

INVESTIGATING CAUSAL BIOLOGIC MECHANISMS INVOLVED IN DIFFERENTIAL
SERUM BONE-SPECIFIC ALKALINE PHOSPHATASE ACTIVITIES IN CANINE
APPENDICULAR OSTEOSARCOMA

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in VMS- Veterinary Clinical Medicine
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2015

Urbana, Illinois

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ABSTRACT

In dogs with osteosarcoma (OS), pre-treatment serum bone alkaline phosphatase (BALP) activity is recognized as a prognostic factor; however, biologically-linked causation for this clinical observation requires further exploration. Recent data demonstrated that BALP correlates with OS burden in dogs; yet, every dog with advanced macroscopic disease will not present with BALP elevations, indicating additional factors contribute to BALP activities. Given that BALP exists as a glycosylphosphatidylinositol (GPI)-anchored membrane protein and serves as an osteoblast differentiation marker, the purpose of this study is to investigate alternative biologic mechanisms that could explain an elevation in BALP in dogs with OS. We hypothesize that 1) canine osteosarcoma cells will possess the endothelin (ET)-1/endothelin A receptor (ET_AR) signaling pathway; 2) blockade of endothelin signaling will attenuate pro-tumorigenic activities in canine OS cells; 3) blockade of endothelin signaling will attenuate BALP activity; and 4) serum BALP activity in naturally-occurring OS-bearing dogs will correlate with tumors that are more osteoblastic in nature than osteolytic as measured via DEXA scan.

The expression of the endothelin axis in canine OS cells was investigated by demonstrating protein expression by western blot analysis. The effects of ET_AR antagonism using ABT-627 on canine OS cells was investigated by characterizing perturbations in 1) cell proliferation by colony forming assays; 2) cell survival using changes in phosphorylation of Akt pathway and Jun/AP-1 pathway by western blot analysis; 3) cell migration by quantitative analysis; 4) BALP activity by cytochemistry; and 5) BALP activity via ELISA and corresponding western blot analysis. Naturally-occurring OS-bearing patients were utilized to determine if serum BALP activity correlated with osteoblastic tumors as determined via DEXA scan.

The endothelin axis is expressed in canine OS cells indicating a possible autocrine/paracrine signaling pathway. Exposing canine OS cells to ABT-627 results in decreased proliferation, survival, and migration of canine OS cells *in vitro*. Exposure to ABT-627 also resulted in decreased BALP expression and activity in canine OS cells. Upper and lower quartile relative bone mineral densities, when accounted for tumor size, correlate with BALP with a propensity for more osteoblastic tumors to have an elevation in BALP. In conclusion, the endothelin axis is expressed in canine OS and, upon attenuation of the pathway, tumor-derived growth and survival advantages are inhibited and BALP expression and activity is decreased. Clinically, patients with more osteoblastic tumors as measured via DEXA scan, have elevated serum BALP activity. This study's findings are the first to provide a link between increased BALP activity and a possible underlying biologic mechanism explaining the poor prognosis associated with this biomarker.

ACKNOWLEDGEMENTS

Most importantly, I would like to sincerely thank my Master's research supervisor, committee head, and long-time mentor, Professor Tim Fan. His support and guidance throughout my entire veterinary career have had a great influence on all of my professional achievements. The completion of this project would not have been possible without him and his commitment to me, especially during periods of diminished morale. Thank you, Tim.

I would also like to thank my research committee members, Professors Laura Selmic and Matt Stewart. Your time, support, and interest in my project are greatly appreciated.

To the members of the Comparative Oncology Research Laboratory who helped with the technical aspects of my project including Holly Pondenis, Dr Katie Wycislo, Mark Byrum, and Alison Masyr, you were all extremely helpful in the development of this project.

Of course, I must express extreme gratitude towards all of the members of the Cancer Care Clinic including Professors Laura Garrett and Jackie Wypij, my oncology resident mates (Drs Roberta Portela, Alycen Lundberg, and Corrine Camero), and the oncology technicians. Thank you for cheering me on and providing endless encouragement.

Finally, I want to express my appreciation to my family, especially my father and mother, who have made me into the man I am today with their infinite love and support.

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CHAPTER 1

INTRODUCTION

Elevated serum alkaline phosphatase is a well-documented negative prognostic factor in both dogs and humans with appendicular osteosarcoma (OS) resulting in shorter disease-free intervals and survival times (1–9). Found on both normal and malignant osteoblasts, bone alkaline phosphatase (BALP) has been identified as a necessary factor for the initiation of mineralization; however, it is not required for continuation of the process (10–12). Serum BALP activity is an index of osteoblastic bone formation with increased measurements in certain human cancers that are characterized by osteoblastic bone metastasis, mainly prostatic cancer (13–19). The precise function of BALP in bone remains to be completely identified as well as the exact biologic explanation for its poor prognostic association with OS bearing patients. Elucidation of the biologic mechanism for its association with a poor prognosis may help to identify novel therapies for the adjuvant treatment of OS in an effort to increase survival and quality of life in these patients.

In humans, and more recently in dogs, a correlation between serum BALP and tumor burden has been identified indicating that larger primary tumors or patients with metastatic disease dictate serum BALP levels, and thus a shorter metastasis-free survival and overall survival (20–24). Clinically, this link between serum BALP and tumor burden is not always evident. Some patients with a great degree of tumor burden, through primary tumor size or presence of metastases, do not show an elevation in serum BALP indicating that there are likely other biologic mechanisms, in addition to tumor size, that contribute to an elevated serum BALP in dogs with OS.

Endothelin-1 (ET-1) is a potent peptide, originally discovered to be expressed by vascular endothelial cells, that has been shown to play a pivotal role in osteoblastic disease processes, mainly through mediating cell differentiation and migration of bone cells (25–28). Endothelin-1 inhibits osteoclasts from resorbing bone, stimulates osteoblast proliferation, and increases expression of osteopontin, osteocalcin, and bone alkaline phosphatase, resulting in promotion of bone formation (29–32). These effects are mediated through endothelin A receptor (ET_AR) and are attenuated through antagonism of this receptor. Selective antagonists of ET_AR are able to block the proliferative and survival effects of ET-1 on osteoblasts and prostate cancer cells (33–38).

The purpose of this study was to investigate additional biologic mechanisms, aside from tumor size, elucidating the prognostic relevance of serum BALP activities in appendicular OS-bearing dogs. The objectives of this study were 1) to identify expression of the endothelin axis in canine OS cells; 2) to evaluate pro-tumorigenic activities *in vivo* of canine OS cell lines following exposure to various concentrations of exogenous ET-1 and endothelin receptor antagonism with ABT-627; 3) to determine the effects of endothelin A receptor antagonism on BALP activity *in vitro*; and 4) to evaluate the relationship between serum BALP activities and relative bone mineral density (rBMD) measured via dual energy x-ray absorptiometry (DEXA) scan. We hypothesized that 1) canine OS cells will possess the ET-1/ET_AR signaling pathway; 2) blockade of endothelin signaling will attenuate pro-tumorigenic activities in OS cells including survival and migration pathways; 3) blockade of endothelin signaling will attenuate BALP activity; and 4) serum BALP activity in naturally-occurring OS-bearing dogs will correlate with tumors that are more osteoblastic in nature than osteolytic as measured via DEXA scan.

CHAPTER 2

LITERATURE REVIEW

2.1. Bone alkaline phosphatase

2.1.1. Origin of alkaline phosphatase

Alkaline phosphatase (ALP) consists of a group of heterogeneous isoenzymes involved in the hydrolysis of monophosphate esters at an alkaline pH (39). There are three measurable isoforms of ALP found in the serum of dogs, including liver, bone (BALP), and corticosteroid isoforms, among other immeasurable isoforms due to extremely short half-lives (39–41). The corticosteroid isoform is coded by the intestinal ALP gene and the liver and bone isoforms are coded by the same gene, the tissue non-specific ALP gene (42). Post-translational modifications, mainly glycosylation, result in these different isoforms of the tissue non-specific isoenzyme of ALP (43).

2.1.2. Function and attachment of BALP via a GPI-anchor

Bone alkaline phosphatase, the bone-specific isoform of ALP, is localized to the outer membrane of normal and malignant osteoblasts via a hydrophobic glycosylphosphatidylinositol (GPI) anchor and is necessary for the initiation of mineralization (10–12,44–46). Release of BALP into circulation, where it is measurable, involves enzymatic cleavage of this GPI-anchor which can be performed by various GPI-specific phospholipases (47–52). Bone alkaline phosphatase activity in serum serves as a biomarker of the rate of osteoblastic bone formation in

mammals (18,53); however, its precise function in bone biology and pathobiology is still undetermined.

Glycosylphosphatidylinositol-anchors are a class of lipid anchors involved in the covalent linkage of proteins, including enzymes, receptors, cell adhesion molecules, and differentiation antigens to the outer leaflet of the plasma membrane of eukaryotic cells (45,49,54). These anchors were first described in studies investigating membrane-bound proteins including tissue non-specific alkaline phosphatase (55), the Thy-1 antigen of rodent thymocytes and neurons, and variant surface glycoproteins (VSG) of *Trypanosoma brucei* (45,56). Because of the tremendous amount of VSGs/organism, attached to the plasma membrane by a GPI-anchor, the African trypanosome was the perfect model for investigation of the structure of the anchor. The core structure of GPI-anchors was determined to be composed of a tetrasaccharide which is glycosidically linked to the 6-hydroxyl group of phosphatidylinositol (PI) on one end and linked via a phosphodiester bond to ethanolamine phosphate. Finally, this ethanolamine phosphate head attaches to the carboxyl-terminal residue of the attached extracellular protein (45), (Figure 2.1).

Synthesis and attachment of the GPI-anchor to proteins occur in the endoplasmic reticulum (ER), with subsequent post-translational modifications being processed in the Golgi apparatus. First, the protein is attached to the ER membrane and the N-terminal leader peptide is split with a peptidase. The carboxyl-terminal of this resulting protein is then bound to the lumen side of the ER membrane and translocated to the site of transamidase complex and presence of the GPI-precursor via the chaperon protein, binding immunoglobulin protein. Through the actions of transamidase complex and processing of the carboxy-termini, the ethanolamine head of the GPI precursor is able to attach to the protein rendering a GPI-anchored protein that is ready for transfer through the Golgi apparatus (57). In the transport process from the ER to the

plasma membrane, GPI-anchored proteins traverse through the *trans*-Golgi network in glycosphingolipid (GSL)-associated forms, or rafts (58–60), (Figure 2.2).

Revelation of the structure and synthesis of this anchor allowed researchers of multiple disciplines to document the presence of over 100 GPI-anchored proteins. These proteins most notably include acetylcholinesterase, exofacial ectoenzymes such as 5'-nucleotidase (5'-NTase), and tissue non-specific ALP (61). Variations in the structure of GPI-anchors of varying membrane-bound proteins were discovered demonstrating species and tissue specificity of these proteins (49). These variations in structure were later discovered to induce resistance from cleavage by various enzymes involved in release of these GPI-anchored proteins (45,62–64).

2.1.3. Release of GPI-anchored proteins

Glycosylphosphatidylinositol-anchors are susceptible to cleavage by various phospholipases including bacterial phosphatidylinositol-phospholipase C (PI-PLC), GPI-specific phospholipase C (GPI-PLC), and GPI-specific phospholipase D (GPI-PLD) (47,50–52,65–69). Phospholipases are enzymes that hydrolyze various bonds within phospholipids into fatty acids and lipid products (Figure 2.3). Glycosylphosphatidylinositol-specificity indicates that activity is directed specifically towards the GPI-anchor with little to no activity towards other phospholipids (45). If released by the activity of a phospholipase, an anchorless form of BALP would be expected to be found in serum; however, *in vitro* studies have identified an anchor-intact, insoluble form that is more commonly released from osteoblasts (70). *In vivo*, BALP circulates in an anchorless form indicating that the released anchor-intact form must be converted to the anchorless form that is soluble and found in circulation. Thus, multiple

pathways for the release of BALP and subsequent entrance into circulation have been accepted including release of an anchorless form into circulation via phospholipases, release of an anchor-intact form which could be generated by membrane fragmentation or matrix vesicle formation, or a combination of the two (71–73).

2.1.3.1. Release of an anchorless form via phospholipases

Phospholipases are divided into four different classes based on the site of catalytic activity with further subdivision into families within each class. Phospholipase A (PLA) is divided into two families, phospholipase A1 (PLA1) and phospholipase A2 (PLA2). These phospholipases hydrolyze the acyl group attached to different positions of the glycerophospholipids liberating free fatty acids and lysophospholipids. Phospholipase B is used to describe a phospholipase with both PLA1 and PLA2 activities (Figure 2.4). Neither of these phospholipases has been shown to be involved in cleavage of GPI-anchors, likely due to the intra-membranous localization of the cleavage site. Phospholipase C (PLC) and phospholipase D (PLD) are both considered phosphodiesterases, enzymes with activity towards phosphodiesterase bonds such as in the GPI-anchor. Phospholipase C cleaves the polar head phosphate from glycerophospholipids resulting in the production of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3) which continue signal transduction through the activation of protein kinase C (PKC) and intracellular calcium release (74–76), (Figure 2.5). Phospholipase D cleaves the terminal phosphodiester bond of the glycerophospholipid near attachment to the cellular membrane between the inositol and the phosphate of the structure (45).

Initially, it was found that cleavage of GPI-anchors was inducible with bacterial PLC (77). An endogenous form of PLC was then discovered in several mammalian cells and tissues where GPI-anchored proteins are expressed which was then termed GPI-PLC due to its similarity to a membrane-associated GPI-PLC found in the parasite *Trypanosoma brucei* (78,79). Due to its low circulating plasma levels, however, it is suspected that GPI-PLC does not have much activity *in vivo* (80). Another difficulty with this specific phospholipase entails its cellular location. In immunogold labeling studies, it was determined that GPI-PLC is located on the cytoplasmic surface of intracellular vesicles. This localization within the cell complicates proposed involvement in cleavage of an extracellular target (81). Alternatively, this phospholipase could be involved in cleavage of the GPI-anchor following its assembly in the endoplasmic reticulum, prior to attachment outside of the cell, leading to formation of the anchorless form of the attached protein released in membrane vesicles.

Instead, GPI-PLD, an enzyme with high specificity for the GPI-anchor and presence in plasma, has been proposed to serve as the enzyme with activity towards this substrate (47,51,68). Studies utilizing the addition of exogenous GPI-PLD purified from bovine serum to cell cultures revealed undetectable changes to alkaline phosphatase levels into the culture medium (66,68). Concurrent treatment with detergents demonstrated alleviation of this inhibition and resulted in a significant release of the GPI-anchored protein into the medium (66). Thus, GPI-PLD was not active against the GPI-anchor of cell surface proteins unless in the presence of a detergent indicating that this anchor may be protected by the phospholipid bilayer of the cell membrane. Potentially, this could be a protective mechanism against the effects of GPI-PLD *in vivo*. With many cells of the body in circulation containing GPI-anchored proteins, it would be detrimental for circulating phospholipase activity to function without some level of regulation. Otherwise,

many of these GPI-anchored proteins would be immediately released as they come in contact with these circulating phospholipases. As with GPI-PLC, an alternative structure into which BALP is released, such as a membrane vesicle or membrane fragmentation, may be able to explain how these phospholipases are able to function *in vivo*.

2.1.3.2. Release of an anchor-intact form of BALP

An alternative process of BALP release results in an anchor-intact, insoluble form to be released (70). As previously mentioned, this insoluble form must then be processed into the soluble form, again through loss of the GPI-anchor that is still intact. Thus, this alternative process would likely still require some phospholipase activity; however, it is suspected that alternative structures in which BALP can be presented to the phospholipases, either through matrix vesicles or membrane fragmentation, may be more susceptible to subsequent cleavage without the need for a detergent. *In vitro* studies have shown that the majority of BALP released from human osteoblasts is in an insoluble form representing an alternative release mechanism aside from GPI-specific phospholipase activity (70,71).

During the process of apoptosis, membrane fragmentation results in the formation of plasma membrane vesicles as part of the regulation of controlled cellular death pathway (82–86), (Figure 2.6). Evaluation of osteoblastic cells undergoing apoptosis has revealed that the controlled release of membrane vesicles containing alkaline phosphatase is an important aspect of calcification (87–91). Consistent with this finding, human OS cells induced to undergo cell death were shown to have an elevated release of BALP while apoptosis inhibitors reversed the rise in BALP (53). Interestingly, human osteoblast-line cells from normal bone demonstrated the same release of BALP under the same conditions. These findings support a hypothesis that

elevations in BALP are associated with apoptosis, and thus, are associated with osteoblast turnover.

2.2. Canine osteosarcoma

2.2.1. Overview

Osteosarcoma (OS) is the most common primary bone tumor of dogs and is analogous to human OS. It is an aggressive, highly metastatic tumor originating from malignant osteoblasts and accounts for approximately 85% of skeletal malignancies (92,93). An estimated 10,000 dogs and 1,000 children per year develop OS in the United States alone (92,94). In dogs, OS occurs primarily in middle-aged to older large to giant breeds with a median age of 7 years and a bimodal age distribution with a second peak around 18 to 24 months (95). Approximately 75% of canine OS lesions occur in the appendicular skeleton at the metaphysis with a 2:1 predilection for the forelimbs to the pelvic limbs (96). The most commonly affected anatomical sites are the distal radius and proximal humerus followed by the distal femur, proximal tibia, and distal tibia (93,97). With a high incidence of approximately 10% of all diagnosed canine tumors and a more aggressive disease course as compared to human OS, canine OS serves as a very valuable comparative tumor model for evaluation of therapeutic targets to improve outcomes for both humans and canines (98,99).

Primary OS lesions grow in an expansile, destructive manner and are associated with a great degree of pain most likely due to microfractures or disruption of the periosteum (92,100). Osteolysis, atypical bone production, or both processes concurrently may be seen radiographically and histologically. At presentation, clinical signs may range from a chronic progressive lameness to acute non-weight bearing lameness, typically associated with pathologic

fracture. A hard swelling may be palpable at the site of the lesion; however, this is not always evident on physical examination, especially depending on location of the lesion. Eventually, 90% of dogs succumb to metastatic disease with the pulmonary parenchyma being the most frequent site of metastasis (95).

Dogs treated with conventional therapy comprised of limb amputation and systemic chemotherapy eventually die of metastatic disease with a median survival time of approximately 10 months. With amputation alone, dogs have a poor prognosis with a median survival of approximately 5 months (101,102). “Limb-sparing” surgical techniques and radioablative methods were developed and can be used for tumors of the distal radius, ulna, and tibia with similar reported survival times (103–105). Palliative radiation therapy with adjuvant bisphosphonate therapy can also be used for dogs in which amputation would not be well tolerated or is not performed due to owner wishes (106). Treatment with pain medications alone inevitably results in poor quality of life from tumor-associated pain or pathologic fracture within a few months of diagnosis.

2.2.2. Prognostic Factors

In dogs and humans with OS, a limited number of poor prognostic indicators have been consistently identified and include advanced stage of disease at presentation (3,107), location of tumor (108–112), tumor size (20,23,24), histologic grade (109,113,114), response to pre-operative chemotherapy (7,107,115), and elevations in serum BALP (1,2,6). Due to heterogeneity and lack of completeness of studies, meta-analyses of prognostic factors in canine and human OS have only identified these limited prognostic factors (4,107,116). Understanding

the biologic mechanism of the cause of these prognostic factors may lead to the discovery of novel therapies for the treatment of OS.

