survival (30). Recently, reduced expression of Argininosuccinate Synthetase (ASS), a required enzyme in the synthesis of the non-essential amino acid arginine, has been correlated with the development of pulmonary metastasis in humans with osteosarcoma. Human osteosarcoma cell lines with low
ASS activity exhibited a complete growth arrest in media lacking arginine suggesting that treatment with an arginine depleting deiminase agent may be effective in osteosarcoma patients (31).

Genomic similarity between dogs and humans has also been identified fairly recently (3). Sequencing of the canine genome allows for genetic comparison across species and such comparison has shown that more than 25% of the canine genome aligns with the human genome. In fact, the degree of alignment or, in other words, the overlap of ancestral genetic sequence is greater between canines and humans than between either of these species and mice (32). This genetic overlap between dog and man, combined with the similarity in clinical disease presentation, supports the use of the dog as an experimental model for human osteosarcoma.

The clinical similarities between canine and human osteosarcoma along with the comparatively higher incidence of canine osteosarcoma and the relative homogeneity of the canine genetic background due to breed stratification provide a unique opportunity to identify potential biologically and clinically relevant molecular subtypes in canine osteosarcoma patients that may then be applied to their human counterparts. A recent gene expression profiling study of dogs with osteosarcoma identified two such molecular subtypes with significantly different overall survival. Overexpression of genes associated with cell cycle disruption and DNA damage was correlated with decreased overall survival and overexpression of genes associated with local tumour microenvironment was associated with increased overall survival. The same molecular subtypes were identified in human osteosarcoma patients and were associated with
similar differences in clinical outcome (33). Recent high resolution array comparative genomic hybridization (aCGH) of canine osteosarcoma tumours found numerous recurrent DNA copy number aberrations, many of which were orthologous with previously reported recurrent aberrations in the DNA from human osteosarcoma tumours. The study identified six genes within these aberrant regions as potentially important in osteosarcoma tumorigenesis. There was recurrent copy number loss in regions encompassing the tumor suppressor genes tumour suppressor candidate 3 (Tusc3) and phosphatase and tensin homolog (Pten). There was recurrent copy number gain in regions encompassing Ras homolog gene family, member C (Rhoc) which is involved in signal transduction pathways and runt-related transcription factor 2 (Runx2) which is involved in osteoblast differentiation as well as the oncogenes tumour sclerosis protein 2 (Tsc2) and Myc (34). Finally, a cross species gene profiling study exploited the more rapid clinical progression of canine osteosarcoma to identify molecular prognostic markers for human osteosarcoma. Two genes, interleukin-8 (IL-8) and solute carrier family 1, member 3 (Slc1a3) were uniformly overexpressed in canine tumours, but overexpressed to a lesser degree in only a proportion of human tumours. High expression of these two genes in human osteosarcoma patients was associated with decreased overall survival (35).

Human osteosarcoma is also frequently studied using murine models, via both surgically induced xenograft tumours and genetically engineered spontaneous tumours. A novel genetically engineered mouse model of human osteosarcoma (MOTO mouse) has recently been generated. In this model, osteoblast specific inactivation of the tumour suppressors protein 53 (Tp53),
retinoblastoma protein (Rb) and protein phosphatase 2 (Pp2a) results in multiple primary osteosarcoma tumours by 21 weeks of age in all mice. The majority of these tumours have histologic features typical of osteoblastic osteosarcoma and develop in the long bones of the limbs and the cranium. Spontaneous pulmonary metastasis occurs in more than 90% of these mice; metastasis to liver is less frequent (36). The early onset of tumours, spontaneous metastasis with pulmonary predilection and primarily osteoblastic histologic phenotype mimics human osteosarcoma fairly closely. Mice heterozygous for both Tp53 and the R1alpha regulatory subunit of cyclic AMP-dependent protein kinase A (Prkar1a) are another potential mouse model of osteosarcoma, developing osteosarcomas of both the axial and appendicular skeleton (37), and will be discussed further later in this chapter.

**Molecular Aspects of Osteosarcoma**

**PRKAR1A**

Cyclic AMP (cAMP)-dependent protein kinase A (PKA) is a tetramer composed of two regulatory and two catalytic subunits. The R1alpha regulatory subunit of PKA (PRKAR1A) is one of four identified regulatory subunits; four catalytic subunits have also been identified to date. The two alpha regulatory subunits are ubiquitously expressed whereas expression of the two beta subunits is limited to a small number of tissues including brain. When cAMP is elevated, it binds to PKA regulatory subunits resulting in their dimerization and the release of PKA catalytic subunits. The catalytic subunits are then free to phosphorylate various downstream targets such as cAMP response element-binding (CREB) thereby initiating
downstream cellular processes such as transcription (Figure 1-1). Regulation of PKA activity via PRKAR1A is important in embryonic development; mice lacking both copies of Prkar1a die in utero due to defects in multiple mesoderm derived tissues. Neural crest specific Prkar1a knockout mice exhibit abnormal intramembranous ossification of craniofacial bones resulting in perinatal mortality due to asphyxiation (38). The role of PKA signaling in embryogenesis may involve cell transitions between epithelial and mesenchymal states. Prkar1a null mouse embryonic fibroblasts express decreased levels of the mesenchymal marker vimentin and increased levels of the epithelial marker E-cadherin (39).

Studies associating PRKAR1A with proliferative conditions and neoplasia focus primarily on a syndrome known as Carney Complex. Approximately two thirds of humans with Carney Complex, an autosomal dominant inherited multiple neoplasia syndrome characterized by cardiac myxomas, spotty skin pigmentation, endocrinopathy, schwannomas and other benign neoplasms, demonstrate inactivating mutations in Prkar1a. Haploinsufficiency of Prkar1a results in increased PKA activity which is postulated to affect cell proliferation and differentiation (40) (41). Contrary to its proposed role in Carney Complex tumorigenesis, increased PKA activity may also decrease cell survival. Recent work demonstrates that PRKAR1A binds with Bim, a pro-apoptotic protein and phosphorylation target of PKA. Phosphorylation of Bim enhances its stability, thereby promoting apoptosis (42). Lesions similar to those reported in human Carney Complex have recently been described in a dog,
including cardiac myxosarcoma, adrenocortical adenoma and pituitary hyperplasia (43), however 

*Prkar1a* gene mutations were not present in this dog.

Mice heterozygous for *Prkar1a* have been genetically engineered as a mouse model for the human Carney Complex syndrome. These mice are prone to tumorigenesis involving cAMP-responsive tissues including schwann cells, osteoblasts and thyrocytes and conditional knockouts within the pituitary and neural crest show local increased proliferation and tumorigenesis. However, conditional cardiac knockouts show decreased cell proliferation and myxomatous changes with subsequent embryonic death (44), suggesting that *Prkar1a* tumor suppressor activity may be tissue specific. Crossing *Prkar1a* heterozygous mice with mice heterozygous for the tumor suppressors *Tp53* and *Rb* results in *Prkar1a+/- Tp53+/-* and *Prkar1a+/- Rb +/-* progeny with decreased overall survival relative to *Prkar1a+/-* and *Tp53+/-* or *Rb+-/* mice respectively. Sarcomas, including osteosarcomas and splenic hemangiosarcomas, are more numerous in *Prkar1a+/- Tp53+/-* mice than *Tp53+/* mice and both pituitary adenomas and thyroid carcinomas are more numerous and larger in *Prkar1a+/- Rb+/-* mice than *Rb+/-* mice. Mice heterozygous for *Prkar1a* also develop more papillomas than wild type mice when exposed to a dermal carcinogen (37). Together, these results suggest that *Prkar1a* functions as a weak tumor suppressor relative to *Tp53* and *Rb* and acts synergistically with other tumorigenic mechanisms.

*Prkar1a* also appears to function as a tumour suppressor in skeletal tumorigenesis. A small proportion of patients with Carney Complex develop benign bone tumors arising from
osteoblasts and termed osteochondromyxomas. These tumours are characterized histologically by lobules of spindle shaped cells within a mixture of osteoid, chondroid and myxoid matrix. Similar tumors arising from incompletely differentiated osteoblasts have been seen in germline Prkar1a heterozygous mice, primarily affecting the tail vertebrae. Increased PKA activity has been demonstrated in these murine tumors (45). Osteoblast specific Prkar1a haploinsufficiency in mice also results in non-metastatic, slow growing bone tumours, but with a wider anatomic distribution and different histologic appearance. These tumours develop in multiple skeletal sites, both long and flat bones, and are composed of thin fibroblast-like spindle cells within a myxoid matrix, lacking both the osteoid and chondroid matrix typical of osteochondromyxomas. They overexpress both phospho-CREB and receptor activator of nuclear factor kappa-B ligand (RANKL) (36).

An association between Prkar1a deficiency and osteosarcoma has been identified in the MOTO mouse model of osteosarcoma. Copy number loss involving the Prkar1a locus and associated decreased Prkar1a expression was detected in more than a third of primary osteosarcoma tumours in these mice. These tumours also demonstrated downstream elevation in expression of RANKL, an initiator of osteoclast differentiation and activation, and reduction in expression of osteoprotegerin (OPG), a decoy receptor for receptor activator of nuclear factor kappa-B (RANK). This alteration in RANKL and OPG expression was mediated by PKA phosphorylation of CREB leading to altered RANKL and OPG transcription by phospho-CREB. Haploinsufficiency for Prkar1a on the MOTO mouse osteosarcoma model background resulted
in very early onset of osteosarcoma (5 weeks versus 18 weeks in control mice) and osteosarcoma
tumours with increased PKA activity, phospho-CREB expression and RANKL expression (36).

Measurement of Prkar1a expression may have some promise as a prognostic indicator in
both canine and human osteosarcoma patients. Recent gene expression microarray experiments
comparing canine osteosarcoma patients with poor versus good response based on disease free
interval following amputation and chemotherapy identified expression differences in genes
associated with PKA signaling. Specifically, expression of PKA regulatory subunits was
downregulated and expression of PKA catalytic subunits was upregulated in poor responders
relative to good responders (46). Somewhat contradictory results from human microarray data
indicate that a good response to chemotherapy, as measured by percent tumour necrosis, is more
likely in osteosarcoma patients with low Prkar1a expression, however there was no correlation
between Prkar1a expression and overall patient survival (36). This correlation between low
Prkar1a expression and good response to chemotherapy may reflect increased tumour cell
proliferation in the face of PKA deregulation and associated increased sensitivity to
chemotherapeutic agents.
Figure 1-1. cAMP binds to the regulatory subunits of PKA(R), releasing the catalytic subunits(C) to phosphorylate downstream targets such as the transcription factor CREB.

IGF Signaling Axis

Insulin-like growth factors (IGFs) act systemically to affect growth and energy metabolism. IGF1 and IGF2 are produced predominantly by the liver and released into systemic circulation, however, they can also be produced locally within neoplastic tissue, and thus are capable of acting in endocrine, paracrine and autocrine fashions. Both IGF1 and IGF2 bind to
tetrameric IGF1 receptors (IGF1Rs), insulin receptors (IRs) and combined hybrid IGF1/insulin receptors, all of which have extracellular binding domains and intracellular domains with tyrosine kinase activity. Both the mitogen-activated protein (MAP) kinase and phosphoinositide 3 (PI3) kinase pathways act downstream of these receptors to mediate intracellular processes (47). The bioavailability of both IGF1 and 2 is affected by association with various high affinity IGF binding proteins (IGFBPs). Proteolytic activity of matrix metalloproteinases (MMPs) has been shown to decrease the affinity of IGFBP-IGF binding, thereby increasing the local concentration of IGFs available for interaction with the IGF1R (48). The bioavailability of IGF2 is also affected by attachment to the decoy IGF2 receptor (IGF2R). Binding of IGF2 to this decoy receptor does not result in intracellular signal transduction, but instead sequesters IGF2 and prevents its attachment to the IGF1R (47)(Figure 1-2). Igf2 is also an imprinted gene with silencing of the maternally inherited allele in normal cells. Loss of this imprinting through changes in Igf2 promotor methylation status and subsequent abnormal expression of the maternal Igf2 allele may result in increased overall IGF2 expression in some neoplasms, particularly human colorectal cancer (49).

Several recent studies have reported increased expression of IGF2 in osteosarcoma tumor tissue. Serum levels of IGF2 are reportedly higher in humans with osteosarcoma than in healthy control individuals and return to normal after surgical removal of the tumor, which implicates tumor tissue as the source of abnormal IGF2 production (50). Increased expression of IGF2 protein has been demonstrated in human osteosarcoma tumours using immunohistochemistry in
conjunction with increased expression of Igf2-p3 and -p4 promotor mRNA. Epigenetic alterations in Igf2 promotor methylation are thought to result in this increased expression of IGF2 by osteosarcoma cells due to loss of imprinting, however, the details of these alterations are not fully understood as both hyper- and hypomethylation of an upstream imprinting control region (ICR) have been reported (51) (52). A zinc-finger CCCTC-binding factor (CTCF) has recently been shown to consistently bind to unmethylated ICRs in human osteosarcoma tumours, but this binding did not regulate IGF2 expression in these tumours (53).

The IGF1R also appears to be important in osteosarcoma pathogenesis. Increased expression of IGF1R has been demonstrated in canine and human osteosarcoma cell lines and in vitro treatment of these cell lines with IGF1 resulted in increased cell proliferation as well as colony formation in soft agar (54). Downregulation of Igf1r expression in human osteosarcoma cells using lentiviral vector siRNA resulted in decreased growth rates with cell cycle arrest, increased apoptosis, decreased invasiveness and enhanced radiosensitivity in vitro as well as decreased tumorigenicity in vivo (55). Along the same lines, increased expression of both IGF1R and IR has been demonstrated in human osteosarcoma tumors as well as human osteosarcoma cell lines (50). Concurrent in vitro blockade of IGF1R and IR using a monoclonal antibody, siRNA or tyrosine kinase inhibitor was found to be more effective in slowing the growth of these osteosarcoma cells than blocking IGF1R alone.
Interference with IGF signaling via blockade of the IGF1R is a promising therapeutic approach in several human pediatric cancers including osteosarcoma. An IGF1R antibody has been shown to slow the rate of mouse osteosarcoma xenograft tumour growth as a single agent treatment and to have a synergistic anti-tumour effect when combined with the chemotherapeutic drugs cisplatin or cyclophosphamide. The antibody inhibited both blood vessel growth and cell proliferation, measured by Ki-67 nuclear proliferation marker immunohistochemical staining, in these xenografts (56). Similar \textit{in vivo} murine osteosarcoma xenograft growth inhibition has been recently demonstrated with IGF1R tyrosine kinase inhibitor treatment (57). Less promising treatment results have been obtained in a prospective, placebo-controlled preclinical study in canine osteosarcoma patients using a long-acting somatostatin analogue (OncoLAR) to suppress serum IGF1. Fifty percent reduction in serum IGF1 levels with OncoLAR treatment in combination with standard chemotherapy did not result in increased tumour necrosis and apoptosis or improved survival compared to patients receiving chemotherapy alone (58). OncoLAR treatment did not suppress local tumour expression of IGF1 suggesting that an autocrine or paracrine signaling loop within the tumour itself may be more important than systemic IGF1 expression in osteosarcoma growth and progression.

Further to the systemic role of IGFs in growth, a single \textit{Igf1} single-nucleotide polymorphism has been reported to be important in determining canine body size (59). This is interesting since, as outlined above (Clinical Aspects of Canine Osteosarcoma), small breed dogs are more likely to develop axial osteosarcomas than large breed dogs and osteosarcoma affecting
any site is much more common in large breed dogs than small breed dogs. The presence of this polymorphism suggests a potential role for IGF signaling in the size-based discrepancies in canine osteosarcoma incidence.

Figure 1-2. IGF2 binds to tyrosine kinase receptors to initiate downstream signalling via the PI3K and MAPK pathways. IGF2 bioavailability is affected by association with IGFBPs and the IGF2 decoy receptor.
TP53

The tumor suppressor gene tumour protein 53 (Tp53) is important in the maintenance of cellular homeostasis and the response of cells to various forms of stress. In the face of genomic damage, it acts to induce cell cycle arrest followed by either DNA repair or cell death depending on the severity of the damage. It thereby protects against genetic mutations that may lead to neoplastic transformation. Several studies have demonstrated Tp53 mutations in canine osteosarcoma. Inactivation of Tp53 was found in five canine osteosarcoma cell lines, all of which were able to grow in soft agar and form tumors in immunodeficient mice (60). Other studies demonstrated point mutations within exons 4-8 of the Tp53 gene in 47% of 15 canine appendicular osteosarcomas (61) and in the primary tumours of 4 out of 17 dogs (62). These mutations were almost identical in location and type to those reported in human osteosarcoma, further supporting the use of canine osteosarcoma as a model for the human disease.

The identification of Tp53 mutations may have prognostic implications in dogs with osteosarcoma. Wild-type TP53 is not detectable using immunohistochemistry due to its short half-life, however abnormalities in the TP53 protein extend its half-life sufficiently to allow for accumulation within cells and subsequent immunohistochemical detection. Retrospective studies have found that TP53 expression as assessed via immunohistochemistry is higher in osteosarcomas than other bone tumours and also higher in appendicular osteosarcomas than axial osteosarcomas (63) (64). Loukopolos et al demonstrated a positive correlation between TP53 expression and histologic grade, mitotic index, degree of tumour necrosis and pleomorphism
suggesting a correlation between $Tp53$ mutation and increased malignancy. More recently, $Tp53$ mutations were detected in 41% of patients in a study of 59 dogs with osteosarcoma; these mutations were negatively correlated with survival time (65).

Mutations in $Tp53$ represent a potential therapeutic target. Wild-type $Tp53$ has been successfully introduced into canine osteosarcoma cells using a plasmid DNA vector and canine osteosarcoma xenograft tumours using an adenoviral vector. The introduction resulted in increased radiation treatment sensitivity in the transfected cell lines (66). Transfection with the adenoviral vector increased apoptosis within the tumours and inhibited tumour xenograft growth (67).

**PTEN/pAKT**

Phosphatase and tensin homolog ($Pten$) acts as a tumor suppressor gene by dephosphorylating phosphatidylinositol 3,4,5-triphosphate (PIP-3) to phosphatidylinositol 4,5-bisphosphate (PIP-2) and thereby preventing the accumulation of PIP-3 within the cytosol. Binding of protein kinase B (Akt) with PIP-3 results in Akt phosphorylation and activation. Loss or mutation of $Pten$ thus leads to an increased level of phospho-Akt (pAkt), which protects the cell from apoptosis and increases cellular proliferation (68).

Loss of $Pten$ tumor suppressor activity occurs fairly commonly in both canine and human osteosarcoma. Absence of $Pten$ expression or mutation of the PTEN protein has been reported in canine osteosarcoma cell lines in conjunction with concurrent increased expression of pAkt (69). Immunohistochemical staining showed complete loss of PTEN expression in ten of fifteen
canine osteosarcoma tumours in the same study. Copy number loss in the region encompassing *Pten* was the most common genomic abnormality detected using array comparative genomic hybridization (aCGH) in a cohort of Golden Retrievers and Rottweilers with appendicular osteosarcoma; the abnormality affected 42% of cases (70). In a study of human osteosarcoma, a smaller proportion of tumors (nine of twenty-eight) demonstrated loss of PTEN expression using immunohistochemistry; single nucleotide polymorphism (SNP) chromosomal analysis revealed copy number loss in the region of the *Pten* locus in at least one third of these tumors (71). No association was found between PTEN loss and clinical outcome in these human patients.

**RB**

The Retinoblastoma protein (RB) acts as a tumour suppressor by regulating the cell cycle. In its active state, it blocks cell cycle progression by binding to transcription factors of the E2F family. Phosphorylation of RB by cyclins and cyclin dependent kinases results in the release of these E2F transcription factors, triggering the transition from G1 into the S phase of the cell cycle. Dysregulation of this pathway or loss of normal RB function via genetic mutation therefore leads to unhindered cell proliferation and contributes to tumourigenesis (72). Osteoblast differentiation also appears to be affected by *Rb* deficiency; mice lacking *Rb* expression exhibit bone malformations and defects in skeletal ossification (73). Recent work indicates that altered adhesion between osteoblasts is correlated with loss of RB function and may contribute to this impaired bone development (74). Loss of *Rb* expression may therefore
promote both osteosarcoma formation, via loss of cell cycle control, and metastasis, via loss of
cell-to-cell adhesions.

The retinoblastoma protein is mutated in numerous human cancers, including
osteosarcoma, and the effects of RB mutation have been studied using mouse models. In one
murine model, genetic deletion of the *Rb* gene was not sufficient to induce osteosarcoma
formation, however, *Rb* loss increased the frequency and malignancy of osteosarcomas
associated with deletion of *Tp53*. A greater percentage of mice with osteoblast precursor specific
knockout of both *Tp53* and *Rb* developed osteosarcomas within the first year of life than those
with knockout of only *Tp53*. Mean survival times were also shorter for double *Tp53* and *Rb*
knockouts than for *Tp53* knockouts. None of the mice with bone specific knockout of only *Rb*
developed osteosarcoma by one year of age (75).

Deficiencies in RB function may also play a role in canine osteosarcoma tumourigenesis.
Abnormalities in the RB pathway have been identified in canine osteosarcoma cell lines. In one
study, one of five such cell lines had decreased RB expression and the remaining four were
deficient in *p16*, an upstream inhibitor of RB phosphorylation (60).

**Histologic Subtypes and Grading**

The microscopic appearance of osteosarcomas varies; however, the histologic diagnosis
of osteosarcoma is based on a single criteria: the disorganized or haphazard production of
osteoid matrix by malignant osteoblasts. An established histological classification scheme for
canine osteosarcoma has been adapted from human pathology and is based on the predominant
pattern identified in representative tumor sections (76) (77). If a single predominant pattern cannot be identified, then the tumor is classified as a combined type osteosarcoma. Sub-types include osteoblastic, chondroblastic, fibroblastic, telangiectatic and poorly differentiated. Osteoblastic osteosarcomas are the most common subtype reported in dogs (76) (6) and can be further subdivided as to productivity based on the amount of osteoid matrix made by the pleomorphic osteoblasts comprising the tumour. Chondroblastic osteosarcomas are characterized by the combined production of both osteoid and chondroid matrix by tumor cells. Fibroblastic osteosarcomas are generally more highly cellular and comprised of pleomorphic spindle-shaped cells similar to those of fibrosarcoma with relatively sparse foci of osteoid production. Telangiectatic osteosarcomas contain multiple, blood-filled spaces lined by neoplastic cells. Accurate tumor sub-typing on small tumor fragments is often not possible due to the great potential for variability within the same tumor and tumor sub-type was correctly predicted in only 3 of 9 core biopsy samples in one canine study (76).

The prognostic significance of canine osteosarcoma histological classification is questionable. The fibroblastic sub-type has been associated with a more favorable prognosis in one study (6), however, another study failed to show any association between histologic sub-type and prognosis (76). Histologic subtype may have greater prognostic significance in human osteosarcoma where a higher proportion of tumors within the fibroblastic sub-type responded well to chemotherapy and a lower proportion of tumors within the chondroblastic sub-type responded well to chemotherapy as compared with tumors of the conventional (osteoblastic) type.
Five year survival for good responders in this human study was 75% compared with only 45% for poor responders. A good chemotherapy response was defined histologically as greater than or equal to 90% tumor necrosis.