Understandably, metastatic disease at the time of presentation is a poor prognostic indicator as treatment options become greatly limited with advanced stage of disease and metastatic cells have the propensity to be more aggressive in biologic behavior. Dogs with proximal humeral lesions tend to have a poor prognosis speculated to be a result of occult disease in a well-muscled area leading to a delay in clinical observation (4). Alternatively, there could be inherent differences in the proximal humeral location such as differences in the microenvironment caused by differences in biomechanics of the area. In humans with OS, the distal lower extremity carries the best prognosis with increased survival times which is similar to the distal radius in canine OS (109,117,118). Histologic subtype has been correlated with prognosis in humans; however, the response to chemotherapy is typically the overriding prognostic factor in these studies (7,113,119). In canine OS, histologic subtype has not been consistently identified prognostic factor and response to chemotherapy is rarely reported because of the high rate of amputation prior to chemotherapy. For patients that are treated with limb-salvage surgery, infection has a positive influence on survival suspected to be due to immune stimulation (120,121). In a retrospective study on pediatric OS, larger tumor size was a poor prognostic indicator that was significantly predictive of overall survival and event-free survival (24). Relative rather than absolute tumor volume was predictive of overall survival in another study in pediatric OS (122). Another retrospective study looking at human OS established an absolute tumor volume cutoff of 150cm^3 as measured radiographically and found that patients with volumes over this cutoff had a poorer prognosis than those with a smaller volume (23).

2.2.2.1 Elevated BALP in humans and canines with OS and metastatic bone disease

The most consistently identified prognostic factor in both humans and dogs with OS is an elevated serum total alkaline phosphatase (SALP). As previously mentioned, there are multiple isoforms of alkaline phosphatase including liver, corticosteroid induced, and bone (BALP) forms. Serum measurement of total alkaline phosphatase is an easy, cheap diagnostic test available in most veterinary clinics. The corticosteroid-induced form may be present in dogs with hyperadrenocorticism, a common concurrent disease process of older dogs, and this form is not routinely reported concurrently with SALP. Thus, measurement of SALP alone cannot distinguish differing isoforms and hence may not provide an accurate assessment of BALP levels in patients with OS and very few studies report serum BALP levels specifically. Bone alkaline phosphatase activity is a more accurate biomarker than SALP in the evaluation and continued monitoring of patients with OS given the restricted osteoblast source of BALP.

Because of its cellular restriction to the osteoblast, BALP is a marker of bone turnover which can be measured in serum and reflects the bone destruction and cell turnover in conditions affecting bone metabolism, including OS and metastatic bone diseases (13–18,53,123). Many human OS studies have reported elevated serum or plasma BALP at diagnosis as a poor prognostic indicator (21,22,124,125). Some of these studies have demonstrated positive correlation between serum BALP activity and local disease recurrence or the presence of metastatic disease. Patients with metastatic OS had higher serum BALP levels than those that did not develop metastatic disease (21,22). In one of these studies, serum BALP in humans was associated with the osteoblastic subtype of osteosarcoma and to the presence of metastatic disease with elevated levels indicating a poorer prognosis; however, in this study, multivariate analysis only found tumor size to be significantly correlated to serum BALP levels (22). In

another study, elevated plasma BALP levels in OS bearing patients at diagnosis was higher in patients who developed disease recurrence than in those who showed no evidence of recurrence (21). Whether in the setting of disease recurrence or the presence of metastatic disease, a poor prognosis with increased BALP is understandable as this enzyme serves as a marker of presence of disease; however, the underlying mechanism by which elevations in BALP activity at diagnosis prior to the development of metastatic disease results in a poorer prognosis is not yet understood.

Multiple canine OS studies have recognized the negative prognostic factor of an increased pre-treatment serum BALP. In a meta-analysis of prognostic factors in canine appendicular OS, elevations in SALP resulted in a shorter survival time by 156 days as compared to dogs with SALP within the reference range. Disease free interval was also affected negatively by elevated SALP levels with a median disease free interval difference of 123 days (4). While most studies report on SALP, few studies in dogs have reported the same prognostic significance of an elevated pre-treatment BALP activity (2,6,20), similar to what has been reported in human literature as discussed previously. In the most recent study, BALP was correlated to tumor burden as measured via plain radiographs and concluded that tumor burden may be one contributing factor in the explanation of differential serum BALP activities noted in dogs with OS (20). Plain radiographs have been used to evaluate tumor burden in a few of the human studies mentioned above as well; however, 3-dimensional magnetic resonance imaging (MRI) provides the most reproducible measurements of tumor volume in human OS patients due to the irregularity and heterogeneous composition of plain radiographs (126,127). One human OS study categorized tumor appearance on MRI into 2 main patterns, homogenous and heterogeneous types, with a 3rd, miscellaneous, category and found a significant correlation between elevated

serum SALP and the heterogeneous type (128). No follow up data on overall survival time or disease free intervals was available. While MRI may provide a reliable tool for OS measurement, it is not the most practical diagnostic test to perform clinically and radiographs are much more accessible in most veterinary practices.

Elevated serum BALP activity is not an exclusive finding in OS and is commonly seen in many non-cancerous pathologic bone diseases, such as Paget's disease (129), osteoporosis (130), fracture healing (19,131,132), hyperparathyroidism (133), and thyrotoxicosis (134), and in metastatic bone disease in non-OS cancers, such as prostate, breast, and lung cancers (14,16,17,123). These observations support a link to a general reparative osteoblast response associated with bone destruction and cell turnover rather than an effect of a malignant osteoblast. As mentioned previously, many human studies have demonstrated a correlation to the presence of metastatic disease with elevated BALP levels in OS and the recent paper in canine OS evaluating elevated BALP activity and tumor burden also demonstrated immunohistochemical BALP staining in soft tissue and visceral metastasis (20). This observation supports an effect of malignant osteoblasts over a general bone reparative response; however, the known contribution, if any, to the serum BALP activity from these soft tissue metastatic lesions is not able to be determined. Further support in the link to purely osteoblastic activity in a general reparative process is demonstrated in studies that show that BALP is not elevated in metastatic bone diseases that are osteolytic in nature (19).

2.3. The Endothelin Axis

2.3.1. Overview

Endothelins (ET) are a family of four 21 amino acid peptides involved in key physiological processes in normal tissues and include modulation of vasomotor tone, tissue differentiation, development, cell proliferation, and hormone production (135–138), (Figure 2.7). The four endothelins are ET-1, expressed primarily in endothelial cells, ET-2, ET-3, and ET-4 (vasoactive intestinal constrictor). Endothelin-2 is found mainly in kidney and intestinal tissue while ET-3 is found in the brain (136,139). These peptides exert their effects by binding to endothelin receptors, endothelin A receptor (ET_AR) and endothelin B receptor (ET_BR), which belong to the rhodopsin-like G-protein-coupled receptor superfamily. Aside from its involvement in normal physiological processes, the endothelin axis has been shown to have a relevant role in various disease conditions including cardiovascular disease (136,140–142), pulmonary disease (143), renal disease (144–146), and multiple cancers (137,138,147).

Endothelin-1 is the only family member to be expressed in endothelial cells and is a very potent vasoconstrictor with similarities to the venom of snakes of the *Atractaspis* family (135,148). Endothelin-1 has been the most extensively studied peptide of this family and has been implicated in the majority of the disease processes in which endothelins are involved. In cancer cells specifically, ET-1 leads to autocrine/paracrine loops that activate aberrant proliferation, escape from apoptosis, new vessel formation, immune modulation, abnormal osteogenesis, alteration of nociceptive stimuli, invasion, and metastatic dissemination (137,138,149). Thus, antagonism of these peptides through receptor blockade provides a viable target for cancer therapy.

2.3.2. Endothelin-1 synthesis and processing

As with the other endothelins, ET-1 is the product of a gene that codes for a large precursor-protein mRNA. The promoter region contains typical CAAT and TATA sequences that enable the regulation of transcription along with additional *cis* elements that provide regulatory sites for important stimuli (150). Within these regulatory sites are locations for GATA-2 protein binding and AP-1 binding, allowing modulation of the transcription of ET-1 gene by a variety of growth factors (151,152). The half-life of the produced mRNA is approximately 15 to 20 minutes (151). Upon translation of the ET-1 gene, a protein of 203 amino acids is produced termed preproendothelin-1 (151). This protein is then selectively processed by an enzyme converting it into a 39-amino-acid prohormone, big ET-1. Big ET-1 is secreted and circulates in plasma; however, this larger peptide has approximately 1/100 the potency of ET-1 and must undergo further enzymatic activity to the more active ET-1. Conversion into this final and active protein, ET-1, is performed by endothelin-converting enzyme (ECE) -1, ECE-2, and chymase (153–159), (Figure 2.8). Quickly following entrance into circulation, in about 4-7 minutes, ET-1 is cleared through pulmonary circulation (160). With such a short half-life and lack of storage in secretory granules, transcription of ET-1 genes occurs very quickly.

Inducing stimuli of ET-1 gene transcription include shear stress, stretch, exercise, hypoxia, and a variety of growth factors and vascular proteins (161–165). A great majority, approximately 75%, of ET-1 secretion from cultured endothelial cells is directed towards the vascular smooth muscle (abluminal) side of the cells (166). Thus, it is unlikely that the majority of the final product contributes to the circulating ET-1 concentration in plasma and suggests more of a paracrine role than as an endocrine hormone. However, it is known that circulating plasma ET-1 measurements are a useful marker of disease severity and of prognostic value in a

variety of diseases as mentioned previously. Prior to its elimination, ET-1 binds to ET_AR and ET_BR enabling activation of the many biologic pathways that it regulates (167), (Figure 2.9)

2.3.3. Endothelin receptors and G-protein coupled signaling

As previously mentioned, the effects of ET-1, along with all other members of the endothelin family, are mediated through binding to two distinct cell surface endothelin receptors, ET_AR and ET_BR. These G-protein-coupled receptors contain seven hydrophobic transmembrane domains with an intracytoplasmic C terminus and an extracellular N terminus. The amino acid structure of both receptors is approximately 50 percent identical with 85 to 90 percent conservation across mammalian species. Endothelin receptor regulation and production is very similar to that of the endothelins. Many of the same factors regulating endothelin production stimulate the production of endothelin receptors including hypoxia and many growth factors (168). Differences in the C terminus of the two receptors allow for networks of divergent intracellular pathways to be activated with different effects. In the vasculature, ET_AR is found on smooth muscle cells and ET_BR is found primarily on endothelial cells and is also expressed on smooth muscle cells (169). Activation of ET_AR and ET_BR found on smooth muscle cells in the vasculature results in the profound vasoconstrictive effects of ET-1. However, ET-1 binding of ET_BR found on endothelial cells results in the release of nitric oxide and prostacyclin which cause vasodilation (160), prevention of apoptosis (149), and inhibition of ECE-1 expression in endothelial cells (170). Thus, ET_BR activation of endothelial cells appears to have counter regulatory effects to ET_AR.

Binding of ET_AR by ET-1 results in G-protein-coupled receptors to associate with intracellular heterotrimeric G proteins, mainly through a pertussin-insensitive G_q subunit associated with ET_AR. This association results in the activation of multiple signaling pathways, concurrently, that are involved in cell survival and proliferation including activation of phosphoinositide phospholipase C (PLC), protein tyrosine kinases, the phosphatidylinositol-3-kinase (PI3K)/Akt pathway, and the RAF/MEK/MAPK pathway (25,138), (Figure 2.10). Cross-signaling occurs between cell surface receptors following ligand binding of growth factors resulting in expansion of the cellular communication network to activate these multiple signaling pathways. In OVCA 433 human ovarian cancer cells, transactivation of epidermal growth factor receptor (EGFR) through phosphorylation by ET-1 resulted in MAPK activation via an intracellular pathway involving ET_AR, β -catenin, and c-Src (171). Because of the multiple signaling pathways affected by the endothelin axis, a combination of drugs may allow for simultaneous inhibition of these pathways.

2.3.4. Involvement in tumorigenesis and targeting for cancer treatment

Endothelin-1 and ET_AR expression have been identified in a multitude of human cancer cell lines and tumors including prostate (29,36,172), ovarian (26), lung (173–175), colon (176,177), renal (178), cervical (179), chronic lymphocytic leukemia (180), and glioma (181–184). Interestingly, in human melanoma cancer cell lines, the ET_BR receptor is recognized as the main receptor in which ET-1 mediates its effects (185–189). Overexpression of this ET-1/ET_AR axis has consistently been identified as a poor prognostic factor amongst these cancers. In the veterinary literature, there are a couple of reports of endothelin expression in canine cancer. One study identified overexpression of ET-1 and ET_AR in canine ovarian cancer patients by western

blotting and immunohistochemistry of samples of their tumors (190). Another study identified ET-1 expression, along with other nociceptive ligands, in canine osteosarcoma cells and patient samples via western blotting and immunohistochemistry, respectively (191). While the exact role of the endothelin axis in cancer is not clearly understood and is likely multifactorial, and blockade of the axis has been found to decrease tumor growth indicating an important role in certain cancers (176). Through the activation of ET_AR and the multiple signaling pathways mentioned above, ET-1 is involved in several aspects of cancer growth and progression including cell proliferation, inhibition of apoptosis, angiogenesis, invasion and metastasis, and modulation of tumor-infiltrating immune cells (137), (Figure 2.11). Specifically in prostate and breast cancer, ET-1 results in proliferation of osteoblasts and the down regulation of osteoclast activity resulting in osteoblastic lesions associated with osteoblastic bone metastases and the alteration of nociceptive stimuli resulting in malignant bone pain (33,38,192), (Figure 2.12). Thus, inhibition of the endothelin axis through degradation of ET-1 or blockade of ET_AR became a large area of interest in cancer patients which led to the development of endothelin receptor antagonists.

2.3.4.1. Endothelin-1 signaling promotes cell proliferation

Mitogenic factors, such as ET-1, are regulated through the activation of cellular receptors and subsequent transmission of a signal through intracellular signaling pathways which effect the nucleus of the cell to promote transcription of genes involved in cellular proliferation. Specific to cellular proliferation, ET-1 activates PLC which leads to the formation of inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG) (Figure 2.13). Inositol 1,4,5-triphosphate increases the intracellular calcium concentration which leads to the vasoconstriction seen with receptor binding of ET-1. Diacylglycerol and calcium stimulate protein kinase C, a known mediator of the

mitogenic action of ET-1 through direct activation of the MAPK (193). As mentioned above, cross-signaling of EGFR by ET_AR activation also results in stimulation of the RAF/MEK/MAPK pathway and subsequent transcription of genes involved in DNA synthesis and cell proliferation. Spontaneous tumor growth has been inhibited in various human tumor cells including prostate, colon, cervical, and ovarian carcinomas through blockade of ET_AR, but not ET_BR (26,176,194–196). Activation of ET_AR has also been shown to induce proliferation in normal endothelial cells in an autocrine manner when ET-1 is upregulated via plasmid transfection (197). *In vitro* work with advanced human prostatic cancer cell lines showed that exogenous ET-1 also enhanced mitogenic effects of multiple growth factors including insulin-like growth factor I, insulin-like growth factor II, platelet-derived growth factor, basic fibroblast growth factor, transforming growth factor (TGF), interleukin-6, and epidermal growth factor (29). These observations demonstrate an autocrine role of ET-1 in cellular proliferation through ET_AR.

2.3.4.2. Endothelin-1 signaling promotes cell survival

A variety of growth factors, receptors, and intracellular signaling pathways are involved in cell proliferation, survival, and the inhibition of apoptosis, a naturally occurring cell death process important to tissue homeostasis and development. Apoptosis transpires through a series of transcriptionally activated events resulting in a cascade of cysteine proteases, called caspases that result in cell fragmentation and subsequent engulfment by phagocytic cells. Dysregulation of apoptosis is a well-known occurrence in cancer development and is recognized as a hallmark of cancer (198). Investigation of the mechanisms of evasion of apoptosis has led to the discovery of a number of disrupted pathways and aberrant expression of growth factors and their receptors in

cancer cells including the anti-apoptotic effects of ET-1 through PI3K activation (178,180,199,200).

The PI3K/Akt pathway includes enzymes involved in cell growth, proliferation, survival, differentiation, and motility. Phosphatidylinositol-3-kinase phosphorylation leads to activation of the serine-threonine kinase, protein kinase B (PKB)/Akt, which affects important mediators of the apoptotic pathways (201). Akt kinase phosphorylates Bad, a member of the Bcl-2 family, and also inactivates caspase 9, both resulting in suppression of apoptosis via the intrinsic pathway (202,203). Akt also blocks the transcription of death-associated genes including Fas ligand (FasL) through phosphorylation and inactivation of a transcription factor of the Forkhead family, FKHRL1 (204). In ovarian carcinoma cells, exogenous ET-1 blocked paclitaxel-induced apoptosis in a concentration-dependent manner and the addition of an ET_AR antagonist was able to attenuate this effect (205). Moreover, it was noted that ET-1 activated the Akt pathway in these cells and the ET_AR antagonist was able to block Akt phosphorylation indicating utilization of the PI3K/Akt signaling pathway by the endothelin axis (199). In human colon carcinoma cells, not only was ET-1 found to inhibit apoptosis mediated by paclitaxel, it also inhibited apoptosis mediated by FasL (186,206).

Endothelin-1 binding to ET_AR results in the upregulation of the PI3K/Akt pathway has also been demonstrated in human prostatic cancer (200,207). Endothelin-1 expression in both malignant and normal prostatic cells was found to be transcriptionally activated by the β -Catenin (β -cat)/T cell factor 4 (Tcf-4) family transcription factors and ET-1 stimulated β -cat/Tcf-4 signaling via the PI3K-dependent pathway. These findings support a positive feedback loop that enhances both signaling pathways to promote proliferation and survival of human prostatic cancer cells (207).

2.3.4.3. Endothelin-1 signaling promotes migration

Invasion and metastasis of tumor cells requires the ability of tumor-associated proteinases that promote the degradation of the surrounding extracellular matrix (ECM) and the basement membrane. Both serine and matrix metalloproteinases (MMPs) have been investigated as key components of this process. Matrix metalloproteinases are able to degrade all layers of the ECM including collagens, fibronectin, laminin, and the basement membrane (208). Urokinase-type plasminogen activator (uPA) is a serine protease involved in tumor progression and metastasis through activation of the urokinase receptor (uPAR) and subsequent plasminogen activation (209–211). In migrating cells, uPAR localizes to the migrating edge of cells in leading lamellipodia and promotes degradation of the ECM (212). Multiple human cancers have demonstrated upregulated activity of uPAR and MMPs, specifically MMP-2 and -9, in association with metastasis and a poorer prognosis (213,214)

Acting through ET_AR, ET-1 is known to mediate tumor invasion through the activity of both of these metastasis-related proteinases (215). Initially in human ovarian cancer patients and later in other solid tumor types as well, studies demonstrated that patients with metastatic disease had higher serum ET-1 levels suggesting that this endothelin axis may be involved in the migration and metastasis process (36,215–217). Endothelin-1 was able to enhance secretion and activation of MMPs -2, -9, -3, -7, and -13 along with the uPA/uPAR axis with blockade of the ET_AR receptor resulting in decreased expression of these proteases and decreased migration and invasion of human ovarian carcinoma cells (215). In human OS cells, ET-1 was shown to increase MMP-2 and -9 synthesis and activity with ET_AR antagonism resulting in decreased cell

invasion (218). These and other studies support a role of the endothelin axis in the invasion, migration, and eventual metastasis of cancer cells.

2.3.5. The endothelin axis is involved in osteoblastic proliferation

Osteoblastic metastasis occurs in a number of human malignancies, most commonly seen in prostatic and breast cancer. To survive in a hostile foreign environment, metastatic cells must be able to establish a microenvironment favoring their own growth. Although there are a few proposed mechanisms of metastasis pathophysiology, the seed and soil analogy of Paget is a commonly accepted method of tumors that metastasize to bone, initially described in studies involving breast cancer. Briefly, tumor cells secrete factors that stimulate osteoblast activity and the formation of new bone which is then enriched with osteoblast-derived growth factors that favor the local growth of tumor cells (219). Many growth factors have been implemented as the supporters of osteoproliferative metastasis including TGF β , insulin-like growth factors-1 and -2, prostate-specific antigen (PSA), uPA, fibroblast growth factors-1 and -2, bone morphogenetic proteins, platelet-derived growth factors, and ET-1 (33,36,220–229), (Figure 2.14). Aside from the proteases PSA and uPA, the majority of factors listed stimulate osteoblast growth and survival directly (33,222,227,229). Through mitogenic and pro-survival effects of these growth factors, osteoblasts are stimulated by tumor cells to create an osteoblastic phenotype and microenvironmental niche permissive for survival.

There is concrete evidence that ET-1 stimulates bone formation which was originally evaluated in human breast and prostate cancer. These tumor types serve as good models as the majority of prostatic bone metastatic lesions are osteoblastic while breast cancer tends to cause

osteolytic lesions; however, approximately 25% of bone metastases in breast cancer are osteoblastic (230–232). The endothelin axis is known to be involved in cellular proliferation and survival of cancer cells as previously discussed; however, in the human prostate cancer model, there is also a clear link to the promotion of osteoblastic bone disease. Endothelin-1 has been consistently identified as a poor prognostic factor as abnormally elevated plasma levels are noted in approximately 58% of men with advanced, hormone-refractory prostate cancer (36). When comparing bone metastases to metastases confined to organs, there was a significant elevation in ET-1 plasma concentration in men with bone metastases. These concentrations did not correlate to tumor burden or serum prostate specific antigen concentrations (37). Contributing to the elevation in ET-1, key components of the clearance pathway of ET-1, ET_BR and neutral endopeptidase (NEP), are downregulated in advanced prostate cancer (29). Neutral endopeptidase is a degradative enzyme involved in the regulation of ET-1 (233).

In a murine model using human breast cancer cell lines, tumor-produced ET-1 was shown to stimulate new bone formation *in vitro* and osteoblastic metastases *in vivo* via ET_AR (37). Human breast cancer cell lines have been shown to have express the enzyme that converts preproendothelin-1 to ET-1, ECE (234,235). Among multiple growth factors investigated, the osteoblastic breast cancer cell line, ZR-75-1, only expresses elevated levels of ET-1 over the osteolytic cell line, MDA-MB-231. To further explore the role of ET-1 in new bone formation, ET-1 stimulated osteoblast proliferation was only blocked by an ET_AR antagonist and not a selective ET_BR antagonist. Thus, an *in vivo* study utilizing female nude mice and the osteoblast ZR-75-1 cell line was performed to induce osteoblastic metastatic bone disease and determine the bone biologic effects of the same ET_AR antagonist. Endothelin A receptor blockade reduced

osteoblastic metastases in these mice while no effect was seen in mice inoculated with the MDA-MB-231 cell line (38).

2.3.6. Endothelin-1 increases ALP activity

Endothelin-1 is a potent peptide that has been shown to play a pivotal role in osteoblastic disease processes, mainly through mediating cell differentiation and migration of bone cells (25–28). Endothelin-1 inhibits osteoclasts from resorbing bone, induces osteoblastic bone formation and increases expression of osteopontin and osteocalcin, resulting in promotion of bone formation (30–33). In a rat osteoblastic OS cell line (ROS17/2.8), ET-1 exposure was found to enhance osteopontin and osteocalcin messenger RNA expression by two-fold whereas, ET-3 had no effect demonstrating the signaling pathway is specific to ET_AR (31). Osteocalcin is a secreted protein that is specific to the osteoblast lineage and osteopontin is a secreted, calcium-binding, glycoprotein found in bone matrix. The gene encoding osteocalcin was upregulated by exposure of fetal rat calvarial cell cultures to ET-1 and this same study also demonstrated an upregulation of alkaline phosphatase genes (32). In another *in vitro* study, incubation of human osteoblastic cells with exogenous ET-1 resulted in a dose-dependent increase in alkaline phosphatase (236). All of these proteins are markers of osteoblast function demonstrating a crucial role of ET-1 in the osteoblast response. Consistent with these genetic markers of increased osteoblast function, ET-1 exposure results in mitogenic effects on osteoblasts via its own direct activity through ET_AR (237,238).

Previously, mice were not a great model to use in the investigation of osteoblastic bone metastases because they rarely developed metastatic bone lesions and these lesions are typically osteolytic. A mouse model in which whole tissue homogenates of canine prostate tissue was

implanted near calvarium of nude mice demonstrated that the canine prostate tissue enhances new woven bone formation (34). Using this model, the same group evaluated the effects of normal canine prostatic tissue homogenates on ALP activity and the effects of ET-1 antagonists on ALP activity *in vitro*. Treatment of cultured rat calvaria with normal canine prostatic tissue was shown to stimulate ALP activity in a time- and dose- dependent manner which could be blocked by ET-1 antagonists. Calvaria treated with homogenates of tissues from other organs did not increase ALP activity (35). These studies suggest that new bone formation and associated ALP activity can be stimulated by prostate tissue which can be abrogated by ET-1 antagonists; thus, providing a link to ET-1 mediated cell-signaling pathways leading to the activation of osteoblasts and associated elevations in ALP activity.

2.3.7. Clinical trials utilizing ET-1 specific antagonists

The effects of ET-1 are mediated through ET_AR and are attenuated through antagonism of this receptor (33,37,38). Selective antagonists of the ET_AR are able to block the proliferative effects of ET-1 on osteoblasts and prostate cancer cells (33). ABT-627, an endothelin antagonist with selectivity for ET_AR, has been evaluated most extensively in the human prostatic carcinoma model. Osteoblastic bone lesions are very common in men with prostatic cancer resulting in severe pain making this aggressive disease a good model for use of ABT-627. *In vitro* studies have shown that this antagonist is able to sensitize prostatic cancer cells to taxane chemotherapy via downregulation of survival pathways, NF- κ B, Bcl-2, and survivin (239). Blockade of these pathways sensitizes cells to be more susceptible to apoptosis; thus, combination therapy with chemotherapeutics may increase the amount of apoptosis induced over monotherapy. The addition of taxane chemotherapy to ET_AR antagonists reveals that this combination of therapies

induces increased levels of apoptosis *in vitro* on human prostatic cancer cells (239,240). When used alone, ABT-627 has no statistically significant improvement in time to progression in men with both metastatic and non-metastatic, hormone-refractory prostate cancer (241,242). Another study in women with relapsed ovarian cancer evaluated the effect of an ET_AR antagonist, zibotentan, in combination with paclitaxel plus carboplatin. Unfortunately, there was no significant improvement in progression free survival compared to women treated with paclitaxel and carboplatin alone (243). Developing novel treatment strategies targeting the endothelin axis and associated pro-tumorigenic activities led by this axis may help to improve survival times in human and veterinary cancer patients.

CHAPTER 3

MATERIALS AND METHODS

3.1. Cell Lines

Three canine OS cell lines including HMPOS (provided by Dr. James Farese, University of Florida), D17 (purchased from American Tissue Culture Collection, Manassas, VA), and Abrams (provided by Dr. Douglas Thamm, Colorado State University) were utilized in this study. Jurkat (human T lymphocyte cells) and NIH/3T3 were used as positive control cell lines. Cells were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine (2 mmol/L), penicillin (100 IU/ml), streptomycin (100 IU/ml), and 10% fetal bovine serum (FBS) in a humidified atmosphere supplemented with 5% CO₂. Cell cultures were maintained in subconfluent monolayers and passaged two to three times weekly as necessary.

3.2. Reagents and Antibodies

Human ET-1 (E7764), Anti-ET-1 antibody (mouse monoclonal; E166), and anti-ET_AR antibody (rabbit polyclonal; E3651) were purchased from Sigma-Aldrich. Atrasentan hydrochloride (ABT-627; HY-15403A) was purchased from MedChem Express. Phosphatidylinositol-specific phospholipase C was purchased from Life Technologies (P-6466). Anti-phosphorylated Akt antibody (rabbit polyclonal, 9271s), anti-Akt antibody (rabbit polyclonal, 9272), and anti-c-jun antibody (rabbit monoclonal, 9165s) were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from GE Healthcare, UK. Anti-β actin antibody

(mouse monoclonal; AC-15) and anti-BALP antibody (mouse monoclonal; ab108337) were purchased from Abcam Biochemicals.

3.3. Cell Protein Collection

Cells were grown in culture until 80-100% confluence was attained. Media was removed and cells were washed twice with PBS. Cells were exposed to trypsin for five minutes to detach from culture plate, followed by addition of complete media to neutralize the effects of trypsin. Collected cells were then centrifuged at 2,000 rpm at 4°C for 5 minutes. Supernatant was removed and the cell pellet was homogenized in 1 ml PBS and transferred to a 1.8 ml eppendorf tube before centrifuging at 10,000 rpm at 4°C for 5 minutes. Supernatant was removed and discarded. Cell pellets were stored at -80°C until protein collection and quantification. Cell pellets were homogenized with 100-150 µl commercially available Mammalian Protein Extraction Reagent (M-PER, Pierce, Rockford, IL) and mixed with fresh Pierce protease inhibitor cocktail solution (diluted 1:100 for final working solution). Homogenate was placed on a shaker at room temperature for 15 minutes and then centrifuged at 10,000 rpm for 15 minutes at 4°C. The supernatant was collected and stored at -20°C until quantification. Cellular protein concentrations were determined using a standard assay kit (Bicinchoninic Acid Protein Assay (BCA), Pierce, Rockford, IL).

3.4. Western Blot Analysis

3.4.1. Endothelin-1 and ET_AR Expression

For each protein expression analysis, 50 µg samples were electrophoresed on 12% polyacrylamide gel and then electrophoretically transferred to nitrocellulose membrane. The membranes were blocked with TBST with 5% milk for 1 hour at room temperature. Western blot analysis was performed using ET_AR and ET-1 antibodies at a concentration of 1:1000 in TBST with 5% milk. The membrane was then washed 3 times with TBST and probed with the secondary antibody diluted 1:5000 in TBST with 5% milk. The ECL blots were developed using ChemiDoc XRS+ molecular imager system (Bio-Rad) with Image Lab software. Band volume analysis was done using Image J software (NIH) and results of each of the proteins were adjusted for β-actin expression levels used as a loading control. Results reported were derived from at least 2 independent experiments.

3.4.2. Akt/phospho-Akt and c-jun/phospho-c-jun expression

The Abrams, D17, and HMPOS cell lines were grown in complete media to 85% confluence. Media was removed and the cells were serum starved for 24 hours. Cells were then exposed to experimental conditions including: 1) serum starved only or serum starved for 24 hours and then stimulated for 1 hour with 2) 10% FBS; 3) DMSO; 4) 100 nM ET-1; 5) 40 µM ABT-627; or 6) 40 µM ABT-627 followed by 100 nM ET-1 for 1 hour. Media was removed and cells were washed with PBS followed by exposure to trypsin for 5 minutes. Upon release of adherence, cells were collected in 0% FBS DMEM to avoid changes in phosphorylation of survival proteins due to exposure to supplemented growth media. Membranes were incubated with either phosphorylated-Akt antibody at 1:500 in TBST with 5% milk, Akt antibody at 1:1000 in TBST with 5% milk, or c-jun antibody at 1:1000 in TBST with 5% milk. The membrane was

then washed 3 times with TBST and probed with the secondary antibody diluted 1:5000 in TBST with 5% milk and developed using a standard chemiluminescence detection kit (Amersham). The ECL blots were developed using ChemiDoc XRS+ molecular imager system (Bio-Rad) with Image Lab software. Band volume analysis was done using Image J software (NIH) and results of each of the proteins were adjusted for β -actin expression levels used as a loading control. Results reported were derived from at least 2 independent experiments.

3.5. Colony Forming Assay

The Abrams, D17, and HMPOS cell lines were seeded overnight in 6-well plates at a concentration of 200 cells per 3 ml of DMEM with 10% FBS. Upon adherence of individual cells, media was discarded and new media containing different experimental conditions were added including DMEM 5% FBS only, DMEM 5% FBS with vehicle (DMSO), or a range of ABT-627 concentrations (10-40 μ M). Cells were allowed to grow undisturbed for 7 (Abrams) or 10 (HMPOS and D17) days. Subsequently, wells containing colonies were gently rinsed with chilled PBS, treated with 3 ml of solution containing 6.0% glutaraldehyde and 0.5% crystal violet for 30 minutes, and then rinsed with tap water. Each of the treatment conditions was performed in 3 separate experiments. The number of visible colonies per experimental condition were quantified 5 times total by 3 different individuals blinded to treatment conditions.

3.6. Migration scratch assay

Qualitative analysis of cell migration was done using the “scratch assay” method (244). The Abrams, HMPOS, and D17 cell lines were grown to 80% confluence in 6-well plates in DMEM supplemented with 10% FBS (complete media). Six experimental conditions for a duration of 24 and 48 hours were evaluated including complete media only, complete media with vehicle (DMSO or H₂O), a range of ET-1 concentrations (100 pM-100 nM), a range of ABT-627 concentrations (10-40 μ M). A standardized acellular gap was created through cell monolayers using a 200ul pipette tip in the middle of each well. Images of the acellular gap were captured at time 0 (maximal gap) and 24 or 48 hours later for each experimental condition using an inverted microscope (Nikon Eclipse TS100) with a mounted digital camera (SPOT Insight QE model #4.2, SPOT Imaging Solutions, Michigan, USA). The average width of 5 representative acellular gaps per experiment conditions and cell line were used for quantitative comparisons at 24 and 48 hours and 2 independent experimental conditions were performed. Each of the treatment conditions was performed in duplicate and the assay results are representative of two separate experiments. Data was analyzed with Image J software (National Institutes of Health, Bethesda, MD).

3.7. Cytologic Detection of BALP Activity

The Abrams, D17, and HMPOS cell lines were all plated into 0.5 ml chamber well slides at a concentration of 25,000 cells per well and allowed to adhere overnight in supplemented media. The media was then removed and the cells were exposed to serum starved media for 48 hours with 1) no additive, 2) DMSO, 3) 40 μ M ABT-627, and 4) 40 μ M ABT-627 for 30 minutes followed by 100 nM ET-1 for 48 hours. The media was then removed and chambers were

washed with PBS. The slides were allowed to dry and were then stained with nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt (NBT/BCIP), an ALP substrate, as previously reported (245). Briefly, slides were incubated for 8-15 minutes at room temperature with sufficient amounts of NBT/BCIP to coat the slide. Slides were rinsed with water, blotted dry, and examined microscopically. Positive staining was indicated by brown to black staining of the cell surface. Three representative images of each chamber containing approximately equivalent cell densities were taken and quantitative analysis was performed on pixel intensity between treated and untreated conditions.

3.8. In vitro BALP ELISA

HMPOS and Abrams cells were plated at a density of 5×10^5 cells/well in 6-well plates in 3 mls of 10% FBS DMEM. After allowing cells to adhere overnight, the media was removed, the plates were washed gently with PBS, and cells were exposed to serum free media with varying conditions for 48 hours including 1) serum starved DMEM only, 2) DMSO, 3) 100 nM ET-1, 4) 40 μ M ABT-627, and 5) 40 μ M ABT-627 for 30 minutes followed by 100 nM ET-1 for 48 hours. Media was removed, and cells were washed gently with 1 ml PBS, then scraped for collection. Supernatant was removed and the cell pellet was resuspended in 0.5 ml PBS with 0.5 Units phosphatidylinositol-specific phospholipase C (PI-PLC) isolated from *Bacillus cereusa* and incubated for 30 minutes at 37°C. After incubation, cells were pelleted and the supernatant was collected for quantitative analysis with a commercial ELISA (MicroVue BAP EIA kit, Quidel, San Diego, CA) test kit utilizing a murine monoclonal anti-BALP antibody.

Protein concentrations were determined in aliquots of each of the cell extracts via BCA analysis. This cell layer protein was used as an index of cell number which could have varied after exposure to the different conditions. BALP activity from the ELISA was corrected for the cell protein layer by dividing the calculated Units by the μg of cell protein ($\text{U}/\mu\text{g}$).

For additional assessment of the amount of membranous cleaved BALP released into cell culture media, a 20 μl aliquot of supernatant derived from the varying conditions after PI-PLC incubation was used for western blot analysis. A rabbit monoclonal anti-human/mouse BALP antibody was utilized at a concentration of 1:1000 incubated overnight at 4°C followed by anti-rabbit secondary antibody at a concentration of 1:1000 for 1 hour at room temperature.

3.9. Clinical Population

The study included dogs with OS that were evaluated at the University of Illinois Cancer Care Clinic between 2003 and 2012. All dogs had a diagnosis of appendicular OS confirmed by either histopathology or cytology with concurrent positive ALP staining (245). All dogs included in the study had orthogonal radiographs of the primary tumor, 3-view thoracic radiographs, DEXA scan, and urine and serum collected at the time of diagnosis. Study candidates were excluded if they received previous treatment including radiation, chemotherapy, or IV bisphosphonates and if they had radiographically apparent pulmonary metastases or metastases to other distant sites at the time of diagnosis. After their initial visits, all dogs were treated palliatively for OS, receiving coarse-fraction radiation treatment with or without an IV aminobisphosphonate. Dogs were re-evaluated at 28-day intervals, at which time serum and urine were collected. When owner consent was given, necropsy was performed at the time of euthanasia.

3.10. Serum BALP ELISA

Serum was collected at the time of initial presentation, and samples were stored at -80°C until analysis. Serum BALP was evaluated with a commercial ELISA (MicroVue BAP EIA kit, Quidel, San Diego, CA) test kit utilizing a murine monoclonal anti-BALP antibody, previously validated for use in dogs. Reference range for dogs ≥ 2 years of age is 0–14 U/L (reflects mean activity ± 2 standard deviations) (246).

3.11. Relative Bone Mineral Density of Primary Tumor

At initial presentation and every subsequent visit, DEXA (QDR-4500W, Hologic, Bedford, MA) scans were performed to measure rBMD of the primary tumor and an equivalent anatomic area of the unaffected contralateral limb. Dogs were sedated (butorphanol, dexmedetomidine, and atropine) and positioned in sternal or lateral recumbency for forelimb or hind limb lesions, respectively. Bone mineral density of the primary tumor was divided by BMD of the contralateral normal limb to determine rBMD as previously described (106). For purposes of this study, a total of 45 dogs were analyzed. The upper and lower rBMD quartiles were utilized as arbitrary cutoffs for the generation of osteoblastic and osteolytic categories, respectively. To account for tumor size, a ratio of rBMD to the longest tumor measurement as defined by response evaluation criteria in solid tumors (RECIST) was formed.