A standardized histologic grading system exists for canine osteosarcoma, however, the clinical and prognostic value of the system is debatable as the vast majority of canine osteosarcomas tend to behave aggressively. The system is based on the following criteria: percentage of pleomorphic cells, mitotic rate, percentage of histologic section occupied by matrix, percentage of histologic section occupied by neoplastic cells and percentage necrosis within the section (76). A score of grade III (high grade) is automatically assigned if there is evidence of hematogenous invasion or lymph node metastasis within the examined section. In a study of 166 canine osteosarcomas, four percent were grade I, 21% were grade II and 75% were grade III. Hematogenous invasion was detected in 71% and lymph node metastasis was detected in 24%. Blood vessel invasion and mitotic index were most predictive of overall tumor grade and grade III designation was predictive of poor outcome.

A subsequent retrospective analysis of 140 primary canine osteosarcomas and their metastases in 2007 looked for associations between tumor grade and its components (mitotic rate, necrosis, pleomorphism) and clinical parameters (79). Mitotic rate was highly variable between sites, but higher overall in appendicular than axial tumors supporting the theory that appendicular osteosarcomas are more aggressive than axial osteosarcomas. Primary tumors that metastasized had a higher grade than non-metastatic primaries suggesting that metastatic
osteosarcoma cells may have a more aggressive phenotype than their primary counterparts and supporting the use of grading in prognostication. Interestingly, younger dogs (<4 years) had more aggressive tumors (higher grade and mitotic index) than older dogs.

Chemotherapy

General Mechanisms of Action: Doxorubicin and Platinum Compounds

Adjuvant or neoadjuvant chemotherapy is part of the standard of care treatment in both canine and human osteosarcoma. Doxorubicin and platinum drugs, typically cisplatin in humans and carboplatin in dogs, are commonly used agents in both species (25) (2). As with any drugs, the efficacy of anticancer agents depends on their pharmacokinetic and pharmacodynamic properties. Pharmacokinetic properties relate to the time course of drug levels in the body and include absorption, distribution, metabolism and elimination. Pharmacodynamic properties relate to drug effects at the cellular level and include cellular uptake and metabolism, binding of drugs to cellular targets and cellular mechanisms for preventing or recovering from drug-induced injury.

Three basic mechanisms are thought to play a role in the anti-tumour activity of doxorubicin. First, doxorubicin can intercalate, or insert itself, between base pairs of the two DNA strands of a double helix resulting in partial unwinding of the helix and potential inhibition of transcription and translation. Second, doxorubicin interacts with topoisomerase II, an important enzyme in DNA and RNA synthesis, promoting DNA strand cleavage and interfering
with the resealing of cleavage sites. Finally, intracellular metabolism of doxorubicin promotes the formation of free radicals which cause oxidative damage to cell membranes (80).

The cytotoxic mechanism of platinum drugs involves interference with DNA synthesis. Two chlorine groups in the cisplatin compound are replaced with water in a chemical reaction within the cell, resulting in a positively charged active drug. The two water ligands then bind to two DNA sites, most commonly on the same DNA strand, forming an adduct, but occasionally on different DNA strands, forming a cross-link. These adducts and cross-links are preferentially formed between the platinum compound and the purine bases, although pyrimidine bases can also be involved (81).

**Resistance in Osteosarcoma**

Despite improvements in clinical outcome with the advent of adjuvant or neoadjuvant chemotherapy, survival of both canine and human osteosarcoma patients with metastatic disease at presentation remains poor compared to those without clinically detectable metastases (13) (82). Resistance to chemotherapy drugs may be one factor contributing to the poor clinical response of these patients. There are very few reported studies examining *in vitro* chemoresistance in canine osteosarcoma. In one such study, *in vitro* treatment with dexamethasone was found to increase the resistance of a canine osteosarcoma cell line to cisplatin and methotrexate. This chemoresistance was not associated with upregulation of either P-glycoprotein or Multiple Resistance Protein (MRP), two cell membrane transporters involved in chemotherapeutic drug efflux (83). Expression of P-glycoprotein at both the mRNA and
protein levels was shown to be induced in the same canine osteosarcoma cell line by prolonged
*in vitro* doxorubicin treatment. The resultant P-glycoprotein expressing cells accumulated
smaller amounts of intracellular doxorubicin with further *in vitro* doxorubicin treatment and were
more resistant to *in vitro* vincristine treatment than the parent cells that did not express P-
glycoprotein (84).

Similar to their canine counterparts, *in vitro* resistance of human osteosarcoma cell lines
to chemotherapy has been linked to transporter proteins associated with drug efflux. A gene
expression microarray study of human osteosarcoma cell lines revealed increased expression of
the gene encoding one such protein known as ATP-binding cassette G2 (*Abcg2*) in a cell line
demonstrating resistance to doxorubicin, cisplatin and etoposide (85).

Antioxidants such as glutathione which are involved in the inactivation of drug-induced
peroxides and free radicals have also been associated with *in vitro* chemoresistance in human
osteosarcoma. Glutathione S-transferase P1 (GSTP1), a catalyst of glutathione detoxification
reactions, was found to be overexpressed in human osteosarcoma cell lines. Its expression was
induced by treatment with doxorubicin and cisplatin. Increased expression of GSTP1 was
associated with resistance to these drugs and inhibition of expression led to increased apoptosis
and DNA injury (86).

Two other studies also point to a role for altered apoptotic signaling in human
osteosarcoma chemoresistance *in vitro*. Overexpression of inosine 5′-monophosphate
dehydrogenase type II (IMPDH2), an important enzyme in guanine nucleotide biosynthesis, in a
human osteosarcoma cell line has been associated with resistance to cisplatin and methotrexate and concurrent overexpression of the anti-apoptotic proteins Bcl-2 and Mcl-1 in vitro (87). Knockdown of the type 1 insulin-like growth factor receptor (IGF-1R) in the same human osteosarcoma cell line has been shown to enhance sensitivity to cisplatin and docetaxel in vitro and has been associated with decreased expression of Bcl-2, increased expression of the pro-apoptotic protein Bax and increased apoptosis (88). The same study claims to have demonstrated similar increased chemosensitivity with IGF-1R knockdown in a mouse subcutaneous xenograft model, however untreated control groups were not included in the study, making the in vivo results questionable.

Finally, recent studies have identified a possible role for microRNAs (miRs), small non-coding RNAs that inhibit mRNA translation, in osteosarcoma chemoresistance. Resistance to doxorubicin, cisplatin and ifosfamide has been associated with expression of miR-140 in a tumour xenograft model. Human osteosarcoma cells overexpressing miR-140 and expressing wild type TP53 showed reduced proliferation rates, induction of both TP53 and P21 and growth arrest in both G1 and G2 phase (89). This suggests that chemoresistance in these cells may be mediated, at least in part, by altered cell cycle control. However, since miRs generally have multiple targets, there may be multiple mechanisms and signaling pathways behind the negative effect of miR-140 expression on chemosensitivity.
Study Rationale and Hypotheses

Recent studies outlined in Chapter 1 (Literature Review) suggest a possible role for PRKAR1A and/or IGF signaling in the molecular pathogenesis of osteosarcoma; however the majority of this work has been in done using tumour tissue or cells from human osteosarcoma patients and mouse models of osteosarcoma. The goal of the second chapter of this thesis was to investigate the immunohistochemical expression of PRKAR1A and IGF2 in canine osteosarcoma and test two hypotheses. The first hypothesis was that PRKAR1A expression correlates with tumour proliferation rate and post-chemotherapy survival. The second hypothesis was that IGF2 expression differs between axial and appendicular tumour locations.

The first aim of the third chapter was to derive novel canine osteosarcoma and normal bone primary cell cultures and undertake initial characterization tests. This derivation and characterization was begun in order to provide a model for future mechanistic in vitro experiments. The first of these experiments is detailed in this chapter and addresses the second aim of this chapter which was to investigate the negative correlation between PRKAR1A expression and post-chemotherapy survival in canine osteosarcoma patients documented in the retrospective immunohistochemical study (Chapter 2). The cell cultures were used to test the hypothesis that PRKAR1A expression is correlated with chemotherapeutic sensitivity and also to examine the downstream signaling pathway of PRKAR1A in the dog.
Chapter 2: Immunohistochemical assessment of PRKAR1A and IGF2 expression in canine osteosarcoma

Abstract

Osteosarcoma is the most common primary bone tumor in dogs and is characterized by aggressive growth and early metastasis. Median survival is approximately one year with amputation and chemotherapy and pulmonary metastases are detectable in approximately ten percent of cases at the time of diagnosis. Recent work in comparative oncogenomics using a mouse model of osteosarcoma has implicated the type 1 alpha regulatory subunit of cAMP-dependent protein kinase (PRKAR1A) in osteosarcoma tumourigenesis. Low Prkar1a expression is associated with a favorable response to chemotherapy in human osteosarcoma but accelerated tumor formation in the mouse model. A polymorphism that alters insulin-like growth factor (IGF) signaling is a major genetic determinant of body size in dogs, and although large size is a risk factor for osteosarcoma development, osteosarcoma in small dogs is more likely to affect the axial skeleton. Immunohistochemistry was used to assess PRKAR1A and IGF2 expression in canine osteosarcoma tissue obtained from Veterinary Teaching Hospital cases over the past ten years. Post-chemotherapy survival was significantly longer in patients with tumours expressing low levels of PRKAR1A, and PRKAR1A expression did not correlate with mitotic index. IGF2 expression did not differ between axial and appendicular sites. Low PRKAR1A expression appears to confer a survival advantage to patients that is independent of mitotic index. Elucidating the mechanism underlying this survival advantage may enable the development of
novel therapeutic modalities. Immunohistochemical staining for PRKAR1A may prove to be a useful prognostic indicator in canine osteosarcoma.

**Introduction**

Osteosarcoma accounts for approximately 85% of canine bone tumours and affects greater than 8,000 dogs in the United States each year. It is a highly aggressive cancer with early metastasis, primarily to lung. Pulmonary metastases are detectable radiographically in less than fifteen percent of cases at the time of diagnosis, however occult pulmonary metastases are present in approximately 95% of canine patients at presentation. One year survival is 50% with surgical resection and chemotherapy (1). Little is known about the molecular pathogenesis of canine osteosarcoma and there is a general lack of clinically useful molecular prognostic markers.

The R1alpha regulatory subunit of PKA (PRKAR1A) is one of four identified regulatory subunits of cyclic AMP (cAMP)-dependent protein kinase A (PKA). Binding of cAMP to PRKAR1A results in the release of PKA catalytic subunits which proceed to phosphorylate various targets, thereby initiating downstream cellular processes. PRKAR1A plays an important role in embryonic development and has been studied extensively in relation to the inherited human Carney Complex multiple neoplasia syndrome which is characterized by cardiac myxomas, spotty skin pigmentation, endocrinopathy, schwannomas and other benign neoplasms occasionally involving bone. Approximately two thirds of Carney Complex patients have inactivating mutations in *Prkar1a*. Studies using mice haploinsufficient for *Prkar1a* as a model
for Carney Complex have identified \textit{Prkar1a} as a weak tumour suppressor or tumour modifier gene; its loss appears to be sufficient to cause benign neoplasms or to synergistically promote the growth of malignant tumours in the presence of other tumourigenic factors (90) (91). In support of these findings, \textit{Prkar1a} haploinsufficiency in a murine osteosarcoma model generated via osteoblast specific inactivation of the tumour suppressor genes \textit{Tp53}, \textit{Rb} and \textit{Pp2a} (MOTO mouse model) results in accelerated osteosarcoma tumour development whereas \textit{Prkar1a} haploinsufficiency on a wild type background results in the growth of non-metastatic, indolent bone tumours (36). Correlations between low \textit{Prkar1a} expression and chemotherapeutic treatment response have been identified in both canine and human osteosarcoma patients, however the results to date are contradictory; low expression has been associated with good response in humans (36) but poor response in dogs (46).