3.12. Statistical Analysis

The distribution of the continuous variable data was evaluated using the Shapiro–Wilk test, skewness, kurtosis, and q–q plots. Data that were not normally distributed were log transformed to meet the assumption of normality. One-way ANOVA was used to evaluate for differences between groups, with Dunnet’s comparison test to detect a difference between control and experimental conditions. A Pearson product-moment correlation coefficient was used to assess the relationship between pretreatment serum BALP and rBMD. Statistical calculations were performed using commercial software programs. $P < 0.05$ was considered statistically significant for all analyses. Statistical analysis was carried out using a commercially available software program (GraphPad InStat, Version 3.10).

CHAPTER 4

RESULTS

4.1 Endothelin-1 and ET_AR are expressed in osteosarcoma cell lines

Immunoblotting analysis demonstrated translation of ET_AR and ET-1 messenger ribonucleic acid (mRNA) into protein in all cell lines (Figure 4.1). Expression of the 23 kDa protein was detected in all experimental cell lines demonstrating ET-1 protein expression. Expression of the 29 kDa protein was detected in all cell lines including the positive control cell line, Jurkat, demonstrating ET_AR protein expression.

4.2 Endothelin A Receptor inhibition affects cell survival signaling

4.2.1. ABT-627 variably attenuates ET-1 induced Akt phosphorylation

Akt is known to protect cells from apoptosis and phosphorylation of this protein must occur for the signaling pathway to be activated. Thus, phosphorylation of Akt would be expected to occur in conditions in which cell survival is stimulated while phosphorylation would not be expected in cell conditions exposed to an inhibitor of cell survival. Immunoblotting analysis identified bands at the expected weights of 57 kDa indicating expression of both Akt and phospho-Akt in the canine OS cell lines Abrams and HMPOS. In both cell lines, increased phosphorylation of Akt was detected after ET-1 exposure to serum-deprived cells and this was equipotent with FBS stimulation (Figure 4.2a,b). ABT-627 treatment alone appeared to have an effect in attenuating phosphorylation of Akt when compared to the control and vehicle conditions while pre-treatment with ABT-627 for 30 minutes prior to ET-1 stimulation resulted in a further decrease in Akt phosphorylation.

4.2.2. ABT-627 attenuates ET-1 induced c-jun phosphorylation

In both the positive control cell line, NIH/3T3, and in Abrams and HMPOS cell lines, immunoblotting analysis identified bands at the expected weight of 43 kDa, as well as, a band at a higher weight, about 55 kDa consistent with the phosphorylated form of c-jun (phospho-c-jun) as this antibody recognizes both the phosphorylated and unphosphorylated forms (according to antibody manufacturer). Incubation of cells with ET-1 resulted in increased phospho-c-jun to c-jun when compared to control conditions. Incubation of cells with ABT-627 alone and ABT-627 followed by ET-1 stimulation resulted in a decreased ratio of phospho-c-jun to c-jun when compared to control and vehicle conditions (Figure 4.3).

4.3. Endothelin A receptor antagonism decreases colony forming ability of canine OS

To determine if antagonism of ET_AR affects cell proliferation and survival, colony forming assays were performed with varying concentrations of ABT-627. After appropriate incubation periods of 7 days (Abrams) and 10 days (HMPOS and D17), there was a consistently identified statistically significant difference in the ability of all OS cell lines to form colonies when exposed to 40 μ M ABT-627 compared to both control and vehicle conditions ($p < 0.01$), (Figure 4.4a-c). The effects of ABT-627 were also statistically significant in the Abrams and D17 cell lines at 30 μ M ABT-627 ($p < 0.01$), (Figure 4.4a,b).

4.4. The endothelin axis affects cell migration of canine OS *in vitro*

Cancer cells must be able to migrate within the microenvironment to vasculature to be able to metastasize to other areas of the body. Thus, we evaluated the effects of ET-1 and ABT-

627 on cell mobility of all three OS cell lines. An acellular gap was created in confluent cultures and the gap was measured at 24 and 48 hours after treatment. Data is represented as a percentage of the original gap (time 0), with 0% being a completely closed gap. In the Abrams cell line, there was a significant difference in gap closure in all ABT-627 concentrations at the 24 hour time point and in both the 30 μ M and 40 μ M ABT-627 wells ($p < 0.01$), (Figure 4.5a). In the D17 cell line, there was a significant difference seen in the 20-40 μ M ABT-627 wells at both 24 and 48 hours ($p < 0.01$), (Figure 4.5b). For HMPOS, the only significant difference was noted at the 40 μ M ABT-627 concentration at both the 24 and 48 hour time points ($p < 0.01$), (Figure 4.5c). In all OS cell lines that were treated with 100 nM ET-1, the gap was more noticeably closed as compared to untreated control and vehicle wells after 24 hours of exposure ($p < 0.05$), (Figure 4.5d-f). There was no significant difference noted in cells treated with ET-1 at any concentration at the 48 hour time point since the gaps were closed completely in all conditions (data not shown).

4.5. Endothelin A receptor antagonism decreases BALP expression and activity

4.5.1. Bone alkaline phosphatase activity is decreased following ET_AR antagonism as measured via cytochemistry

Exposure of the HMPOS and Abrams cell lines to ABT-627 alone and ABT-627 followed with ET-1 stimulation resulted in a subjective visual decrease in BALP activity compared to the control and vehicle conditions. When quantitative analysis was performed with pixel intensity of images taken of the different conditions, there was a statistically significant difference in the HMPOS cell line in chambers treated with ABT-627 alone or ABT-627

followed by ET-1 compared to control and vehicle conditions ($p < 0.001$), (Figure 4.6). There was no significant difference between any of the conditions in the Abrams cell line.

4.5.2. Bone alkaline phosphatase activity is decreased following ET_AR antagonism as measured via ELISA

In an additional method to measure BALP activity following exposure to ET_AR antagonism, an ELISA was performed on both the Abrams and HMPOS cell lines. Following 24 hours of treatment with ABT-627 and the combination of ABT-627 followed by ET-1, cells from the HMPOS cell line demonstrated decreased BALP activity that was statistically significant when compared to control and vehicle treated cells ($p < 0.01$) (Figure 4.7a). While both of these treatments decreased the cell protein layer, there was still a statistically significant difference in BALP activity after normalization of the BALP activity to the amount cell protein layer ($p < 0.01$), (Figure 4.7b). Treatment of cell lines with ET-1 did not cause significant changes in BALP activity.

Western blot analysis of protein expression of BALP correlated with ELISA findings with a decrease in band expression at the expected weight of 75 kDa in the ABT-627 and ABT-627/ET-1 combination treated cells (Figure 4.7c).

4.6. Serum BALP activity in appendicular OS bearing dogs correlates with bone mineral density when accounting for tumor size

Clinical patients with appendicular OS had DEXA scans of the affected and contralateral limbs measured at diagnosis along with serum BALP activity. The upper and lower quartiles of dogs with DEXA scans were separated out into osteoblastic (rBMD greater than 1.3) and

osteolytic (rBMD less than 0.7) groups (Figure 4.8). When BALP activity was log transformed to normalize data, the osteoblastic group demonstrated elevated serum BALP activity when compared to the osteolytic group ($p < 0.01$), (Figure 4.9). To account for tumor size, a ratio of rBMD to RECIST was formed and this ratio was correlated with the log BALP activity. A statistically significant difference was still appreciated between groups with the osteoblastic group having elevated BALP activity compared to the osteolytic group ($p < 0.01$), (Figure 4.10).

CHAPTER 5

DISCUSSION AND CONCLUSIONS

In normal physiologic processes, the endothelin axis actively participates in key cellular pathways including cell proliferation, apoptosis, angiogenesis, vasomotor tone, and hormone production (136). Endothelin-1 signaling through ET_AR coordinates various tumorigenic properties including cell proliferation, survival, migration, invasion and metastases, and angiogenesis (137,138). In regard to malignant bone pathology, ET-1 has been identified as a key driver in perpetuating the successful development and progression of skeletal metastases particularly for prostatic and breast cancer in humans (37,38,192,230–232). Endothelin-1 released by malignant cells that have reached a bone microenvironment results in proliferation of osteoblasts and the down regulation of osteoclast activity resulting in osteoblastic bone metastases (33,37,38,137). Inhibition of ET_AR has been shown to decrease the ability of metastatic bone lesions to form and has resulted in dampening the effect of ET-1 induced pro-tumorigenic properties (33,35,176,199,240).

Accompanying the results derived from studies of human OS cells, the findings of the current investigation demonstrate that canine malignant osteoblasts similarly possess ET-1/ET_AR signaling machinery (174,218). Based upon the co-expression of both receptor and cognate ligand, it is plausible that canine OS cells have the potential to exploit autocrine and paracrine ET-1 signaling as a mechanism favoring pro-tumorigenesis. Differences in expression were minimal between cell lines and HMPOS appeared to have lower ET-1 expression than Abrams and D17.

It has been well documented in other studies that ET-1 utilizes the PI3K/Akt survival pathway as well as the mitogen-activated protein (MAP) kinase pathway and induces expression of expression of immediate early response genes (149,199,200,247–249). Activation of PI3K through multiple signaling cascades leads to the phosphorylation of Akt. Phosphorylated Akt can regulate cell growth and survival through negative regulation of cell cycle inhibitors and effects on p53 (250–252). In human prostatic carcinoma cells, it was demonstrated that ET-1 exposure resulted in increased expression of pAkt in a time- and dose-dependent manner suggesting survival signaling occurs through a PI3K pathway (200). Inhibitors of other survival pathways, in that study, were not successful in blocking the effects of ET-1. In another study investigating human ovarian carcinoma cells, ET-1 was once again shown to cause phosphorylation of Akt which could be attenuated by pre-treatment with an antagonist of ET_AR (199). Endothelin-1 induced phosphorylation of Akt was prevented by treatment with wortmannin, a PI3K inhibitor in that study. The results from these studies provide strong evidence that ET-1 signaling through ET_AR results in a pro-survival effect utilizing the PI3K/Akt survival pathway.

In an attempt to connect our findings of the pro-tumorigenic properties of ET-1 to signaling pathways, we investigated both the PI3K/Akt and JNK survival pathways following exposure to ET-1 and ET_AR antagonism through immunoblotting analysis of pAkt/Akt and phospho-c-jun/c-jun, respectively. Our findings demonstrate activation of Akt by ET-1 which could be blocked by treatment with ABT-627 suggesting that ET-1 signals through ET_AR to induce phosphorylation of Akt. We showed similar findings in Abrams and HMPOS cells when evaluating the expression of phospho-c-jun and c-jun, a key transcription factor involved in the formation of AP-1 and consequent cell proliferation. Endothelin-1 induced increased expression of phospho-c-jun to c-jun while ABT-627 was able to block the phosphorylation of c-jun. These

findings suggest that blockade of ET-1 signaling might reduce cell proliferation signals. Induced phosphorylation of Akt and c-jun by ET-1 which can be attenuated with ET_AR antagonism suggests that, similar to human prostatic and ovarian carcinoma, canine OS cells utilize the endothelin axis as both pro-survival and mitogenic signaling pathways.

Given the potential for the existence of an autocrine/paracrine signaling loop in canine OS as well as the known role of the endothelin axis in osteoblast proliferation and survival in skeletal metastases, the current study explored the effects on canine OS cell migration, proliferation, and survival following exposure to ABT-627, a selective antagonist of ET_AR. With regard to OS cell proliferation and survival, a dose-dependent anti-mitogenic effect of ABT-627 was demonstrated in the Abrams, D17, and HMPOS cell lines through the use of colony forming assays. Maximal reduction of colony formation was noted at the highest concentration of ABT-627 (40 μ M) and correlated well with attenuation of Akt and c-jun phosphorylation. The activity of ABT-627 was also effective at the lower concentration of 30 μ M. Along with inhibition of cell proliferation, we evaluated the effects of the endothelin axis on migration as multiple other studies have demonstrated pro-migratory effects of ET-1 (30,208,215,218,253). ET-1 signaling blockade with ABT-627 reduced canine OS cell migration in a dose-dependent manner with the most profound effect noted at the highest concentration used (40 μ M). A dose-dependent pro-migratory effect of ET-1 was also demonstrated in all three cell lines. Thus, our results show that the endothelin axis is involved in multiple pro-tumorigenic processes in canine OS cells.

Aside from its involvement in pro-tumorigenic processes, the endothelin axis has been shown to be an important player in the production of metastatic osteoblastic bone lesions in humans (37,192,230–232,254). Upregulation of genes including osteopontin, osteocalcin, and

BALP, has been identified following stimulation with ET-1 supporting the peptide's involvement in osteoblastic proliferation and differentiation (31,32). Taking these findings one step farther, one group demonstrated that canine prostatic tissue increased ALP activity in cultured rat calvaria which could be attenuated by ET_AR blockade. With these findings in mind, we evaluated the effects of ET_AR blockade on BALP activity of canine OS cell lines as measured via cytochemistry and ELISA. Corroborating the previous data, in canine OS cell lines, there was a strong, negative correlation between cellular BALP activities following exposure to ET_AR antagonism supporting the hypothesis that ET-1 signaling is linked directly to BALP activity. Subjectively, cytochemistry of BALP activity showed a strong difference in staining intensity between treated and untreated cells. To account for possible differences in cell survival between chambers, and thus the strictly subjective visual appearance of a difference in staining intensity, we performed pixel quantification of inverse images of representative areas of each chamber. This more quantitative analysis still supported a significant difference between treated and untreated chambers in the HMPOS cell line. There was no statistically significant difference in the Abrams cell line and this could be due to the decreased basal BALP activity noted in this cell line resulting in difficulties to detect changes in intensity of BALP activity. To further substantiate the link between BALP activity and the endothelin axis, we performed an ELISA measuring BALP activity on the Abrams and HMPOS cell lines. HMPOS cells that were treated with ABT-627 alone or ABT-627 followed by ET-1 stimulation demonstrated a marked decrease in BALP activity compared to control and vehicle wells. Media from these conditions were analyzed for BALP expression via immunoblotting analysis and the results mirrored the results of the ELISA analysis. These consistently identified decrease in BALP activity and expression in

cells treated with an antagonist of ET_AR lends additional credence to the hypothesis that ET-1/ET_AR signaling has the capacity to directly contribute to BALP activity in canine OS cells.

Multiple studies have identified various prognostic indicators in both dogs and humans with OS (1–3,6–9,23,107,109,114–117,119,255). In humans, and more recently in dogs, a positive correlation between tumor size and serum BALP activity has been identified with elevated tumor size being a poor prognostic predictor of metastasis-free survival and overall survival (20,23,24). In our study, we separated dogs with naturally-occurring appendicular OS into two groups based upon DEXA imaging. Using the upper and lower quartiles of all dogs scanned, thus, dogs with more osteoblastic and osteolytic tumors, respectively, we selected for patients with a difference in bone microenvironment. Our hypothesis was that dogs with more osteoblastic tumors have an increased number of osteoblasts, a known contributor to BALP expression, would have higher levels of serum BALP. Furthermore, we propose that, with our evidence that ET-1 is positively correlated to both BALP activity and osteoblast differentiation and survival, the elevation seen in serum BALP seen in patients with OS could be an epiphenomenon caused by elevated levels of tumor-associated ET-1. We identified that dogs with more osteoblastic tumors had significantly higher levels of log serum BALP. To account for the previously identified relation to absolute tumor burden, and thus tumor size, we created a ratio of rBMD to RECIST and compared this ratio to log serum BALP levels in these patients. Once again, there was a statistically significant difference in dogs with more osteoblastic tumors when accounting for tumor size to log serum BALP. Thus, differences in bone microenvironment, specifically more osteoblastic OS tumors, contribute to the observed differential serum BALP activities in dogs with OS.

Our study has several limitations to address including the use of a few OS cell lines to represent the heterogeneous disease of canine OS. Many of the experiments were *in vitro* in nature which limits the immediate translational significance. While biologically achievable human levels were utilized for this study, a pharmacologic study is necessary to determine if the same dose is achievable in dogs. Only one ET_AR was selected for use in this study; however, there are many other compounds that target this specific receptor or both endothelin receptors. Thus, ABT-627 may not be the most effective compound to have evaluated. We evaluated a couple of survival pathways that are well-documented to be affected by ET_AR antagonism and it is important to recognize that there are still other survival pathways that could be involved in endothelin signaling that were not evaluated (199,247–249). While we provide evidence that the endothelin axis plays a role in osteoblast survival and BALP activity, and that serum BALP in clinical patients is linked to osteoblastic tumors, we have not specifically evaluated the effect that ET_AR blockade could have on serum BALP *in vivo* in naturally-occurring OS-bearing dogs. Further investigation of the effect that ET_AR blockade has on serum BALP activity and prognosis is necessary to obtain a better understanding of our findings.

In conclusion, our results showed that canine OS cells express both ET-1 and ET_AR which are involved in pro-tumorigenic processes including cell migration and survival. To explain the relation to underlying signaling pathways of these processes, we evaluated and found changes in phosphorylation of survival pathways, PI3K/Akt and JNK, when exposed to ABT-627. Endothelin A receptor antagonism also demonstrated a negative effect on BALP activity in canine OS cells. Utilizing naturally-occurring appendicular OS-bearing dogs, this study provides new information in the biologic explanation of elevations in serum BALP and the identified poor prognosis, specifically the relation between rBMD and serum BALP when accounting for tumor

size, another recognized contributing factor to serum BALP. Additionally, we have provided a plausible link between the endothelin axis, a known osteoblast survival signaling pathway, to the production of BALP activity. These findings could help elucidate, as part of a multifactorial explanation, the biologic mechanism to explain the poor prognostic significance of elevated BALP levels in patients with OS.

CHAPTER 6

LIST OF FIGURES

Figure 2.1 Structure of GPI-anchored proteins. (From <http://cms.unige.ch/sciences/biochimie/Reika-Watanabe,124.html>)

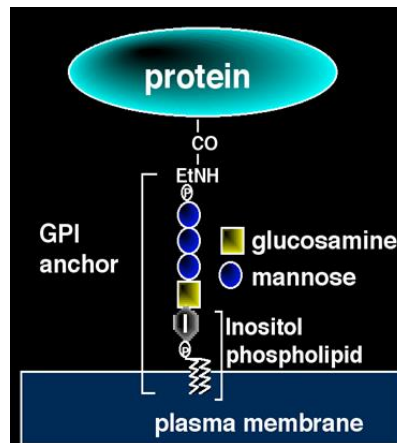
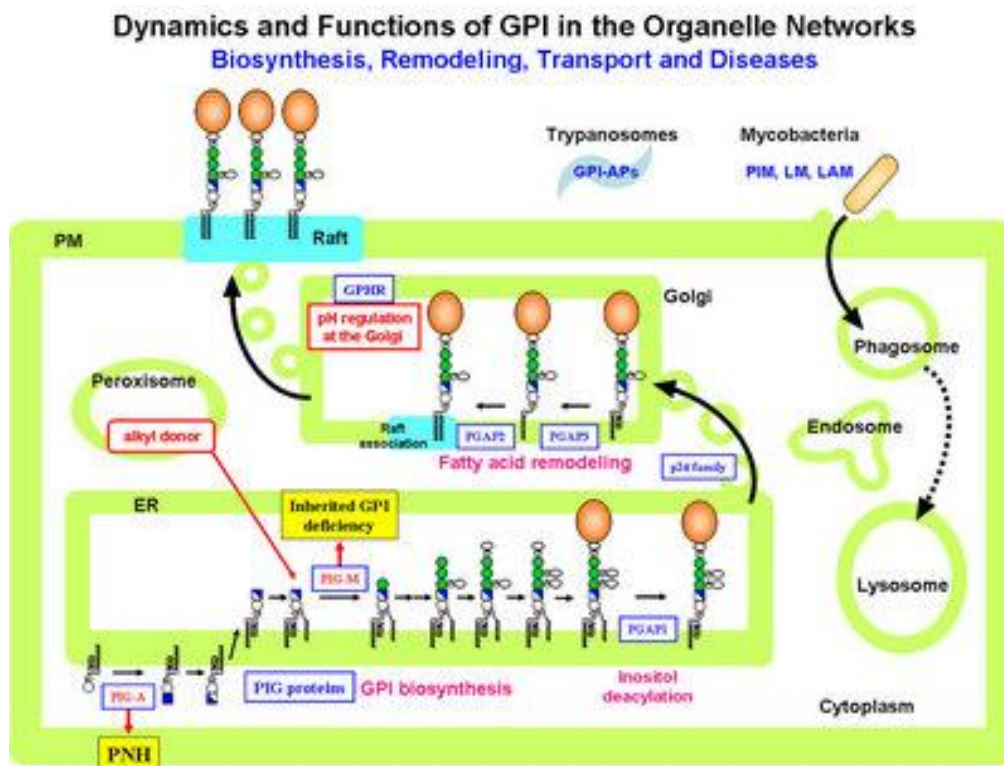


Figure 2.2 Process of GPI-anchor protein formation and trafficking to the plasma membrane. (From <http://www.fbs.osaka-u.ac.jp/organelle-network/eng/research/22/>)



(<http://www.cureffi.org/2013/06/29/proteolytic-shedding-of-prion-protein/>)

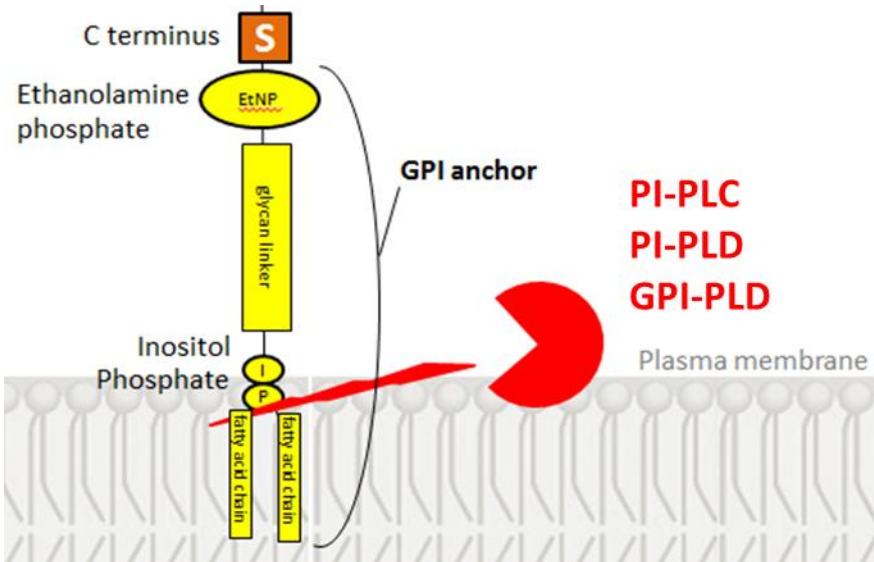


Figure 2.4 Sites of cleavage of phospholipases. PLB is characterized as an enzyme with activity on both PLA1 and PLA2. (*Molecular Cell Biology, Sixth Edition*. 2008. W. H. Freeman and Company. Figure 10-14.)

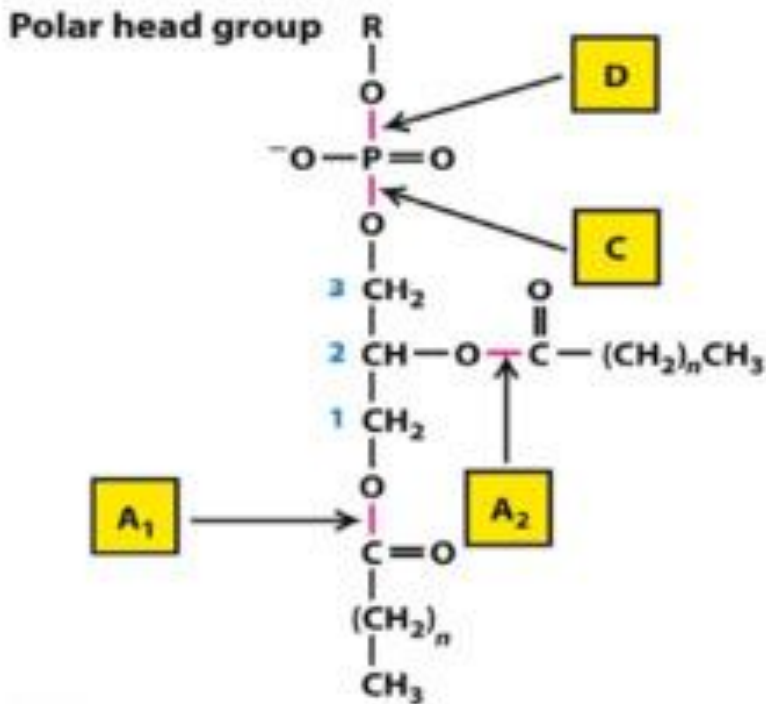


Figure 2.5 Phospholipase C activation results in conversion of PIP_2 to DAG and IP_3 . (From <http://www.endocrinesurgeon.co.uk/index.php/what-are-cell-surface-receptors-and-second-messenger-systems>)

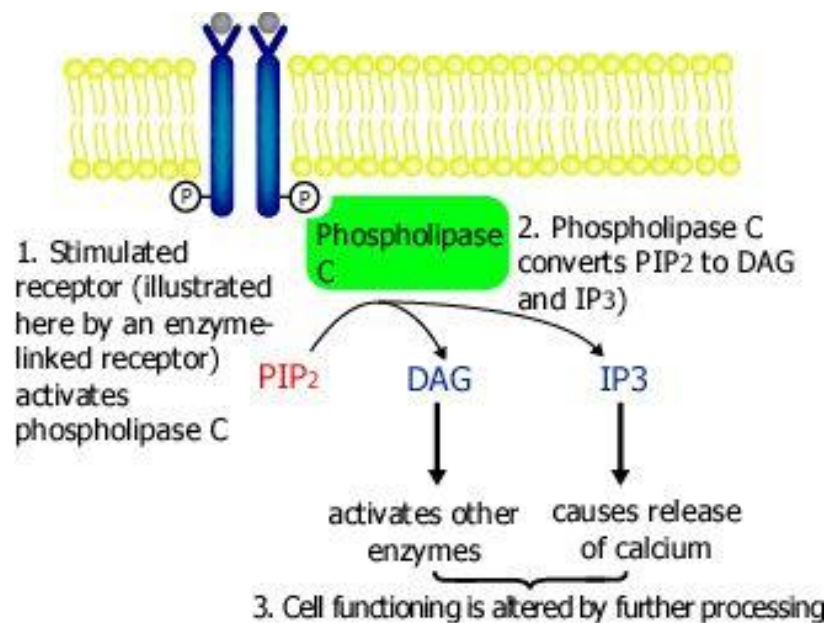


Figure 2.6 Formation of extracellular vesicles including exosomes created by resting or activated cells, microvesicles created from tumor cells, and apoptotic bodies from apoptotic cells. (<http://www.extracellularvesicles.org/>).

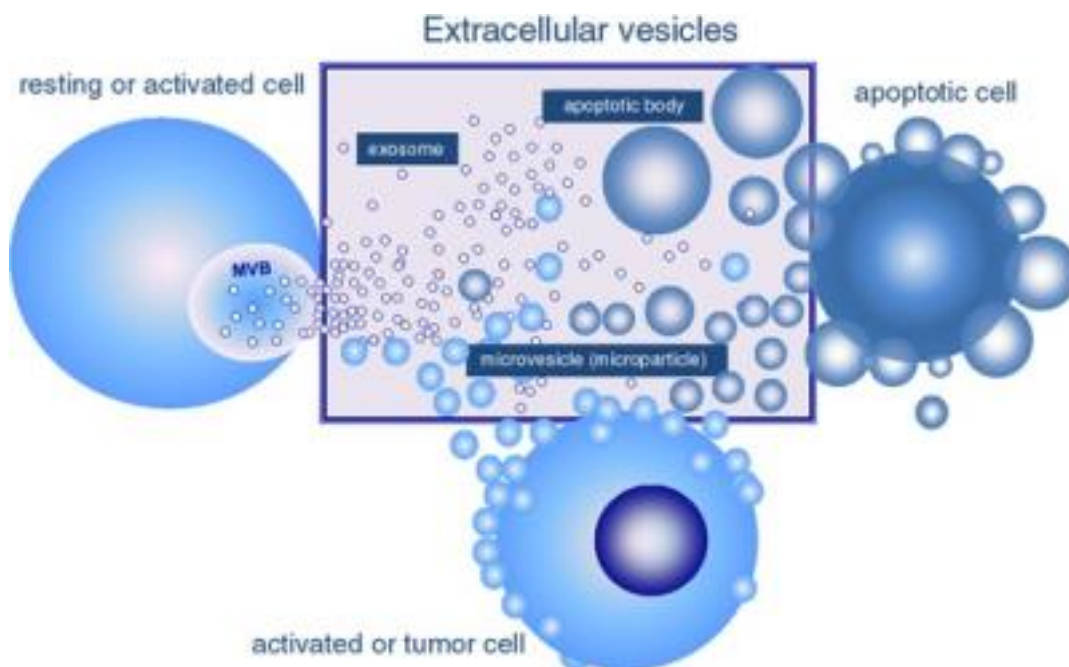


Figure 2.7 Amino-acid structure of ET-1. ([http://www.europeanurology.com/article/S1569-9056\(08\)00169-3/fulltext/endothelin-receptors-as-therapeutic-targets-in-castration-resistant-prostate-cancer](http://www.europeanurology.com/article/S1569-9056(08)00169-3/fulltext/endothelin-receptors-as-therapeutic-targets-in-castration-resistant-prostate-cancer)).

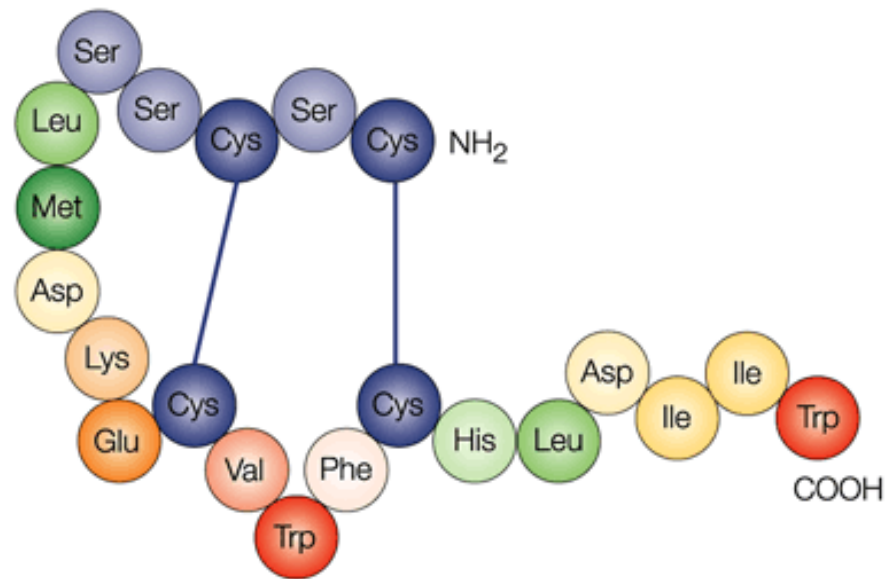


Figure 2.8 Regulation and processing of ET-1 from the ET-1 gene in endothelial cells. (<http://www.endothelins.com/Endothelin%20Biology/>)

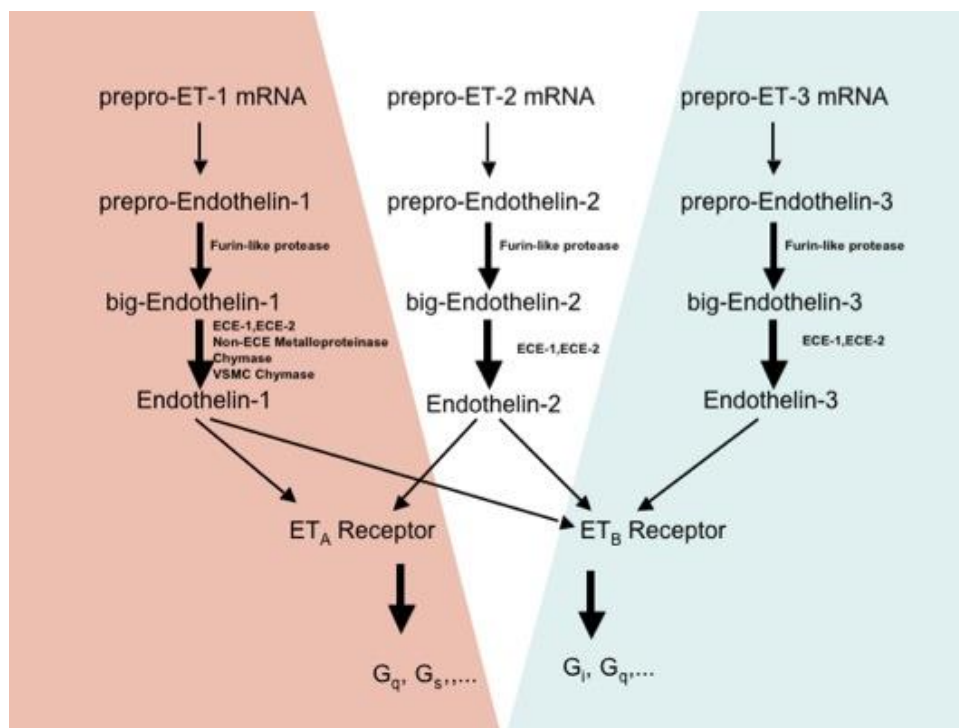


Figure 2.9 Downstream functions of endothelin receptors. Parentheses indicate functions infrequently mediated by corresponding receptor.
(http://www.nature.com/nrc/journal/v3/n2/fig_tab/nrc990_F2.html)

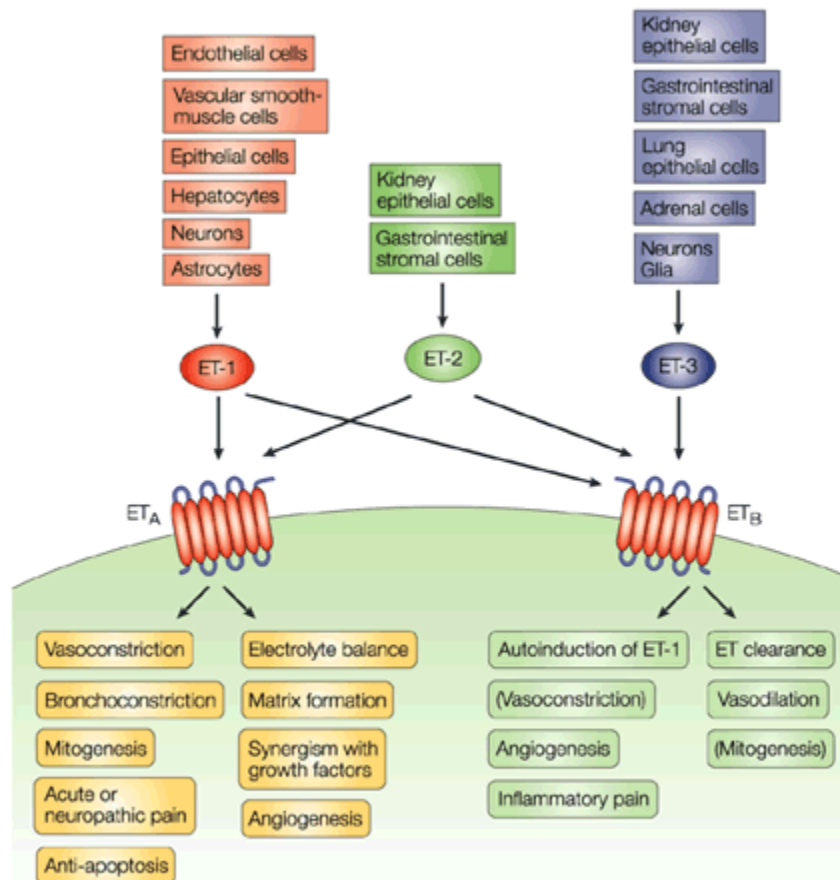


Figure 2.10 Endothelin A receptor binding by ET-1 triggers activation of multiple signal transduction pathways in cancer cells. Activation of PLC results in mobilization of calcium through IP₃ and PKC activation through DAG. Along with PTKs, FAK and paxillin, PLC activation ultimately results in activation of the RAF/MEK/MAPK pathway. Signaling through ET_AR also activates PI3K, which results in Akt survival pathway stimulation and increased protein translation by the mTOR signaling pathway. PI3K also initiates ILK-dependent GSK-3 β phosphorylation which results in stabilization of SNAIL and β -catenin ultimately resulting in transcription of genes involved in EMT. Cross-signaling through activation of c-Src and subsequent transactivation of EGFR is partly responsible for MAPK activation. Finally, ET-1 promotes COX-1 and -2 expression, PGE₂, and VEGF production.
(<http://www.nature.com/nrc/journal/v13/n9/full/nrc3546.html>)

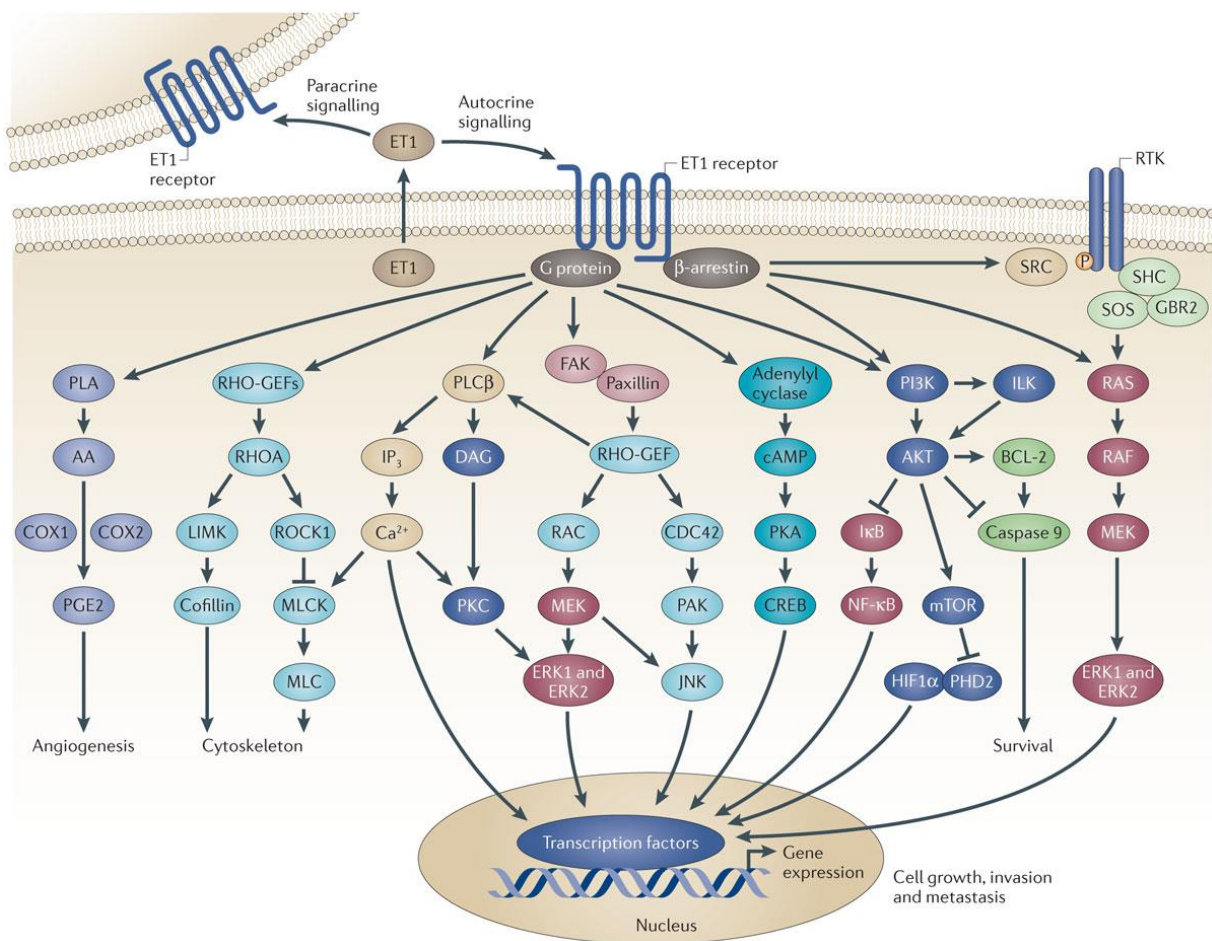


Figure 2.11 Endothelin-1/ET_AR signaling in tumor cells enables several aspects of cancer growth and progression. In many cancers, ET-1 results in cell proliferation, cell survival through inhibition of apoptosis, angiogenesis, invasion and metastatic spread, and modulation of tumor-infiltrating immune cells. In prostate and breast cancer, ET-1 induces osteoblast proliferation and decreases osteoclastic bone resorption resulting in osteoblastic bone metastases. (Bagnato and Rosanò, *International Journal of Biochemistry & Cell Biology*, 40(8), 1443-1451, 2008).