IGF2 (insulin like growth factor 2) and the IGF signaling axis are key regulators of growth and energy metabolism. IGF2 binds to IGF1, insulin and hybrid IGF1/insulin receptors thereby activating the downstream mitogen-activated protein (MAP) kinase and phosphoinositide 3 (PI3) kinase pathways. Bioavailability of IGF2 is regulated by association with IGF binding proteins as well as binding with the decoy IGF2 receptor. As an imprinted gene, the maternally inherited \textit{Igf2} allele is silenced in normal cells. Loss of this imprinting via epigenetic changes in \textit{Igf2} promotor methylation is thought to result in increased IGF2 expression in some human tumours such as colorectal cancer (49) and osteosarcoma (51) (52). Increased IGF2 serum concentration which normalizes after tumour excision (50) and increased
IGF2 expression in tumour tissue (51) have both been reported in human osteosarcoma. Decreased \textit{Igf2} expression in the MOTO mouse model of osteosarcoma is associated with the development of smaller appendicular osteosarcomas (Wood, G et al, unpublished data) which is interesting considering the size-based discrepancies in canine osteosarcoma skeletal distribution. Osteosarcoma affecting any skeletal site is more common in large breed dogs, however osteosarcoma is more likely to affect axial sites in small breed dogs than in large breed dogs. Furthermore, a single-nucleotide polymorphism in \textit{Igf1} is critical in canine body size determination (59) suggesting a possible role for IGF signaling in these size based differences in tumour location.

The aim of this study was to assess the expression of PRKAR1A and IGF2 retrospectively in canine osteosarcoma cases seen at the Ontario Veterinary College teaching hospital using immunohistochemistry, to quantify proliferation rate in conjunction with the evaluation of PRKAR1A expression and to corroborate PRKAR1A immunostaining levels with western blotting on cases with available fresh tissue. The first hypothesis was that PRKAR1A expression would correlate with proliferation rate and chemotherapeutic treatment response, with lower expression associated with a higher mitotic index, increased chemosensitivity and longer post-chemotherapy survival. The second hypothesis was that IGF2 expression would differ between appendicular and axial tumour locations, with higher expression in appendicular than axial tumours.
Materials and Methods

Case Selection

One hundred and forty Veterinary Teaching Hospital biopsy and necropsy canine osteosarcoma cases were identified from January, 2000 to July, 2010. All cases had a histologic diagnosis of osteosarcoma. Sixty-four cases were selected for PRKAR1A immunohistochemical staining based on the availability and quality of paraffin blocks and these cases are summarized in Appendix 1. Cases were included regardless of treatment history and tumour location and included seventeen axial tumours (27%). A small subset of these cases (18 in total) were also stained immunohistochemically for IGF2. Approximately equal numbers of axial and appendicular tumours (10 axial and 8 appendicular) were selected for IGF2 staining. An attempt was made to restrict IGF2 staining to cases affecting a single breed of dog, resulting in seven Golden Retriever cases, three of which affected the axial skeleton. However, due to the relatively low numbers of axial osteosarcoma cases in the database, other, primarily large breed, dogs were also included.

Immunohistochemistry

Sections were deparaffinized in three changes of xylene, rehydrated through an ethanol series and placed into 3 % hydrogen peroxide for 30 minutes to block endogenous peroxidase activity. Slides were then rinsed in water. Antigen retrieval was performed by boiling slides in 10 mM citrate buffer, pH 6.0 for 2 minutes in a pressure cooker at 20 psi. After rinsing with phosphate buffered saline (PBS), slides were incubated in Serum Free Protein Block (Dako,
Glostrup, Denmark) for 30 minutes at room temperature in a humidified chamber. Slides were then incubated overnight at 4°C with either mouse monoclonal anti-human PRKAR1A (1:100 dilution; BD Transduction Laboratories, Franklin Lakes, NJ) or goat polyclonal anti-mouse IGF2 (1:100 dilution; R and D Systems, Minneapolis, MN). Identical overnight incubation with antibody diluent alone and mouse monoclonal anti-human smooth muscle actin (1:100 dilution, Dako, Glostrup, Denmark) served as negative controls for PRKAR1A immunostaining. For IGF2 immunostaining, slides were incubated overnight at 4°C with normal goat serum as a negative control. After a PBS rinse, all slides were incubated for 30 minutes at room temperature with horseradish peroxidase conjugated rabbit anti-mouse or rabbit anti-goat antibodies respectively (1:200 dilution; Dako, Glostrup, Denmark). After a final PBS rinse, the slides were treated with diaminobenzidine (DAB) (Dako, Glostrup, Denmark) for 10 minutes at room temperature and then rinsed with water. Tissues were counterstained with Gill’s hematoxylin #3 (Polysciences Inc., Warrington, PA) and then dehydrated through a reverse series of ethanol and xylene. Cover slips were mounted with Cytoseal (Electron Microscopy Sciences, Fort Washington, PA).

**Immunohistochemical Scoring**

There was negative immunostaining of tumour cells on all negative control slides for both PRKAR1A and IGF2. There was strong positive immunostaining of smooth muscle cells within artery and arteriole walls on control slides incubated with anti-smooth muscle actin as a negative control (Figure 2-1).
Figure 2-1. Negative controls for (A) PRKAR1A and (B) IGF2 immunohistochemistry. Application of the primary mouse monoclonal anti-human PRKAR1A antibody has been omitted in A1. The primary antibody has been replaced by a different mouse monoclonal anti-human antibody (anti-smooth muscle actin) in A2. The primary goat polyclonal anti-mouse IGF2 antibody has been replaced with normal goat serum in B.

Each slide was assigned a PRKAR1A and IGF2 expression level based on the observation of one of three distinct immunohistochemical staining patterns. In the high staining pattern, all tumour cells exhibited strongly positive cytoplasmic staining. In the low staining pattern, all tumour cells exhibited weakly positive cytoplasmic staining (Figure 2-2). In the
patchy staining pattern, there were distinct areas of both strongly positive and weakly positive staining cells within the same tumour section.

Figure 2-2. Immunohistochemical staining of canine osteosarcoma tumour sections for (A) PRKAR1A and (B) IGF2 demonstrating (1) high and (2) low staining intensity.
Mitotic Index

Mitotic index was counted for each PRKAR1A stained slide as the total number of mitotic figures identified over 10 contiguous 400x fields (field of view diameter = 0.55 mm) when available, selecting the viable cellular areas within the tumour with the highest mitotic activity. A mitotic index was then assigned for each case as the highest mitotic index counted over multiple tissues for a given case.

Cell and Tissue Western Blots

Primary cells derived from canine osteosarcoma and chondrosarcoma tissue were grown to 80-90 % confluency in 75 cm$^2$ flasks then rinsed with cold PBS. RIPA lysis buffer with added anti-protease (10 % v/v) and anti-phosphatase (1 % v/v) was then added (1 mL of lysis buffer per flask) and flasks were placed on ice for 5 minutes. Cell protein lysates were centrifuged at 14,000 x g for 10-15 minutes at 4°C and the supernatants were frozen at -80°C.

Fresh tissue was collected from Veterinary Teaching Hospital canine osteosarcoma cases from the spring of 2009 until the present, flash frozen in liquid nitrogen and then stored at -80°C. Fresh tissue from several cases representative of a range of PRKAR1A immunohistochemical expression levels was frozen in liquid nitrogen and then pulverized prior to incubation for 45 minutes in RIPA lysis buffer with added protease (10 % v/v) and phosphatase (1 % v/v) inhibitors (5 mL of buffer per gram of tissue). Tissue protein lysates were centrifuged at 14,000 x g for 10-15 minutes at 4°C and the supernatants were frozen at -80°C.
After thawing the protein lysates, total protein concentrations were quantified spectrophotometrically in 96-well plates using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA).

Cell and tissue protein lysates were incubated for 15 minutes in a 70°C water bath to denature proteins prior to loading 20 µg of each sample into a 10% acrylamide:bisacrylamide gel and running them alongside a protein ladder at 100-150 volts for 2-3 hours. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Biorad, Hercules, CA) at 100 volts for 2-2.5 hours. Membranes were blocked with 5% milk and then incubated with anti-PRKAR1A antibody (1:1000 dilution; BD Transduction Laboratories, Franklin Lakes, NJ) in 5% milk overnight at 4°C. After washing, the membranes were incubated at room temperature with horseradish peroxidase conjugated rabbit anti-mouse antibody (1:10,000 dilution; Dako, Glostrup, Denmark) in 5% milk for 45 minutes prior to chemiluminescent detection of protein bands using radiographic film. Membranes were then stripped prior to overnight incubation with anti-Beta-actin antibody (1:2,000 dilution; Cell Signaling, Danvers, MA) at 4°C. After washing, the membranes were incubated at room temperature with horseradish peroxidase conjugated swine anti-rabbit antibody (1:10,000 dilution; Dako, Glostrup, Denmark) in 5% milk for 45 minutes prior to chemiluminescent detection of protein bands using radiographic film. Probing for beta-actin served as a loading control. Primary cell cultures were run as a positive control.
Statistical Analysis

Each case was assigned a PRKAR1A expression level based on the staining intensity of the primary tumour. Survival of patients with high, patchy and low PRKAR1A expressing tumours was compared using a Kaplan-Meir survival curve and adjustments were made for multiple comparisons using the Bonferroni method (Bonferroni corrected threshold p value of 0.05/3 = 0.0167). Patients with no history of chemotherapy treatment were excluded as were those receiving chemotherapy treatment for less than 2 months (see Appendix 1 for chemotherapy drug details). An overall survival curve was created incorporating all patients (total of 44).

Mitotic indices of cases with high, low and patchy PRKAR1A expression were compared using a single factor ANOVA.

IGF2 staining of tumours at axial versus appendicular sites was compared using a Fisher’s exact test.
Results

Median survival for the population of canine osteosarcoma patients receiving chemotherapy and immunohistochemical staining for PRKAR1A was 210 days with a range of 60 to 1,300 days (Figure 2-3). Post-chemotherapy survival was significantly longer for patients with PRKAR1A low expressing tumours than those with PRKAR1A high expressing tumours (p=0.011). There was no significant difference in post-chemotherapy survival between patients with patchy expressing tumours and those with either low or high expressing tumours. Median survival was 207 days for patients with high expressing tumours and 762 days for patients with low expressing tumours (Figure 2-4).
Figure 2-3. Overall survival curve including all canine osteosarcoma patients receiving chemotherapy for at least 2 months and tissue immunostaining for PRKAR1A. Median survival is 210 days (n = 44).

Figure 2-4. Survival curve comparing post-chemotherapy survival of patients with high (n = 28), patchy (n = 9) and low (n = 7) PRKAR1A expressing tumours. Survival of patients with low expressing tumours was significantly longer than those with high expressing tumours (p<0.0167).
Mitotic index was highly variable, ranging from zero to 200 mitotic figures counted in ten 400x fields. There was no significant correlation between mitotic index and PRKAR1A staining pattern (p=0.869, Figure 2-5).

![Graph showing mitotic index and PRKAR1A staining patterns](image)

**Figure 2-5.** Mitotic index (total number of mitotic figures in ten 400x microscopic fields) was counted to compare tumour proliferation rate with PRKAR1A immunohistochemical staining pattern. There were no significant differences in mitotic index between patterns (single factor ANOVA, p>0.05, n = 69). Error bars represent the standard error of the mean.

Fresh tumour tissue was available for a small group of cases representing a range of PRKAR1A immunohistochemical expression levels. Western blots were run for PRKAR1A in an effort to corroborate the PRKAR1A immunohistochemical data. PRKAR1A expression levels varied between canine osteosarcoma tissue from different tumours (Figure 2-6). Western blot PRKAR1A expression was assessed qualitatively and categorized in a similar manner to the
immunohistochemical PRKAR1A expression to allow for comparisons between PRKAR1A expression in the same tumour using the two different detection methods. Agreement between PRKAR1A immunohistochemical expression levels and Western Blot expression levels in tissue from the same tumour was inconsistent (Table 2-1). PRKAR1A tended to be detected at a slightly higher molecular weight in tumour tissue (51 kD) than in cells derived from tumour tissue (49 kD) and the expression bands tended to be thicker; double bands at 49 and 51 kD were also evident in a few of the tissue wells (Figure 2-6). This likely reflects post-translational modification with the addition of phosphate, acetyl or ubiquitin groups to PRKAR1A protein within tissue.