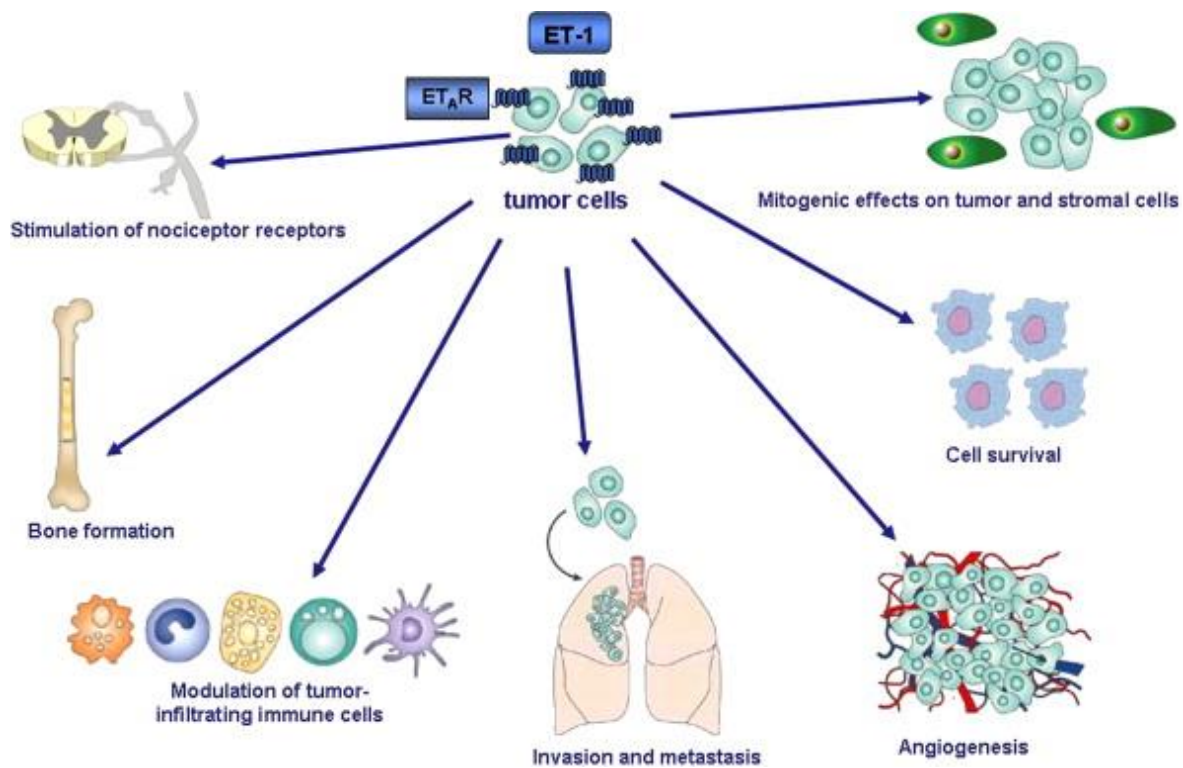


Figure 2.12 Endothelin-1 stimulates osteoblast proliferation and new bone formation while decreasing osteoclast functions. Multiple growth factors are affected and effect ET-1 signaling. (http://www.nature.com/nrc/journal/v3/n2/fig_tab/nrc990_F4.html)

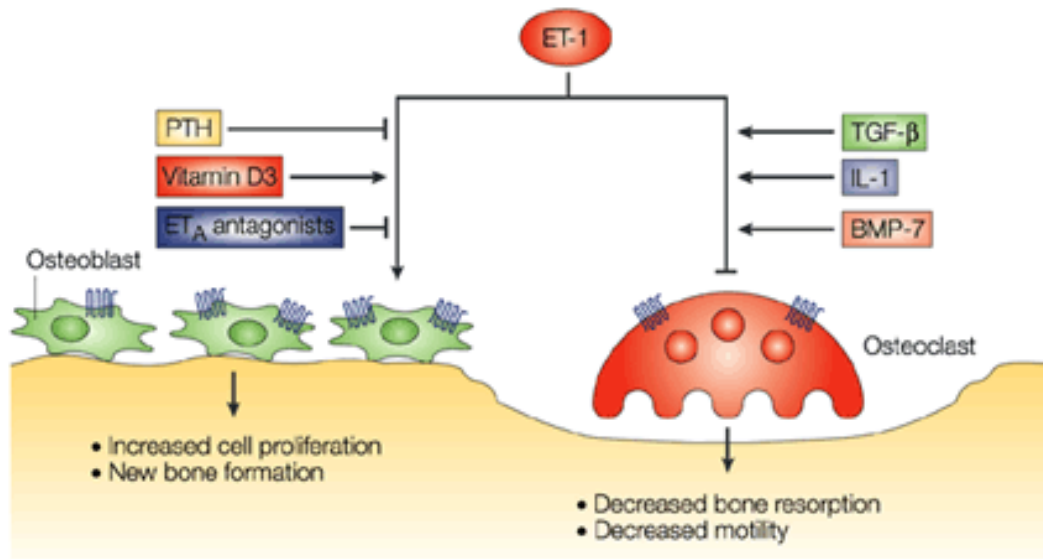


Figure 2.13 Endothelin-1 binding to ET_AR results in activation of intracellular PLC and subsequent activation DAG and IP₃. (http://www.nature.com/nrc/journal/v3/n2/fig_tab/nrc990_F3.html)

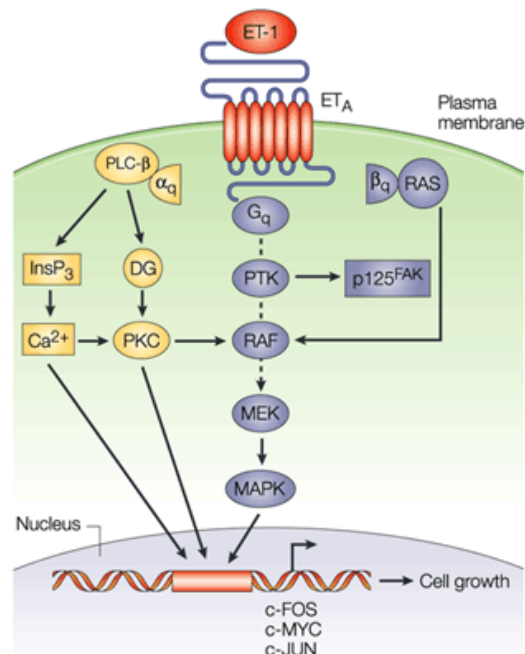


Figure 2.14 Tumor cells produce multiple growth factors that support the development of osteoblastic metastases.
(<http://img.medscape.com/fullsize/migrated/editorial/cmecircle/2005/4898/images/489803.gif>)

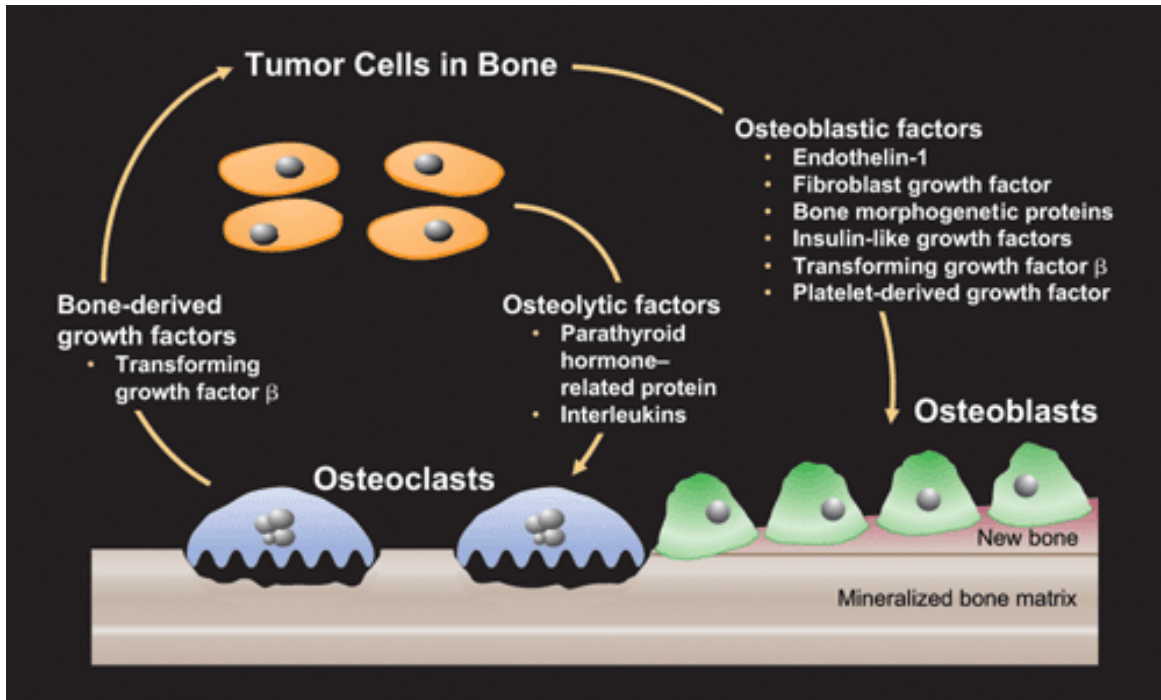


Figure 4.1 Endothelin-1 and ET_AR are expressed by canine OS cell lines. Jurkat is a positive control for ET_AR.



Figure 4.2 Effects of ET-1 and ABT-627 on the expression levels of pAkt and Akt in serum starved Abrams and HMPOS cells. **(a)** Increased phosphorylation of Akt in HMPOS cells treated with ET-1 and 10% FBS can be seen visually with darkening of the bands. Cells treated with ABT-627 and the combination of ABT-627 and ET-1 show decreased band weight. **(b)** Treatment with ABT-627 alone resulted in mild reduction in the ratio of pAkt/Akt while incubation with ABT-627 followed by ET-1 stimulation resulted in more marked reductions in the Abrams cell line. Mild increases in pAkt/Akt ratio are noted with ET-1 and FBS treated cells.

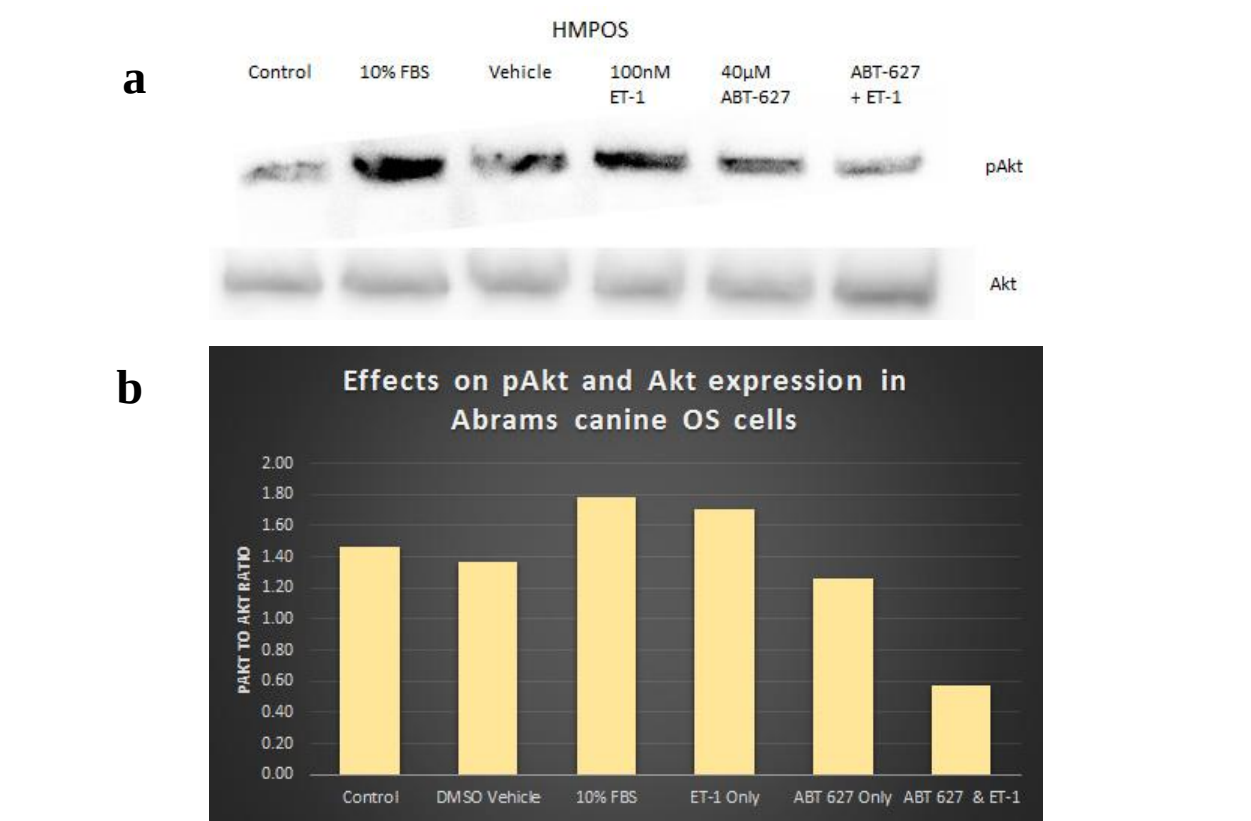
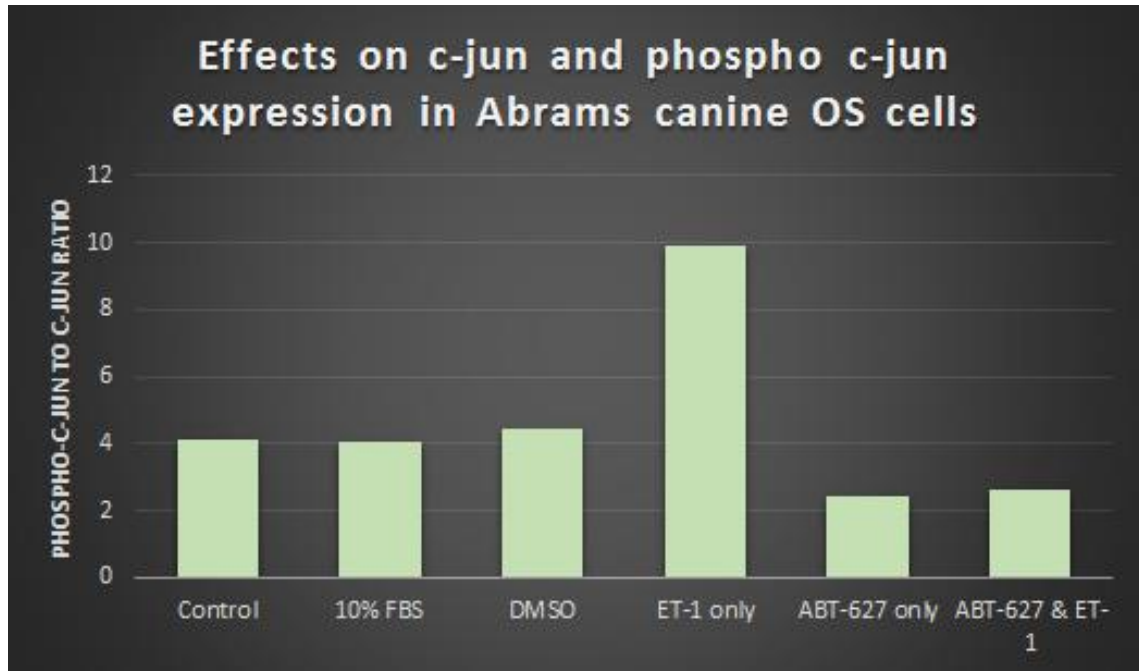


Figure 4.3 Effects of ET-1 and ABT-627 on the expression levels of phospho-c-jun and c-jun in **(a)** Abrams and **(b)** HMPOS cells. Treatment with ET-1 resulted in production in the expression of phospho-c-jun compared to expression levels of c-jun. Treatment with ABT-627 alone or followed by ET-1 stimulation resulted in reduction in the ratio of phospho-c-jun/c-jun.

a



b

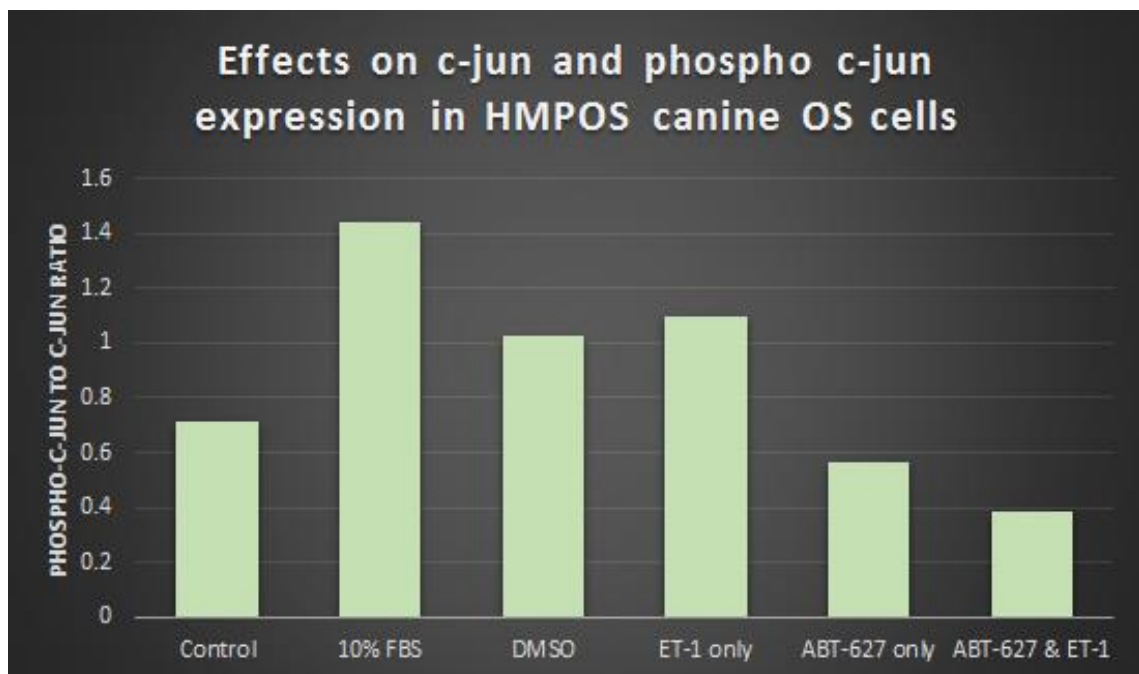


Figure 4.4 Effects of ABT-627 and ET-1 treatment on colony forming ability of canine OS cells. **(a-b)** In the Abrams and D17 cell lines, there was a statistically significant difference in number of colonies formed at 30 μ M and 40 μ M ABT-627 compared to control and vehicle wells ($*p < 0.01$). **(c)** In the HMPOS cell line, there was a statistically significant difference in number of colonies formed at 40 μ M ABT-627 ($*p < 0.01$).

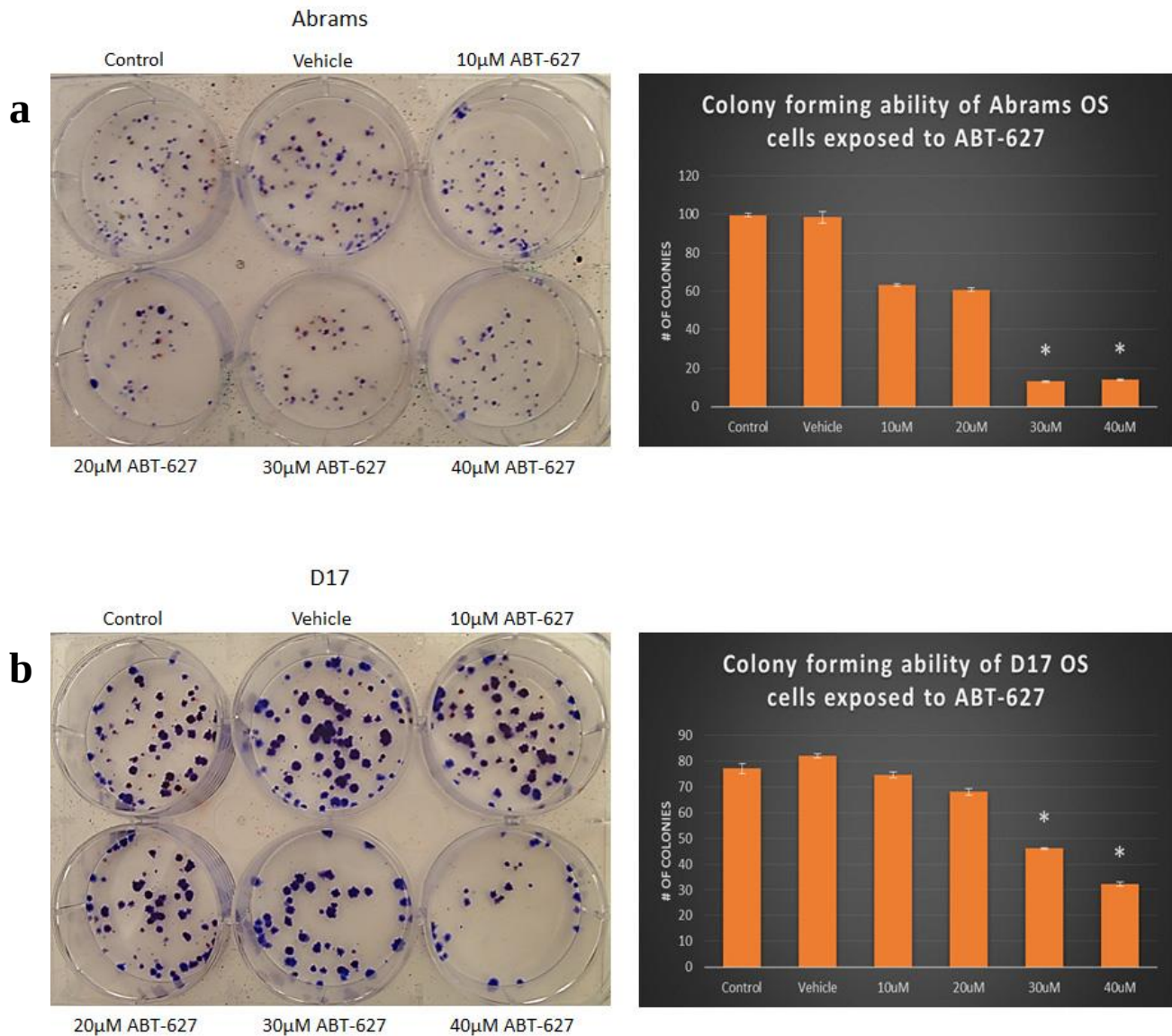


Figure 4.4 (cont.)

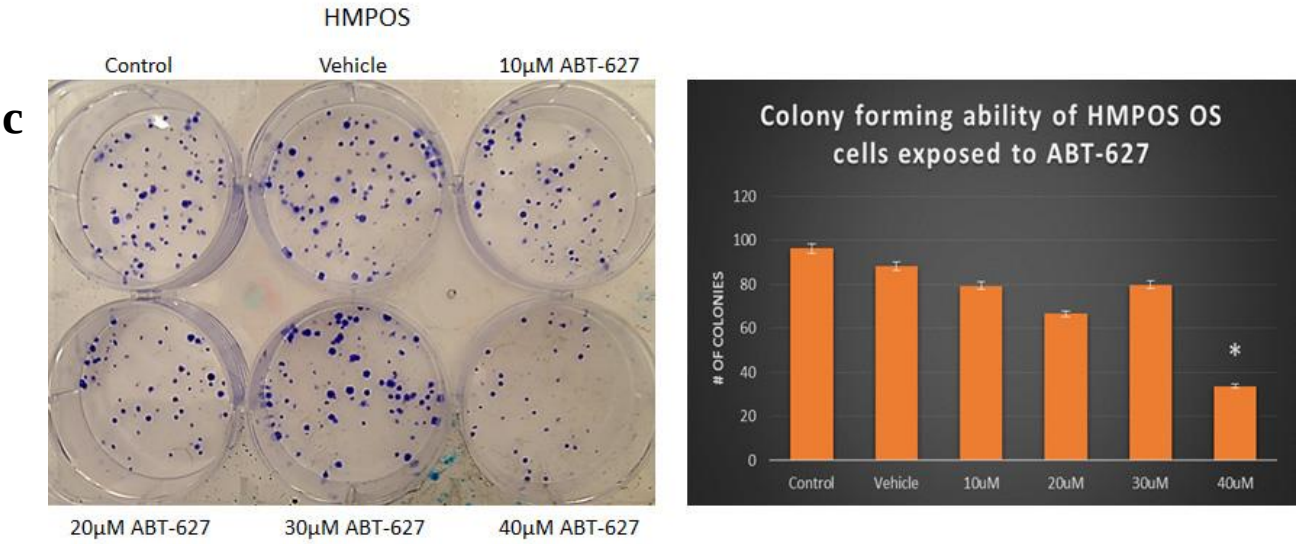


Figure 4.5 Treatment with ABT-627 inhibits while ET-1 promotes cell migration in a scratch assay. A monolayer of cells was scratched and cells were then treated with ET-1 or ABT-627 at indicated concentrations. Coverage of defect cells was documented by photomicrographs after a 24hr period incubation. **(a)** Treatment with ABT-627 inhibited scratch closure in the Abrams cell line at all concentrations after 24 hours exposure and at 30 μ M and 40 μ M ABT-627 when incubated for 48 hours ($*p < 0.01$). **(b)** Treatment with ABT-627 significantly inhibited the D17 cell line at 20-40 μ M ABT-627 at 24 hours and at 30 μ M and 40 μ M ABT-627 when incubated for 48 hours ($*p < 0.01$). **(c)** In the HMPOS cell line, inhibition of scratch closure was seen at 40 μ M ABT-627 at both the 24 and 48 hour exposures ($*p < 0.01$). **(d)** Treatment with ET-1 promoted scratch closure at higher concentrations of 10 nM and 100 nM in the Abrams cell line, **(e)** at concentrations of 1 nM-100 nM in the D17 cell line, **(f)** and at 100 nM in HMPOS cell line ($*p < 0.05$). Images are representative of 2 separate experiments.

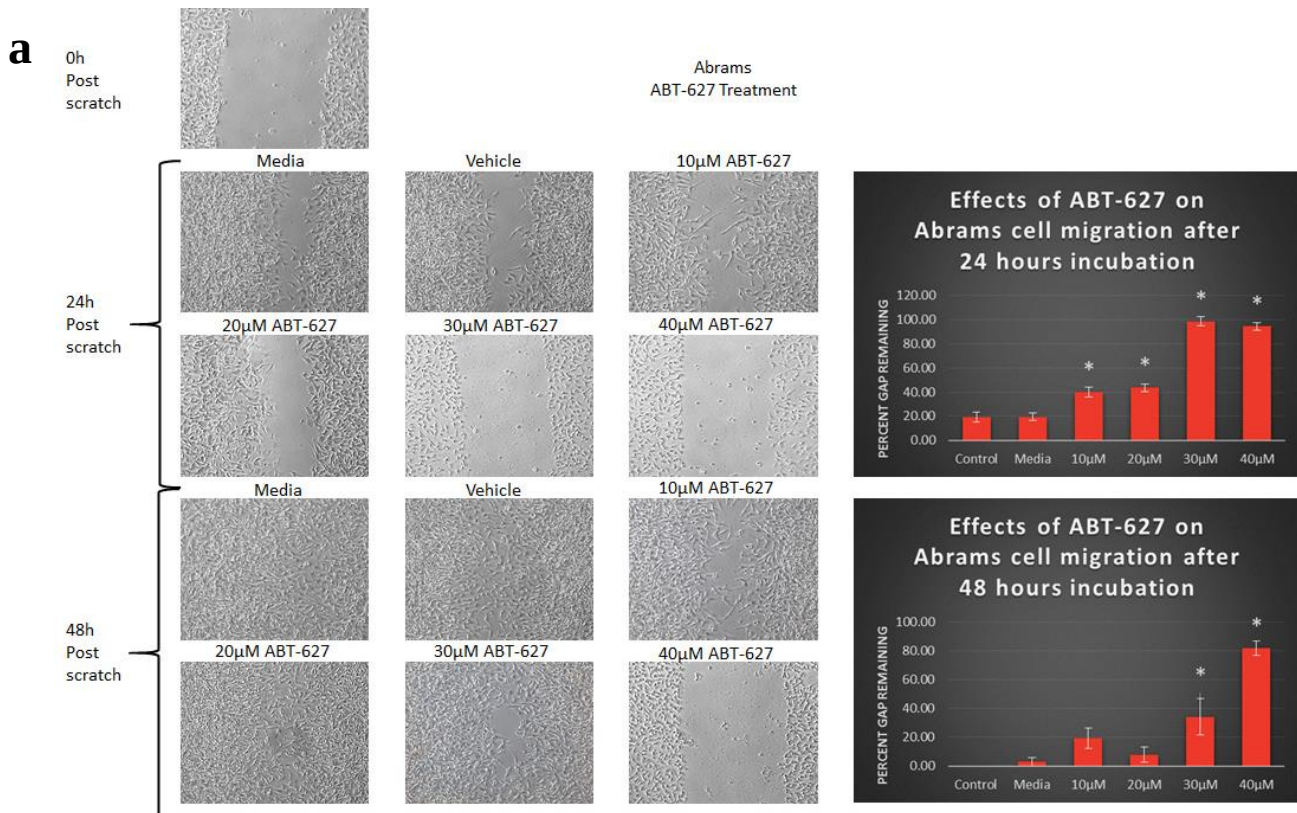


Figure 4.5 (cont.)

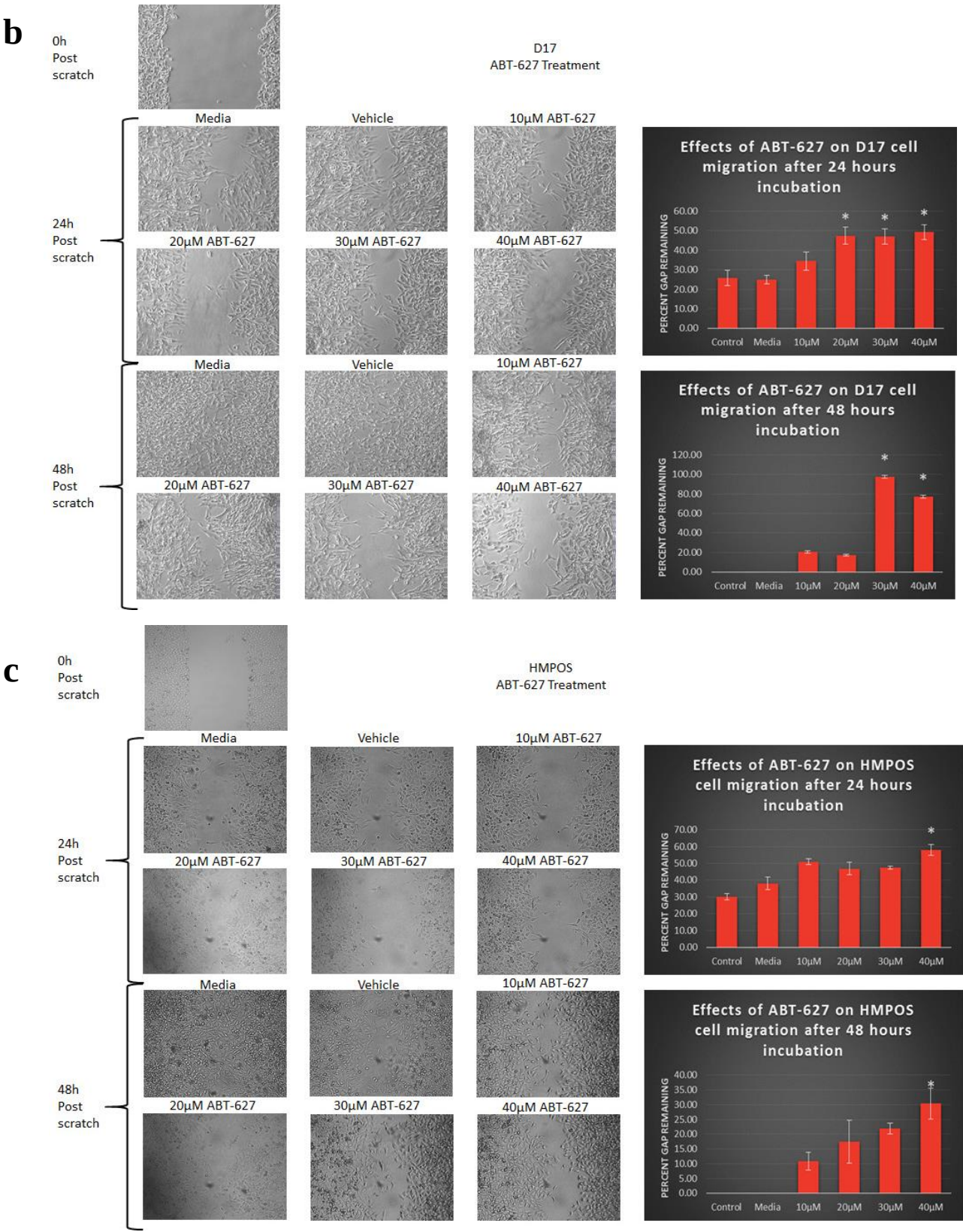


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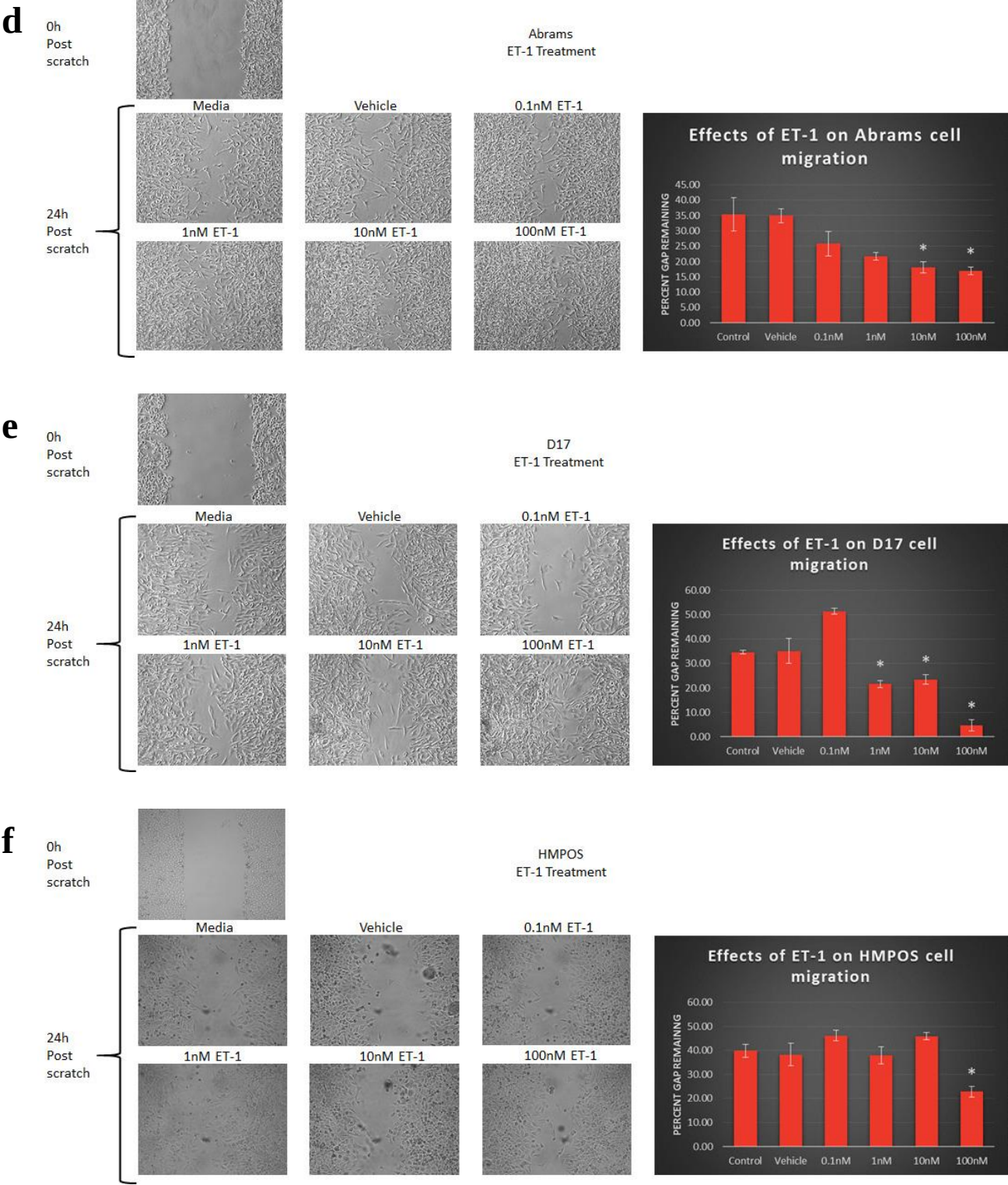


Figure 4.6 Cytochemistry of OS cell lines following exposure to ABT-627 and combination therapy of ABT-627 followed by ET-1. Chamber slides were exposed to varying conditions in serum free medium for 48 hours prior to 8 and 15 minute NBT/BCIP incubation times for the HMPOS and Abrams cell lines, respectively. Pixel quantification of BALP staining of representative areas of each chamber. **(a & c)** Pictures of BALP ICC slides and corresponding inverse images utilized for pixel quantification. **(b)** The HMPOS cell line demonstrated significant decreases in BALP staining after treatment with ABT-627 and combination treatment of ABT-627 followed by ET-1 ($*p < 0.001$). **(d)** There was no significant difference between treated and untreated conditions in the Abrams cell line.