Figure 2-6. Western blot for PRKAR1A (49 kD). The first nine wells represent canine osteosarcoma tumour tissue, all from primary tumour sites except JL-31TF (31T) which is from a lung metastasis. The last two wells represent primary cell cultures derived from a canine chondrosarcoma primary tumour (15 = JL15) and the same pulmonary metastasis (31T = JL-31TF). The cells were run as positive controls. Probing for beta-actin (45 kD) was performed as a loading control.
Table 2-1. Summary table of correlations between PRKAR1A expression level as measured by immunohistochemistry versus western blotting for several canine osteosarcoma cases with available fresh tissue and variable immunohistochemical staining patterns.

Most of the cases (15/18 = 83 %) stained for IGF2 showed high expression. IGF2 staining intensity did not differ between axial and appendicular tumour sites based on the small numbers of cases evaluated (p=0.411, Figure 2-7).

<table>
<thead>
<tr>
<th>CASE</th>
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<tr>
<td>JL-1</td>
<td>patchy</td>
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<tr>
<td>JL-21</td>
<td>patchy</td>
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Table 2.2. Contingency table of canine osteosarcoma cases from axial and appendicular sites showing low or high staining for IGF2. Fifteen of eighteen (83 %) cases demonstrated high expression. There were no significant differences in staining intensity between sites (Fisher’s exact test, p>0.05, n = 18).
Discussion

A small subpopulation of canine osteosarcoma patients with prolonged post-chemotherapy survival has been identified using immunohistochemical staining for PRKAR1A; the median survival of patients with low PRKAR1A expressing tumours is 555 days longer than that of patients with high PRKAR1A expressing tumours. Despite the low numbers of low PRKAR1A staining cases, the survival advantage for patients with low expressing tumours receiving chemotherapy treatment is statistically significant. In the MOTO mouse model of osteosarcoma, Prkar1a haploinsufficiency results in more rapid tumour development which may be due to an increased rate of tumour cell proliferation or a decreased rate of tumour cell death. Low expressing PRKAR1A canine tumours may be similarly more aggressive than their high expressing counterparts, but more sensitive to chemotherapy. In the MOTO model, low PRKAR1A expression in osteosarcoma tumours was associated with increased expression of receptor activator of nuclear factor kappa-B ligand (RANKL), a promoter of osteoclastogenesis, and decreased expression of osteoprotegerin (OPG), a decoy receptor of RANKL. Interestingly, a recent gene expression study found downregulation of genes associated with osteoclastogenesis in human osteosarcoma tumours relative to normal bone. Fewer osteoclasts were also counted in tumour biopsies when compared to biopsies of normal bone. These differences in gene expression and osteoclast numbers were more pronounced in patients that responded poorly to chemotherapy suggesting a possible role for osteoclastogenesis in chemosensitivity (92). PRKAR1A downstream signaling pathways are not currently known in the dog, but if they are
similar to those in the MOTO mouse model of osteosarcoma, then prolonged post-chemotherapy survival of canine osteosarcoma patients with low PRKAR1A expressing tumours may be associated with RANKL-induced increased osteoclastogenesis.

At a more intuitive level, low PRKAR1A expressing canine osteosarcomas may be more sensitive to chemotherapy due to a higher rate of tumour cell proliferation. However, assessment of proliferation rate using mitotic index did not reveal a correlation between PRKAR1A staining pattern and mitotic index, suggesting that the survival advantage afforded by low PRKAR1A expression is not associated with increased cell proliferation. In veterinary diagnostic pathology, mitotic index is routinely calculated as the number of mitoses per area and does not directly take into account cell density, however, mitotic figures are counted in the areas with the greatest mitotic figure density and areas with a large proportion of stroma or matrix are avoided in the enumeration. This is arguably less accurate than counting the number of mitotic figures in a given number of tumour cells. The number of mitotic figures per ten 400x fields was used as a measure of tumour proliferation rate in this study in order to achieve results that would be practical and applicable in a veterinary diagnostic laboratory setting. Staining for other, possibly more sensitive, proliferative markers such as the Ki67 protein, which is present in all stages of the cell cycle except G0, and proliferating cell nuclear antigen (PCNA), which is expressed in the nuclei of cells during S phase, or, alternatively, measuring tumour proliferation rate as the number of mitoses in a given number of tumour cells, might still reveal an association between proliferation rate and PRKAR1A immunostaining. Immunohistochemical staining for the
apoptotic markers caspase 3 and annexin v could also be illuminating pending the results of Ki67 and PCNA staining.

Results linking low PRKAR1A expression with increased post-chemotherapy survival contradict those of O’Donoghue et al. (46) in which Prkar1a expression was downregulated in canine osteosarcoma patients with a post-chemotherapy DFI (disease free interval) \(< 100 \text{ days}\) relative to those with a DFI \(> 300 \text{ days}\). Here, Prkar1a expression is measured at the protein level whereas the O’Donoghue et al. study measured Prkar1a expression at the RNA level. It is possible that differing results therefore reflect post-translational modifications in the PRKAR1A protein which could affect protein stability, however no such modifications have been documented. Binding of microRNA to Prkar1a mRNA could also theoretically inhibit translation and result in contradictory expression patterns at the RNA and protein levels. Differences in the results of these two studies may also be explained, at least in part, by differences in study design. O’Donohue et al. included only patients with appendicular osteosarcoma receiving both amputation and chemotherapy in their prospective study and used DFI as an endpoint. Here, a larger spectrum of patients were involved, including all patients receiving chemotherapy with or without amputation/surgical resection for either appendicular or axial osteosarcoma and overall survival was used as a measure of chemotherapy response. Disease free interval, calculated based on the detection of metastatic disease, may be a more biologically relevant measure of cancer progression than overall survival in veterinary medicine, a field in which euthanasia is not uncommon. However, radiographic detection of suspected
pulmonary metastases in dogs with osteosarcoma without histologic confirmation could falsely shorten DFI. Furthermore, in a retrospective study such as ours, variability in case management and lack of complete record keeping makes the comparison of patients using DFI difficult.

Inconsistent agreement between PRKAR1A expression as measured by immunohistochemistry and western blotting was found and may reflect a heterogeneous tumour microenvironment. Although formalin fixed and frozen tissues were collected in tandem from the same tumour locations, one sample immediately adjacent to the other, PRKAR1A expression may vary greatly from one small area to another within a given tumour. Indeed, the patchy staining pattern described with immunohistochemistry for PRKAR1A is testament to this heterogeneity. PRKAR1A is ubiquitously expressed throughout the body in non-neoplastic tissues and PRKAR1A expression is non-existent in necrotic tissues. Osteosarcoma is a highly invasive neoplasm and it is common to see large areas of necrosis within an osteosarcoma tumour. It is therefore likely that the frozen tissue samples used for Western blotting contained non-neoplastic and/or necrotic tissue which would artificially skew tumour PRKAR1A expression levels.

The number of cases stained for IGF2 is too small to allow for definitive conclusions, however, the trend does suggest that high IGF2 expression is common in canine osteosarcoma. There does not appear to be an association between IGF2 staining intensity and tumour location. The IGF signaling pathway is a complex network of interacting ligands, receptors and binding proteins; other members of this pathway such as the IGF1R may prove to be more important in
breed size-related tumour site predilections in canine osteosarcoma, however further studies are necessary to pursue this hypothesis.

Based on our preliminary work, PRKAR1A has promise as a prognostic marker and therapeutic target. However, prospective studies including larger numbers of cases as well as studies into the mechanism by which PRKAR1A expression influences post-chemotherapy survival are needed to validate and expand on our results. Nevertheless, immunohistochemical screening for PRKAR1A in canine osteosarcoma patients may prove to be a promising clinical test to predict response to chemotherapy and targeting PRKAR1A therapeutically to reduce its expression may prolong the lifespan of dogs with this aggressive cancer.
**Chapter 3: Derivation and characterization of primary canine osteosarcoma and osteoblast cells and in vitro investigation of the role of PRKAR1A in chemosensitivity**

**Abstract**

Primary cell cultures and cell lines serve as valuable models of neoplastic disease in both humans and animals by enabling mechanistic *in vitro* and *in vivo* experimentation. To initiate an ongoing cell culture derivation and characterization project, primary cell cultures were derived from both canine osteosarcoma tissue and normal bone. Preliminary characterization of these cultures, including doubling time, BrdU incorporation, anchorage independent growth in soft agar and immunocytochemistry was undertaken and attempts were made to isolate clonal cell lines. Expression levels of the type 1 alpha regulatory subunit of cAMP-dependent protein kinase (PRKAR1A), a protein encoded by a tumour modifier gene, were assessed in several of these primary cultures using Western Blotting. PRKAR1A expression varied between cell cultures, but did not appear to be related to expression levels of phosphorylated cAMP response element-binding (phospho-CREB), a downstream target of cyclic AMP (cAMP)-dependent protein kinase A (PKA). Low PRKAR1A expression has been associated with a good response to chemotherapy in human osteosarcoma and preliminary experiments reported in Chapter 2 of this thesis identify a correlation between low PRKAR1A expression in canine osteosarcoma patients and significantly prolonged post chemotherapy survival times. Chemotherapeutic sensitivity of primary canine osteosarcoma cell cultures with variable innate PRKAR1A expression was compared using an MTT assay. Chemosensitivity to both carboplatin and
doxorubicin did not correlate with PRKAR1A expression, but faster growing cells with shorter
doubling times and higher rates of BrdU incorporation tended to be more chemosensitive. These
preliminary results suggest that the post-chemotherapy survival advantage in dogs with low
PRKAR1A expressing tumours may not involve downstream CREB phosphorylation or be
directly related to chemosensitivity at the cellular level.

Introduction

Canine osteosarcoma is a locally invasive and highly metastatic primary bone tumour
with a poor prognosis. The current standard of care for canine osteosarcoma patients is
amputation or surgical resection in combination with chemotherapy, usually either carboplatin or
doxorubicin. Although the addition of chemotherapy to surgical treatment increases survival in
these patients, median overall survival with standard of care treatment remains low at less than
one year (2) (13) (12). A better understanding of the molecular pathogenesis of canine
osteosarcoma may enable the development of new, and possibly more effective, molecularly
targeted therapies.

The R1alpha regulatory subunit of cyclic AMP (cAMP)-dependent protein kinase A
(PKA) (Prkar1a), has been identified as a weak tumour suppressor gene in mice (91) (90) (44)
and may play a similar role in humans with Carney Complex, an inherited syndrome of multiple
benign neoplasms often associated with Prkar1a mutations (41) (40). Prkar1a has also recently
been implicated in osteosarcoma tumourigenesis. Low Prkar1a expression in a murine
osteosarcoma model (MOTO mouse model) leads to more rapid osteosarcoma tumour
development. Human osteosarcoma patients with low Prkar1a expressing tumours respond better to chemotherapy than those with high Prkar1a expressing tumours as measured by percentage tumour necrosis (36). Recent data reported in Chapter 2 of this thesis correlates low PRKAR1A expression in canine osteosarcomas with significantly prolonged post-chemotherapy patient survival. The mechanisms behind these observations are poorly understood thus far, however, studying downstream PKA signaling may provide clues. Binding of cAMP to PRKAR1A results in the release of PKA catalytic subunits which phosphorylate downstream targets such as the transcription factor cAMP response element-binding (CREB). In the MOTO murine osteosarcoma model, tumours with low Prkar1a expression demonstrate increased PKA activity along with elevated phospho-CREB expression (36).

The first aim of this project was to initiate an ongoing derivation and characterization of cell cultures from primary and metastatic canine osteosarcoma tumours as well as normal bone to serve as models and tools in current and future experiments. Further to this aim, the isolation of genetically identical clonal cell populations from each primary cell culture was undertaken, thereby facilitating future genetic manipulations. The second aim was to assess the expression of PRKAR1A and phospho-CREB, a downstream target of PKA, in these cell cultures using western blots. The third aim was to exploit any identified innate variability in PRKAR1A expression in these cell cultures to investigate the role of PRKAR1A in chemosensitivity using an MTT assay to assess post-chemotherapy cell viability. The first hypothesis was that PRKAR1A expression would vary between primary cell cultures and would be inversely related
to phospho-CREB expression. The second hypothesis was that PRKAR1A expression would correlate with chemosensitivity, with increased post-chemotherapy survival in low expressing cells.

**Materials and Methods**

*Primary Cell Culture Derivation*

Both normal bone and osteosarcoma tumour tissue was collected from all fresh veterinary teaching hospital biopsy and necropsy canine osteosarcoma cases from June, 2009 until August, 2010. Sterile instruments were used and tissue explants were submersed in 70 % ethanol for 30 seconds, rinsed with PBS and incubated overnight at 4°C in PBS containing 10 times the cell culture concentration of gentamicin and fungizone after collection. Explants were minced into 1-2 mm diameter fragments and cultured in DMEM (osteosarcoma) or AMEM (normal bone) (Fisher Scientific, Waltham, MA) with 10% FBS (Invitrogen, Carlsbad, CA), 50 µg/mL gentamicin and 1 µg/mL fungizone (Fisher Scientific, Waltham, MA) at 37°C with 5 % CO₂ until cells growing out from explants reached 50-60 % confluency. The cells were then passaged twice, suspended in the above media with 5-10 % DMSO at a concentration of 1-5 x 10^6 cells/mL and frozen in liquid nitrogen.

One primary cell culture (JL-31TF) was derived from thoracic fluid collected immediately after euthanasia from a canine osteosarcoma patient with extensive pulmonary metastatic disease. The thoracic fluid was centrifuged for 5 minutes at 11,000 rpm and pelleted cells were washed three times in PBS. The cells were then suspended in DMEM media with 10
% FBS, 50 µg/mL gentamicin and 1 µg/mL fungizone, cultured at 37°C with 5 % CO₂, passaged twice and frozen as above.

**Clonal Isolation**

Cells were thawed and passaged twice before plating at densities of 10 cells per well, 1 cell per well and 1 cell per 10 wells in 96 well plates. Plates were examined bi-weekly for growth; cells in wells exhibiting growth were expanded to 24 well plates, 6 well plates and finally 25 cm² flasks. Cells derived from 10 cell per well plates were re-plated at 1 cell per well and 1 cell per 10 wells. Cells derived from 1 cell per well and 1 cell per 10 well plates were frozen in liquid nitrogen as described above.

**Characterization**

**Doubling Time**

Cells were grown to 80-90 % confluency in two 60 mm petri dishes and then subcultured into 9 dishes each for a total of 18 dishes. Cells were harvested and counted in triplicate every 8-48 hours, depending on their rate of growth, until cells in the remaining uncounted dishes reached 100 % confluency. Prism software was used to fit an exponential curve to the data and generate doubling times.

**Anchorage Independent Growth in Soft Agar**

Base agar media (0.5 %) was made by melting 1 % DNA grade agar in the microwave then cooling to 40°C in a water bath and mixing with an equal volume of warm (40°C) double strength AMEM with 20 % FBS and double the desired cell culture concentration of gentamicin
and fungizone. Three milliliters of base agar was added to each 60 mm cell culture dish, allowed to set and stored for up to a week at 4°C. Base agar media was warmed to room temperature for 30 minutes prior to addition of top agar media. Top agar media (0.35 %) was prepared in a similar manner using 0.7 % DNA grade agar. Cells were suspended in top agar media and plated in triplicate on top of the base agar at a density of 2,500 cells/mL; 3 mL of top agar were added to each dish. Top agar without cells was added to a single dish of base agar as a negative control for each assay. Dishes were incubated at 37°C with 5 % CO$_2$ for 10-14 days and fed 1-2 times per week by adding 0.75 mL of AMEM with 10 % FBS, 50 µg/mL gentamicin and 1µg/mL fungizone. Each dish was stained with 0.5 mL of 0.0005 % Crystal Violet for 1-2 hours at room temperature. Colonies were identified using a dissecting microscope.

**BrdU Labeling and Immunocytochemical Detection**

Cells were plated in quadruplicate at 50,000 cells per well on four well chamber slides and incubated overnight at 37°C with 5% CO$_2$. BrdU labeling reagent (Invitrogen, Carlsbad, CA) was filter sterilized and then diluted 1:100 in DMEM media with 10 % FBS, 50 µg/mL gentamicin and 1 µg/mL fungizone. Spent media was removed from the wells and replaced with BrdU labeling solution. Complete culture media without BrdU labeling reagent served as a negative control. Cells were then incubated for 2-17 hours, depending on their rate of growth, at 37°C with 5% CO$_2$. Slower growing cells were incubated for longer than faster growing cells. Cells were then gently rinsed with PBS, fixed in 80% ethanol for 20-30 minutes at room
temperature and rinsed with 1% Triton X-100 in PBS. Slides were stored at 4°C with PBS in the chambers.

To denature the cellular DNA, the fixed cells were incubated in 1N HCl for 10 minutes on ice, then 2N HCl for 10 minutes at room temperature followed by 20 minutes at 37°C. Cells were then incubated in 0.1M Borate buffer for 12 minutes at room temperature and finally rinsed with 1% Triton X-100 in PBS. To block endogenous peroxidase activity, cells were treated with 3% hydrogen peroxide for 30 minutes at room temperature. Cells were then rinsed with 1% Triton X-100 in PBS. After a one hour room temperature incubation in Serum Free Protein Block (Dako, Glostrup, Denmark), cells were incubated overnight with mouse anti-BrdU antibody (1:100; Invitrogen, Carlsbad, CA). Overnight incubation with primary antibody diluent alone at 4°C served as a negative control. Cells were then rinsed with 1% Triton X-100 in PBS prior to incubation with horseradish peroxidase conjugated rabbit anti-mouse secondary antibody (1:200 dilution; Dako, Glostrup, Denmark) for 45 minutes at room temperature. Following a final PBS rinse, the cells were treated with diaminobenzidine (DAB) (Dako, Glostrup, Denmark) for 10 minutes at room temperature and then rinsed with water. Cells were lightly counterstained with Gill’s hematoxylin #3 (Polysciences Inc., Warrington, PA) and coverslips were mounted with Cytoseal (Electron Microscopy Sciences, Fort Washington, PA). One hundred cells were counted per cell line/primary cell culture and the number of positively staining cells was divided by the BrdU label incubation time for that particular cell line/primary cell culture to give a BrdU index (% of cells going through S-phase per hour).
**Immunocytochemistry**

Cells were grown to 50-80 % confluency on coverslips. Cells were then rinsed with PBS, fixed in 10 % neutral buffered formalin for 10 minutes, rinsed again with PBS and finally incubated in 0.5 % Triton X-100 for 10 minutes. Cover slips were placed into 3 % hydrogen peroxide for 30 minutes at room temperature to block endogenous peroxidase activity. They were then rinsed in PBS prior to a 30 minute room temperature incubation with Serum Free Protein Block (Dako, Glostrup, Denmark) in a humidified chamber. Cover slips were then incubated overnight at 4°C with either anti-PRKAR1A (1:100 dilution; BD Transduction Laboratories, Franklin Lakes, NJ), anti-Osteocalcin (1:100; Invitrogen, Carlsbad, CA) or anti-Osteonectin (1:100; Invitrogen, Carlsbad, CA). Overnight incubation with primary antibody diluent alone at 4°C served as a negative control. After rinsing in PBS, coverslips were incubated at room temperature with horseradish peroxidase conjugated rabbit anti-mouse antibody (1:200 dilution; Dako, Glostrup, Denmark). After yet another PBS rinse, the slides were treated with diaminobenzidine (DAB) (Dako, Glostrup, Denmark) for 10 minutes at room temperature and then rinsed with water. Tissues were counterstained with Gill’s hematoxylin #3. Cover slips were dried and then mounted on microscope slides with Cytoseal (Electron Microscopy Sciences, Fort Washington, PA).

**Cell Western Blots**

Cells were grown to 80-90 % confluency in 75 cm² flasks then rinsed with cold PBS. RIPA lysis buffer with added anti-protease (10 % v/v) and anti-phosphatase (1 % v/v) was then
added (1 mL of lysis buffer per flask) and flasks were placed on ice for 5 minutes. Cell protein lysates were centrifuged at 14,000 x g for 10-15 minutes at 4°C and the supernatants were frozen at -80°C.

After thawing the protein lysates, total protein concentrations were quantified spectrophotometrically in a 96-well plate using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA).

Cell protein lysates were incubated for 15 minutes in a 70°C water bath to denature proteins prior to running them alongside a protein ladder through either a 10 or 12 % acrylamide:bisacrylamide gel at 100-150 volts for 2-3 hours. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Biorad, Hercules, CA) at 100 volts for 2-2.5 hours. Membranes were blocked with 5 % milk and then incubated with anti-PRKAR1A antibody (1:1000 dilution; BD Transduction Laboratories, Franklin Lakes, NJ) or anti-CREB antibody (1:1000 dilution; Cell Signaling, Danvers, MA) in 5 % milk or anti-phospho-CREB antibody (1:1000 dilution; Cell Signaling, Danvers, MA) in 5 % bovine serum albumin overnight at 4°C. After washing, the membranes were incubated at room temperature with either horseradish peroxidase conjugated rabbit anti-mouse antibody (1:10,000 dilution; Dako, Glostrup, Denmark) or swine anti-rabbit antibody (1:10,000 dilution; Dako, Glostrup, Denmark) respectively in 5 % milk for 45 minutes prior to chemiluminescent detection of protein bands using radiographic film. Membranes were then stripped prior to overnight incubation with anti-Beta-actin antibody (1:2,000 dilution; Cell Signaling, Danvers, MA) at 4°C. After washing, the
membranes were incubated at room temperature with horseradish peroxidase conjugated swine anti-rabbit antibody (1:10,000 dilution; Dako, Glostrup, Denmark) in 5 % milk for 45 minutes prior to chemiluminescent detection of protein bands using radiographic film. Probing for beta-actin served as a loading control.

Chemotherapy treatment and MTT Assay

D17 and primary osteosarcoma cells (JL-25, JL-32LM, JL-31TF, JL-36) were harvested at 80 % confluency, plated at 10,000 cells per well in 96 well plates and incubated overnight at 37°C with 5 % CO₂ before chemotherapy treatment. The last column of the plates was filled with media alone as a control. Spent media was removed and cells were treated in quadruplicate with incremental doses of Carboplatin (0 µM to 10,000 µM) and Doxorubicin (0 µM to 500 µM) diluted in cell culture media. After 72 hours of incubation with the chemotherapeutic agents, an MTT assay (Cell Titer 96 Non Radioactive Cell Proliferation Assay; Promega, Madison, WI) was performed to assess cell viability. Briefly, the media was changed in all wells prior to the addition of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye and a four hour incubation at 37°C with 5 % CO₂. Solubilization solution was then added to all wells to solubilize the formazan reaction product and the plates were incubated for 1 hour. The absorbance was measured at 570 nm and 630 nm using a spectrophotometric plate reader. Finally, the plates were incubated overnight and the absorbance readings were repeated at the same wavelengths. Overall absorbance was calculated as the difference between the readings at 570 nm and 630 nm.
Results

Primary Cell Culture Derivation and Clonal Isolation

Currently, we have accumulated 15 canine osteosarcoma primary cell cultures including three derived from pulmonary metastases, two of which can be paired with cells from the primary tumour in the same patient. Two primary cell cultures have been derived from canine chondrosarcoma tissue (JL-15), one slow growing and one fast growing variant. Eight canine primary cell cultures have been derived from non-neoplastic bone. Seven clones have been isolated from the fast growing JL-15 variant.