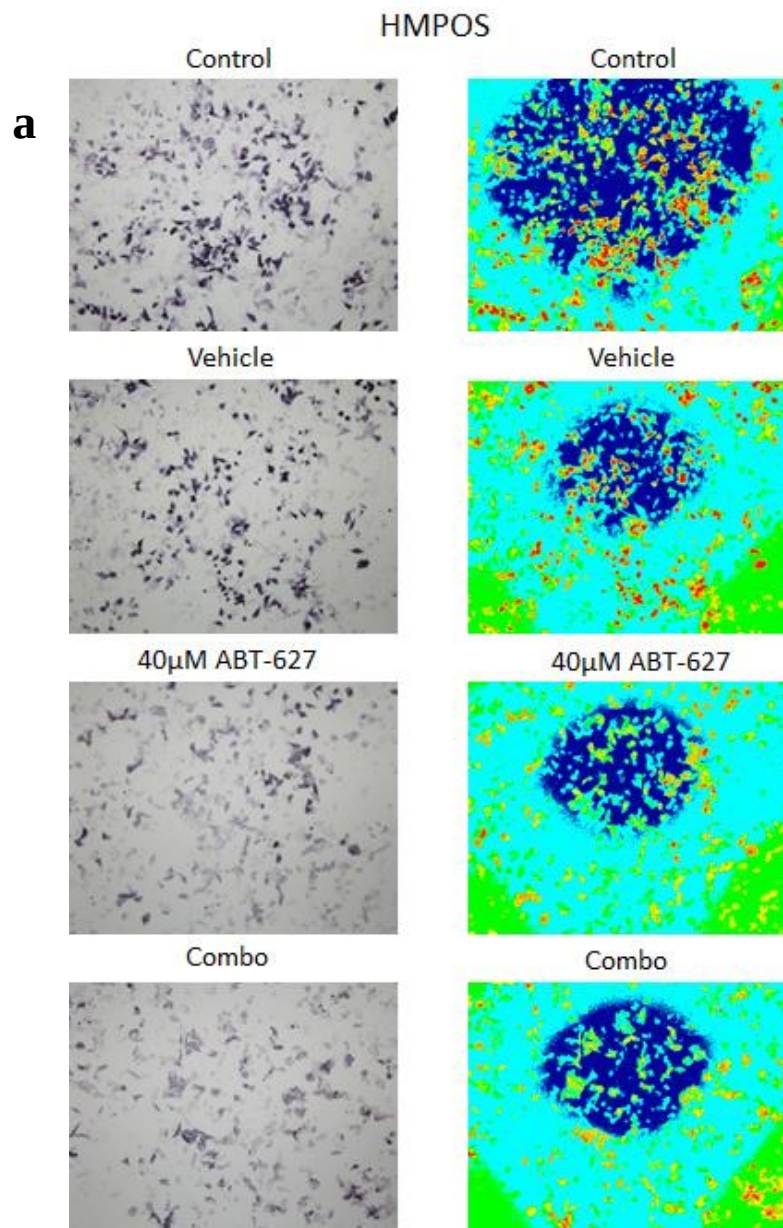


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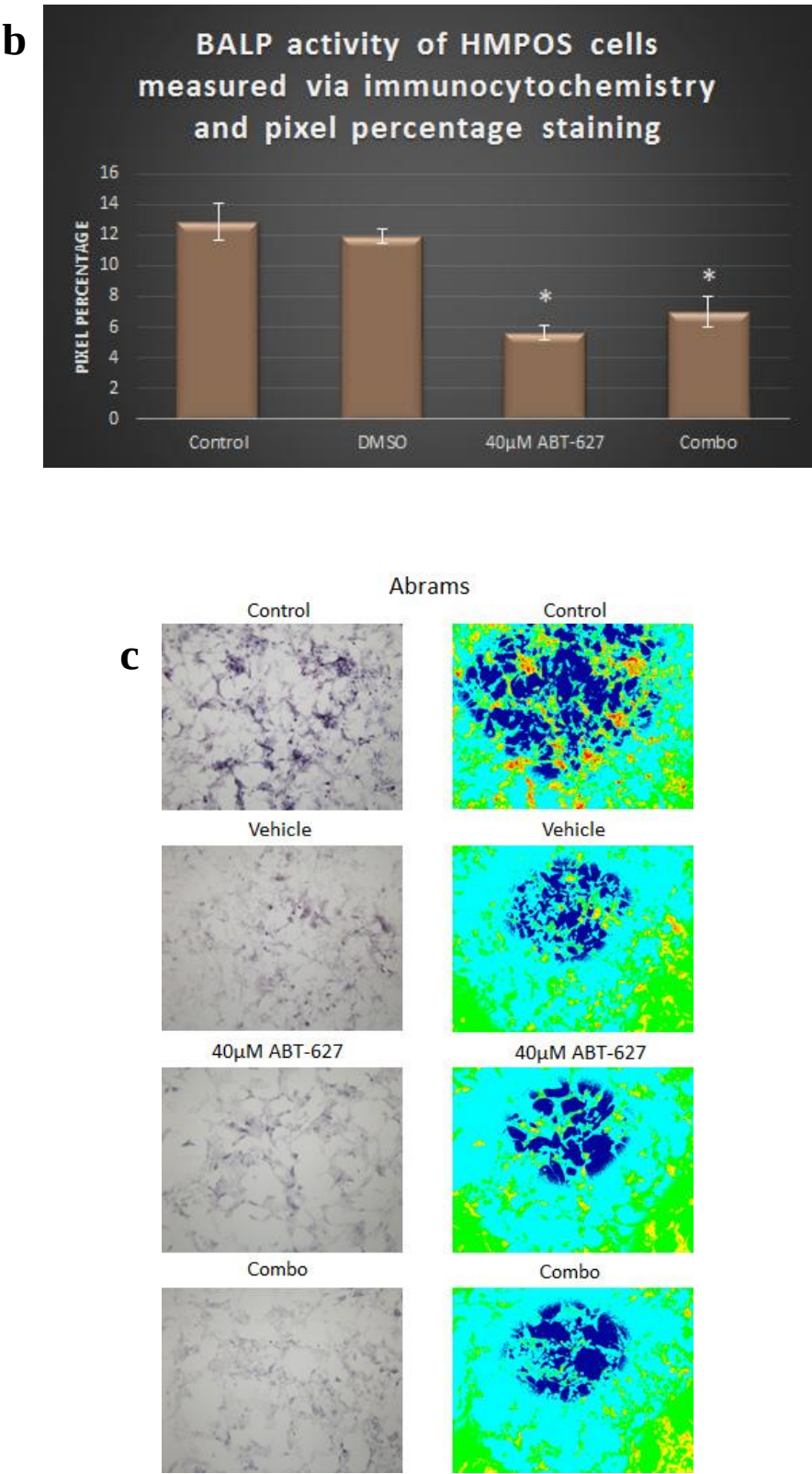


Figure 4.6 (cont.)

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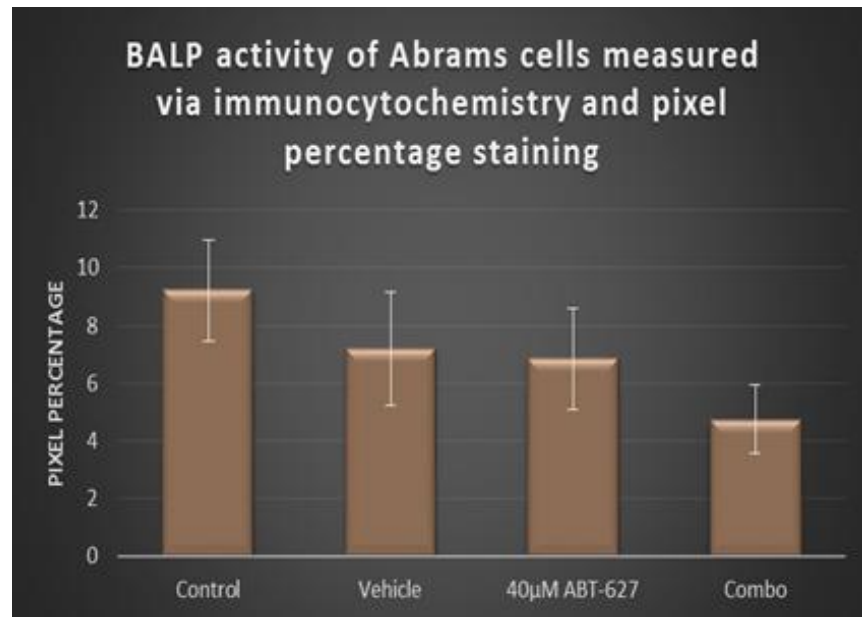


Figure 4.7 Effects of ABT-627 and combination therapy of ABT-627 followed by ET-1 stimulation on net release of BALP activity from HMPOS cells as measured via ELISA. Replicate cultures (n=2) of HMPOS cells were exposed to varying conditions in serum free medium for 24 hours. Bone alkaline phosphatase activity (U/L) was measured in duplicate aliquots of the (a) cell-conditioned medium and (b) normalized to cell layer protein. (c) Immunoblotting analysis of cell-conditioned medium correlating with BALP activity from ELISA. Data shown as group mean values $\pm$ SEM. Significant difference compared to control and vehicle (* $p < 0.01$).

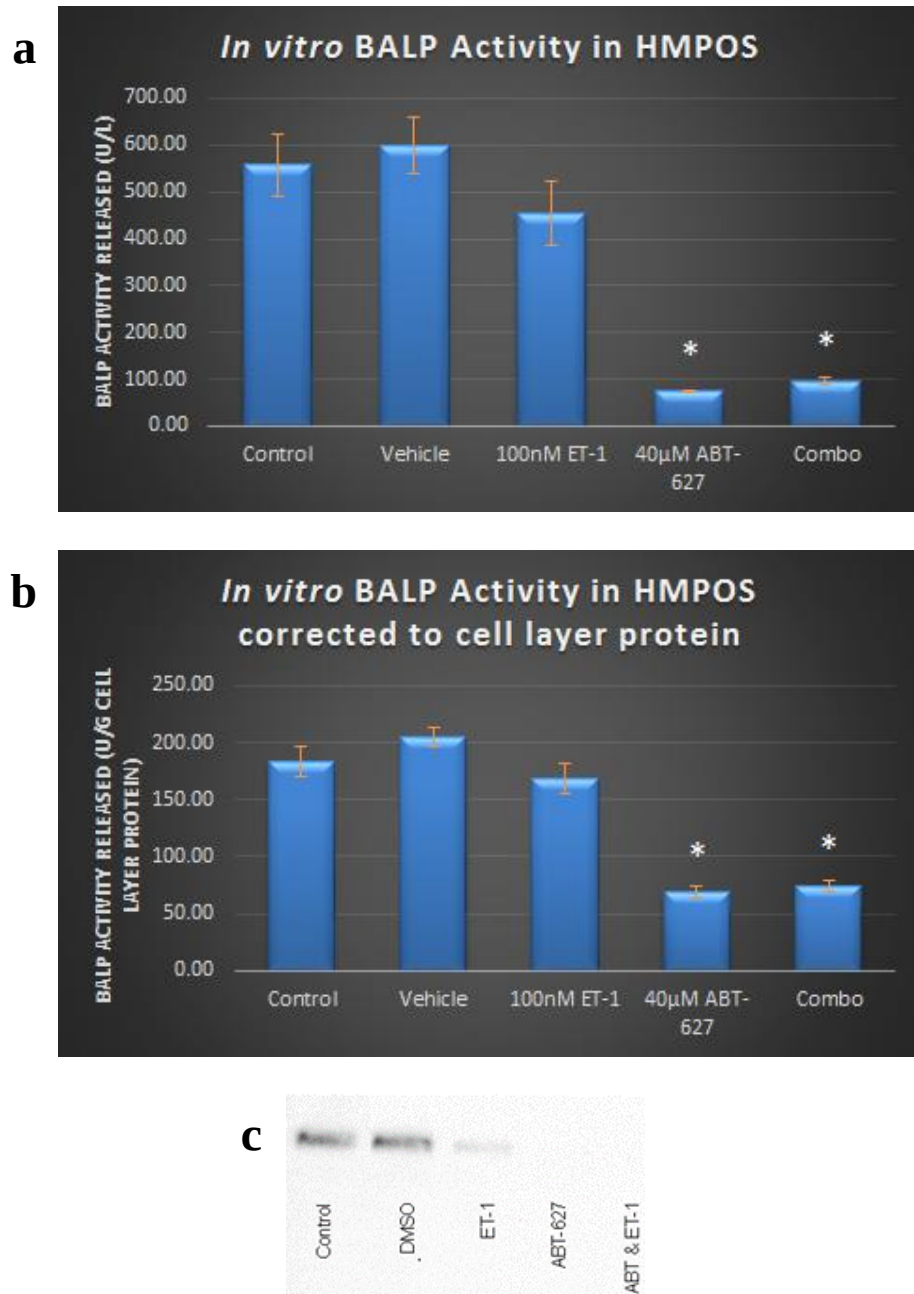


Figure 4.8 Separation of clinical canine OS patients into upper 25% quartile (osteoblastic) and lower 25% quartile (osteolytic) categories as measured by rBMD via DEXA scan.

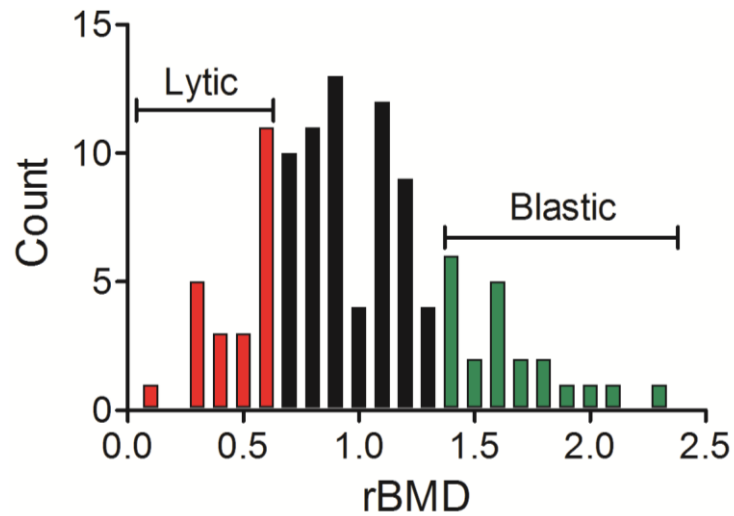


Figure 4.9 Osteoblastic tumors have significant increases in serum BALP activity when compared to osteolytic tumors. (* $p < 0.01$)

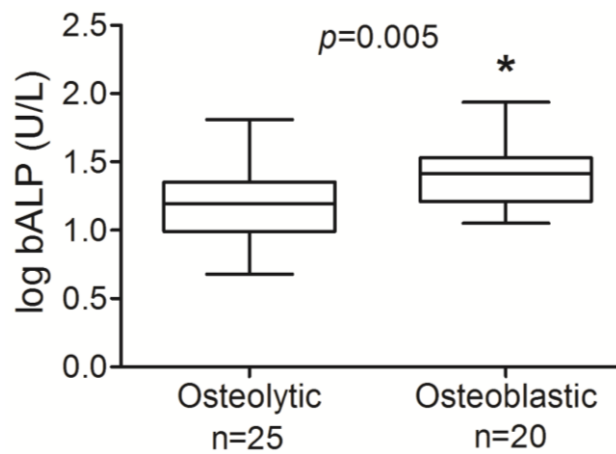
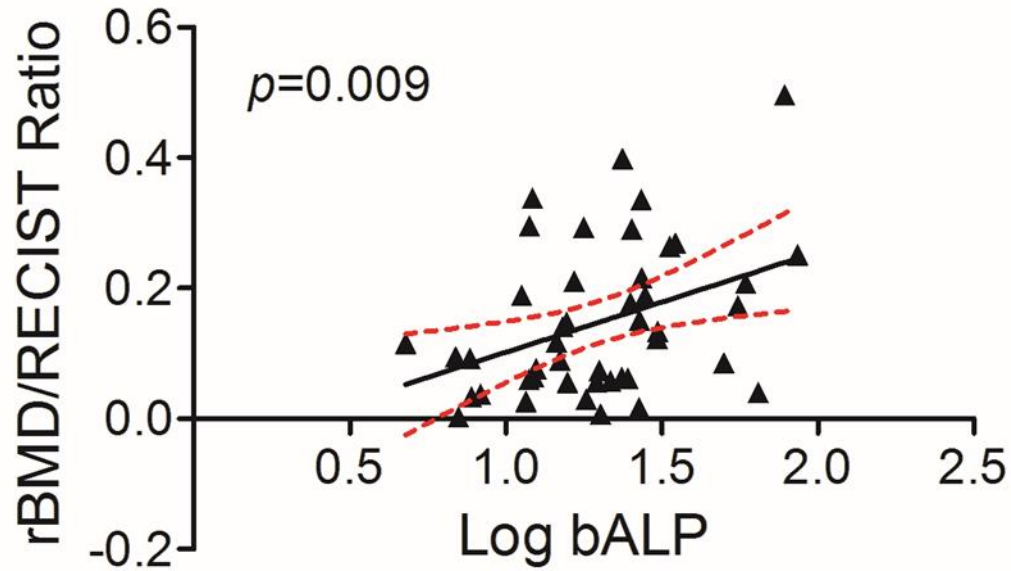


Figure 4.10 To account for tumor size as a cause of elevated BALP activity, a ratio of rBMD to RECIST was performed and compared to log transformation of serum BALP activity. There was a significant correlation between this ratio and serum BALP activity with an increased ratio (more osteoblastic tumors) having elevated BALP activity. ($*p < 0.01$)



CHAPTER 7

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