Characterization

Doubling Time

Doubling times ranged from 17.16 to 63.68 hours. Doubling time was shortest in the D17 commercially available canine osteosarcoma cell line (ATCC, catalog # CCL-183) followed by the JL-31TF and then JL-32LM cells, both derived from pulmonary metastases. Doubling time was longest in the JL-25 cells. Doubling times for these four cell populations correlate with empirical observations of their growth rates relative to one another.
**Figure 3-1.** Exponential growth curves and doubling times for D17, JL-31TF, JL-32LM and JL-25 cells. Doubling time is fastest for (A) D17 commercially available canine osteosarcoma cells and slowest for (D) JL-25 cells. Error bars represent SEM.
Growth in Soft Agar

At this point in time, only one of the primary cell cultures, JL-32LM from a pulmonary metastasis, has formed colonies when grown in soft agar. Colony formation was also observed after incubation of the D17 commercially available canine osteosarcoma cell line in soft agar (Figure 3-2). The D17 cells formed fewer colonies in soft agar than did the JL-32 LM cells.

Figure 3-2. (A) Numerous colonies of JL-32LM primary canine osteosarcoma cells growing in soft agar. (B) A few colonies of D17 cells also formed in soft agar.

BrdU Labeling and Immunocytochemical Detection

Four primary osteosarcoma cell cultures (JL-31TF, JL-32 LM, JL-25 and JL-36) have been labeled with BrdU for immunocytochemical staining to assess cell proliferation rate. Nuclear staining was not observed with omission of BrdU label or primary antibody incubation (Figure 3.3).
Figure 3-3. Immunocytochemistry of JL-31TF cells for BrdU. (A) Positive nuclear staining of actively dividing cells, (B) negative control with BrdU label omitted, (C) negative control with primary antibody omitted.

The BrdU index (% of cells going through S-phase per hour) was highest for the D17 commercially available canine osteosarcoma cell line (Figure 3-4), followed by the JL-31TF and JL-32LM cells, both derived from pulmonary metastases and finally the JL-36 and JL-25 cells, both derived from primary tumours (Table 3-1). BrdU indices for these five cell populations correlate with empirical observations of their growth rates relative to one another.
Table 3-1. Summary of BrdU incorporation rates (BrdU labeling indices per hour) for the D17 canine osteosarcoma cell line and selected primary canine osteosarcoma cell cultures. Cells are derived from osteosarcoma primary tumour tissue unless otherwise specified (TF= thoracic fluid, LM= lung metastasis).

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</tr>
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</tr>
<tr>
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<td>JL-25</td>
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Figure 3-4. Comparative immunocytochemical staining for BrdU in the (A) D17 cell line and (B) JL-31TF canine osteosarcoma primary cell culture. The D17 and JL-31TF cells were incubated with BrdU label for almost identical lengths of time (2.6 hours for D17, 2.7 hours for JL-31TF) and a greater proportion of D17 cells stain positively for BrdU over this time period indicating that D17 cells proliferate faster than JL-31TF cells.
Immunocytochemistry

Immunocytochemical staining for osteonectin and osteocalcin, two proteins expressed by osteoclasts, was undertaken to aid in identifying the lineage of our primary cell cultures. Ten of fourteen primary cell cultures assessed using immunohistochemistry stained positively for osteonectin (Figure 3-5) while only 2 of 14 stained positively for either osteocalcin or PRKAR1A. The D17 cell line exhibited positive staining for only osteonectin. One primary cell culture (JL-35) was positive for all three markers (Table 3-1).

![Image A](image1.png) ![Image B](image2.png)

**Figure 3-5.** Immunocytochemistry for osteonectin, (A) positive cytoplasmic immunostaining of JL-18 cells and (B) corresponding negative control cover slip.
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**Table 3-2.** Summary of immunocytochemistry on the D17 canine osteosarcoma cell line and multiple primary canine osteosarcoma cell cultures. Cells are derived from osteosarcoma primary tumour tissue unless otherwise specified (NB= normal bone, TF= thoracic fluid, LM= lung metastasis).
**Cell Western Blots**

PRKAR1A expression varied between primary cell cultures. JL-31TF demonstrated consistently high Prkar1a expression and JL-25 demonstrated consistently low PRKAR1A expression. PRKAR1A expression in JL-13 cells was inconsistent, both low and intermediate levels were detected. The remainder of the canine osteosarcoma primary cell cultures showed intermediate PRKAR1A expression (Figure 3.3). Phospho-CREB expression was detected in the D17 commercially available canine osteosarcoma cell line and at very low levels in both the JL-31TF and JL-32LM cells (Figure 3.4, A). CREB expression was also detected in the D17 cell line as well as the JL-31TF cells (Figure 3.4, B). Phospho-CREB expression did not correlate with PRKAR1A expression.
Figure 3-6. Western blot for PRKAR1A (49 kD). All wells represent primary canine cell cultures from osteosarcoma tumours except 15 (JL-15) which is from a chondrosarcoma and D17 which is a commercially available canine osteosarcoma cell line. Two of the cell cultures were derived from pulmonary metastases (31T = JL-31TF and 32L = JL-32LM). Probing for Beta-actin (45 kD) was performed as a loading control. PRKAR1A expression varied between different cell cultures and also within the same cell culture in one instance (JL-13); JL-15 and JL31TF were high expressing, JL-25 was low expressing, JL-13 demonstrated inconsistent expression (both low and intermediate) and the remainder showed intermediate expression. Faint 49 kD bands for JL-15, JL-31TF and JL-32LM with probing of the upper membrane for Beta-actin result from incomplete stripping of the membrane after probing for PRKAR1A.
Figure 3-7
Figure 3-7. Western blots for (A) Phospho-CREB (43 kD) and (B) CREB (43 kD). The wells represent primary canine osteosarcoma cell cultures except for D17 which is a commercially available canine osteosarcoma cell line and PymT which is a cell line from a mouse mammary adenocarcinoma derived from a transgenic mouse with mammary gland specific polyomavirus middle T oncogene expression. Two of the cell cultures were derived from pulmonary metastases (31T = JL31TF and 32L = JL-32LM). The murine cell line was run as a known positive control and probing for Beta-actin (45 kD) was performed as a loading control. Phospho-CREB expression was only detectable in the D17, JL-31TF, JL-32LM and murine cells with the uppermost band in each well running at approximately 43 kD. CREB was detectable in the D17, JL-31TF and murine cells with the lower D17 band running at approximately 43 kD. The single approximately 80 kD band within the D17 well with probing for CREB most likely represents non-specific binding of the primary antibody. Multiple 30-37 kD bands detected in the D17 and mouse positive control wells for both CREB and phospho-CREB most likely represent protein degradation products.

Chemotherapy Treatment and MTT Assay

Chemosensitivity of the primary cell cultures and the D17 cell line varied, but did not correlate with PRKAR1A expression (Figure 3.8). Instead, chemosensitivity tended to be correlated with doubling time and BrdU incorporation; faster growing cells with shorter doubling times and higher rates of BrdU incorporation tended to be more chemosensitive than slower growing cells with longer doubling times and lower rates of BrdU incorporation (Figure 3-9).
Figure 3-8

A  Carboplatin Chemosensitivity

B  Doxorubicin Chemosensitivity
Figure 3-8. Logarithmic dose response curves of % cell viability with increasing (A) carboplatin and (B) doxorubicin concentrations in µM, comparing the relative chemosensitivity of the commercially available canine osteosarcoma cell line (D17) and four primary osteosarcoma cell cultures, two of which were derived from pulmonary metastases (JL-32LM and JL-31TF). The IC₅₀ values for carboplatin are as follows: JL-31TF=109.7 µM, D17=123.9 µM, JL-32LM=266.8 µM, JL-25=325.6 µM and JL-36=349.7 µM. The IC₅₀ values for doxorubicin are as follows: D17=1.29, JL-32LM=1.53, JL-31TF=2.75, JL-25=10.00 and JL-36=10.00. Error bars represent SEM.
Figure 3-9. Linear regression graphs plotting cell proliferation rate as measured by (A and B) doubling time and (C and D) BrdU incorporation versus chemosensitivity. Chemosensitivity tends to be positively correlated with proliferation rate.
Discussion

To date we have derived a large collection of primary canine osteosarcoma and normal osteoblast cell cultures. Our attempts at isolating clonal cell populations have been unrewarding for the most part as growth of our primary cells is generally very slow when the cells are plated at very low densities. Irradiated, non-multiplying, but still metabolically active, fibroblast cells can be used as feeder layers to promote the growth of co-cultured cells via secretion of growth factors. Repeating the clonal isolation procedure using a fibroblast feeder layer may prove more successful in the future.

Characterization of primary canine osteosarcoma and normal osteoblast cell cultures is an ongoing project. Growth in soft agar is a commonly used assay to detect malignant transformation based on the premise that non-neoplastic cells generally require a solid surface for growth. As might be predicted, the D17 commercially available canine osteosarcoma cell line formed colonies in soft agar, a testament to its recognized neoplastic phenotype. The JL-32LM primary cell culture also formed colonies in soft agar, providing strong initial evidence that it is composed of neoplastic cells.

Immunocytochemical characterization has provided few clues as to the identity of the primary cell cultures. Osteocalcin is a relatively specific osteoblast marker and is expressed by osteoblasts late in differentiation, but can also be expressed by chondrocytes, odontoblasts, cementoblasts and cementocytes. Positive osteocalcin immunostaining therefore suggests that the JL-25 and JL-35 cells are most likely either normal or neoplastic osteoblasts. Primitive
neoplastic osteoblasts may not be sufficiently differentiated to express osteocalcin; negative osteocalcin immunostaining of many of our primary cell cultures is therefore less informative. Osteonectin is a glycoprotein secreted by osteoblasts which links collagen to mineral in bone matrix, but can also be expressed by fibroblasts in the context of tissue repair and remodeling (93) and by neoplastic cells of non-osteoblastic lineage where it is thought to play a role in cell-matrix interactions and the deposition of extracellular matrix (94). Positive staining in the majority of our cell cultures is therefore not a particularly discriminating result. The preponderance of negative immunocytochemical staining for PRKAR1A was surprising considering that PRKAR1A expression was detected in all of the cell cultures tested using western blotting. One possible explanation is that immunocytochemistry is a less sensitive detection method for PRKAR1A expression than western blots. PRKAR1A expression could also be temporally unstable, varying over time within a given primary cell culture, or heterogeneous within a given primary cell culture, differing between different subpopulations. Variations in PRKAR1A expression were detected by western blot between different populations of JL-13 cells which were derived from the same primary canine osteosarcoma tumour and may also reflect such temporal instability or spatial heterogeneity in PRKAR1A expression.

The BrdU indices may be somewhat difficult to interpret in isolation. Due to the wide range of doubling times calculated for the different primary cell cultures, these different cell cultures were each incubated with BrdU for different lengths of time instead of for a constant short time period. Labeling for variable lengths of time resulted in a reasonable number of
positively staining cells for manual counting, even in the slow growing cell cultures, thus minimizing counting error. However, over a long incubation period, it is possible that some proportion of cells could have passed through S phase of the cell cycle more than once, leading to an overestimation of the actual proliferation rate of the primary cell cultures. Although the BrdU indices may not be quantitatively accurate as calculated, they likely give a qualitative estimate of proliferation rate as evidenced by their correlation with doubling times and empirical growth rate assessments.

Ultimately, establishing the nature of the cells and their usefulness for future experimentation will rely on the results of many different tests and, although preliminary testing has begun, several key tests are still pending. In the near future, canine osteosarcoma cells will be injected into immunodeficient mice to test their tumorigenicity. Genetic stability will also be assessed using array comparative genomic hybridization.

In the MOTO mouse model of osteosarcoma, decreased Prkar1a tumour expression was associated with increased downstream phosphorylation of CREB by PKA. CREB expression was detected at very low levels in only one of our primary canine osteosarcoma cell cultures and phospho-CREB was detected at even lower levels in only two of our primary canine osteosarcoma cell cultures. The barely detectable or non-existent levels of phospho-CREB expression in our primary cell cultures, even those with low PRKAR1A expression, can therefore be explained by a paucity or complete lack of CREB substrate. The majority of the CREB detected in the D17 cell line exists in a phosphorylated state as phospho-CREB. Intermediate
levels of PRKAR1A may be sufficiently low to result in increased PKA activity and downstream CREB phosphorylation in this cell line.

The innate variability in PRKAR1A expression between different primary osteosarcoma cell lines was exploited to investigate the role of PRKAR1A in chemosensitivity *in vitro* using an MTT assay. Interestingly, chemosensitivity was not correlated with PRKAR1A expression, but with cell proliferation rate as measured by doubling time and BrdU incorporation. This result is not unexpected since, in general, chemotherapy drugs target actively dividing cells; rapidly dividing cells should therefore be more chemosensitive than slowly dividing cells. However, it does not explain the results reported in Chapter 2 of this thesis which showed significantly prolonged post-chemotherapy survival in canine osteosarcoma patients with low PRKAR1A expressing tumours. Use of a colony formation assay to assess *in vitro* chemosensitivity may have provided different results as it is arguably a more biologically relevant test. The MTT assay measures cell survival after chemotherapeutic treatment whereas the colony formation assay assesses the ability of cells that survive chemotherapy treatment to replicate in culture. The MTT assay was chosen for use in this study for its relative rapidity and also because of the poor colony forming ability of the slower growing canine osteosarcoma primary cell cultures. It is also possible that the canine osteosarcoma primary cell cultures do not represent the heterogeneous population of cells existing within the primary tumour from which they were derived; tissue culture conditions may have selected for cells with differing chemosensitivity than the tumour as a whole.
General Discussion and Further Work

A subpopulation of canine osteosarcoma patients with low PRKAR1A expressing tumours and significantly prolonged post-chemotherapy survival was identified. Based on this result, canine osteosarcoma cells with low PRKAR1A expression were hypothesized to be more sensitive to chemotherapy treatment \textit{in vitro} than those with high PRKAR1A expression. However, initial experiments exploiting innate variability in PRKAR1A expression between different canine osteosarcoma primary cell cultures failed to find a correlation between PRKAR1A expression and chemosensitivity. There are many factors other than PRKAR1A expression that could potentially affect chemosensitivity and explain, at least in part, these conflicting results. One is the phase of cell growth \textit{in vitro}. Cells in the plateau phase of growth tend to be more resistant to chemotherapy than those in the exponential phase of growth (95). Despite efforts to treat cells while they were growing exponentially, it is possible that different cells were treated during slightly different growth phases due to the complicating factor of highly variable cell proliferation rates.

The tumour microenvironment, particularly decreased oxygen concentration and decreased pH, can also negatively affect the sensitivity of tumour cells to chemotherapeutic agents. Hypoxia and acidosis are common in solid tumours as a result of imbalances in oxygen delivery and consumption which are related to disorganized and ineffective vasculature as well as increased tumour cell metabolism and rapid tumour growth (96) (97). There are multiple mechanisms underlying the chemoresistance observed in hypoxic and acidic tumour
microenvironments. These range from decreased drug delivery by disorganized microvasculature and decreased uptake of basic drugs in an acidic extracellular microenvironment to hypoxia inducible factor 1 (HIF-1) transcription factor mediated downregulation of pro-apoptotic proteins and upregulation of drug efflux transporter proteins such as P-glycoprotein (98) (99) (100). Therefore, in the absence of the influences of the microenvironment, the in vitro results may not accurately reflect clinical chemosensitivity.

Specifically, cells with high PRKAR1A expression may be more resistant to chemotherapy in vivo than in vitro due to the effects of the tumour microenvironment which are unrelated to PRKAR1A expression. Furthermore, although histologic examination of canine osteosarcoma clinical cases does not suggest a relationship between PRKAR1A and tumour vasculature, it is also possible that PRKAR1A expression directly alters the tumour microenvironment in such a way as to increase clinical chemosensitivity without affecting the response of individual cells to chemotherapy treatment in vitro. Low PRKAR1A expression could be involved in regulating or normalizing tumour vasculature and thereby improving both drug delivery to neoplastic cells and blood flow to relatively slowly dividing nutrient deprived cells in vivo, an effect that would not be measurable in an in vitro setting. This possibility could be explored in the future using stable PRKAR1A knockdown in mouse xenograft tumours by assessing tumour blood flow with Doppler imaging and vascular density with immunohistochemical staining of endothelial cell markers.
Other potential explanations for the disparity observed between *in vivo* and *in vitro* responses to chemotherapy treatment may involve the characteristics and identity of our primary cell cultures. In chapter 3, variability in PRKAR1A expression between different populations of JL-13 cells derived from the same osteosarcoma tumour was documented. Due to prohibitively low cell numbers, PRKAR1A expression was not measured in the cells subjected to chemotherapy treatment immediately prior to this treatment and therefore we cannot rule out similar PRKAR1A expression variability in these cells. Furthermore, our primary cell cultures have not been completely characterized. Non-neoplastic osteoblasts and fibroblasts are less sensitive to chemotherapeutic agents than neoplastic osteoblasts due to their comparatively slow proliferation rate. PRKAR1A is ubiquitously expressed and during the retrospective immunohistochemical examination of PRKAR1A expression, low to moderate intensity positive cytoplasmic immunostaining for PRKAR1A in fibroblasts and osteoblasts was noted. It is therefore possible that cells such as JL-25 which are relatively chemoresistant despite low PRKAR1A expression are actually non-neoplastic.

Contrary to what has been reported in cells derived from MOTO mouse osteosarcoma tumours (36), preliminary investigation of PRKAR1A downstream signaling in canine osteosarcoma primary cell cultures did not demonstrate increased phosphorylation of CREB by PKA in association with low PRKAR1A expression. CREB expression was almost non-existent in our primary cell cultures and therefore unavailable for phosphorylation. The post-chemotherapy survival advantage associated with low expression of PRKAR1A in canine
osteosarcoma tumours may therefore be mediated by phosphorylation of another more plentiful, and as yet unidentified, protein. The fact that the mechanism behind this survival advantage remains elusive should not discourage the potential use of PRKAR1A expression as a prognostic marker in canine osteosarcoma. If further large scale prospective studies of canine osteosarcoma patients corroborate our findings, then immunohistochemical assessment of PRKAR1A expression would provide valuable prognostic information especially for pet owners. Owners of canine osteosarcoma patients with low PRKAR1A expressing tumours might be more likely to pursue chemotherapy for their dog despite side effects and financial expenditure based on the predicted increased post-chemotherapy survival time associated with low expression of this marker.

Greater understanding of PRKAR1A downstream signaling in the future will increase the number of associated targets available for therapeutic intervention; however, solely based on the results herein, potential novel therapeutics can be proposed. PRKAR1A is a negative regulatory subunit of PKA and binding of cAMP to PRKAR1A triggers the release of PKA catalytic subunits, thus increasing PKA activity (101). One potential therapeutic strategy would be to use gene therapy to either decrease the expression of the gene encoding PRKAR1A via delivery of genes encoding microRNA targeted against Prkar1a or to increase the expression of PKA catalytic subunits via delivery of genes encoding these subunits. Gene therapy has traditionally resulted in nonspecific whole body gene expression, however, recently the use of a C-X-C chemokine receptor type 4 (Cxcr4) promotor has enabled tumour specific transgene expression
with systemic therapy (102). CXCR4 expression has been associated with metastatic progression and decreased overall survival in human osteosarcoma (103) (104). Use of this promotor might therefore facilitate therapeutic targeting of metastases in patients with aggressive disease.

Drugs could also potentially target molecules upstream of PKA. The level of PKA activity within a given cell is controlled in part by negative regulation of cAMP levels by phosphodiesterases (105). Therapeutic interference with this enzymatic negative regulation would increase cAMP levels resulting in increased PKA activity. One potential problem with therapeutic targeting of cAMP is that, as a ubiquitous second messenger, the effects of upregulation would likely be pleiotropic, leading to alterations in many intracellular signaling pathways.

The preliminary results presented in this thesis prompt additional experiments. Further characterization of our primary osteosarcoma cell cultures is necessary in order to confirm their neoplastic phenotype. Tumourigenic potential of the cell cultures will be assessed by injecting them into immunodeficient mice and the genomic stability of the cell cultures will be assessed using aCGH. Pending the results of this characterization, specific cell cultures will be selected to serve as *in vitro* models of canine osteosarcoma, allowing for the generation of *in vivo* murine xenograft models of canine osteosarcoma and generally providing tools for future mechanistic studies.
One of these studies will focus on further investigating the mechanisms behind the post-chemotherapy survival advantage associated with low PRKAR1A expressing canine osteosarcoma tumours. To this date, differences in chemosensitivity between different primary canine osteosarcoma cell cultures with high versus low innate PRKAR1A expression have not been found. However, these different cell cultures are genetically heterogeneous and vary markedly in proliferation rates, both of which confound interpretation of these results. A stable knockdown of PRKAR1A in one or a few of our primary canine osteosarcoma cell cultures will therefore be established using shRNA technology in order to control for these confounding variables. This will allow for examination of the effect of variations in PRKAR1A expression within the same primary cell culture on chemosensitivity to doxorubicin and carboplatin, two commonly used drugs in the treatment of canine osteosarcoma, in vitro, and also possibly in vivo using a mouse xenograft model. Successful subcutaneous and intramuscular murine xenotransplantation of canine osteosarcoma cells has been reported (106) (107). The generation of an orthotopic mouse model via intra-femoral injection of canine osteosarcoma cells may also be attempted in an effort to better mimic spontaneously developing appendicular tumours. If these further experiments demonstrate an increase in chemosensitivity with knockdown of PRKAR1A, then the expression of molecules classically associated with variations in chemosensitivity such as P-glycoprotein and glutathione S-transferase could be investigated.
Summary and Conclusions

Canine osteosarcoma is clearly a disease for which a better understanding of the molecular pathogenesis would aid in developing new treatment strategies. The identification of critical molecules, receptors or pathways involved in canine osteosarcoma tumourigenesis through mechanistic studies will hopefully enable the development and use of molecularly targeted drug therapy such as monoclonal antibodies and small molecule inhibitors.

Our retrospective study of PRKAR1A expression has identified a small population of low PRKAR1A expressing canine osteosarcoma patients with significantly longer post-chemotherapy survival. This survival advantage appears to be unrelated to tumour proliferation rate and preliminary in vitro experiments suggest that it may not be directly related to chemosensitivity. Further studies, including larger prospective clinical studies, are required to confirm this promising preliminary finding and elucidate the mechanisms underlying the effects of PRKAR1A expression on survival in canine osteosarcoma patients. Corroborating evidence may eventually lead to the use of PRKAR1A immunohistochemical staining to predict treatment outcome in canine osteosarcoma patients and/or the future development of treatments inhibiting PRKAR1A expression.
References


45. Pavel E, Nadella K, Towns WH, Kirschner LS. Mutation of prkar1a causes osteoblast neoplasia driven by dysregulation of protein kinase A. Molecular Endocrinology 2008;22(2):430.


## Appendix 1

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<td>33.4</td>
<td>AP</td>
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<td>NA</td>
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<td>Mitotic Index</td>
<td>Prkar1a</td>
<td>IGF2</td>
<td>Survival (days)</td>
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<td>AP</td>
<td>S, C (Doxo)</td>
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<td>AX</td>
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<td>Site</td>
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<td>Prkar1a</td>
<td>IGF2</td>
<td>Survival (days)</td>
</tr>
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<td>S</td>
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<td>AP</td>
<td>S</td>
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<td>AX</td>
<td>S</td>
<td>6</td>
<td>patchy</td>
<td>high</td>
<td>43*</td>
</tr>
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<td>St. Bernard</td>
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<td>AP</td>
<td>S, R</td>
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**Appendix 1.** Summary of canine osteosarcoma cases stained for PRKAR1A and IGF2. Cases highlighted in blue were included in the PRKAR1A post-chemotherapy survival curve. (m = male, mc = male castrated, f = female, fs = female spayed, AP = appendicular, AX = axial, S = surgical resection, C = chemotherapy, R = radiation, Carbo = carboplatin, Doxo = doxorubicin, NR = not recorded, NA = not applicable, * = censor)