CHARACTERIZATION AND INHIBITION OF CANINE OSTEOSARCOMA TUMOR-INITIATING CELLS

A Dissertation

by

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ABSTRACT

Controlling metastatic and invasive tumors is the challenging part of treating cancer. The overall aim of this dissertation was to target these aggressive cells, called tumor-initiating cells (TICs). They exploit embryonic stem cell genes that should not otherwise be activated, allowing them their aggressive capabilities. Osteosarcoma is the most common bone cancer of children. Canine osteosarcoma was selected as the type of cancer to study because of its predictable and similar behavior to human osteosarcoma. Human osteosarcoma has an analogous gene signature to canine osteosarcoma, but a slightly prolonged length of remission due to more aggressive therapy. The goals accomplished in this dissertation were: 1) Optimized a method using epirubicin to enrich for TICs; 2) Used molecular techniques to determine the phenotypic differences in canine osteosarcoma TICs compared to typical cancer cells; 3) Evaluated two classes of drugs that specifically target the unique phenotypes in TICs *in vitro* and *in vivo*. These drug classes were smoothened-inhibitors and Erb-B2 receptor tyrosine kinase 2 (HER2)-inhibitors.

Epirubicin reliably enriched for phenotypically different TICs. Embryonic stem cell genes were expressed in TICs but not the differentiated tumor cells. A second embryonic stem cell pathway, the hedgehog pathway, is also upregulated in TICs. Two smoothened-inhibiting drugs, cyclopamine and

vismodegib, were used to block the hedgehog pathway in canine osteosarcoma cell lines in both *in vitro* and *in vivo* studies.

The second target, HER2, was upregulated in both differentiated osteosarcoma cells and TICs. Lapatinib was used to block HER2 of TICs *in vitro* resulting in a significant cell death, which was demonstrated in viability assays. HER2 was also downregulated in RT-qPCR. Canine osteosarcoma mouse xenografts treated with lapatinib showed inhibition of tumor growth and tumor cell death.

In conclusion, these studies have demonstrated the benefit of using therapies that target specific cancer cell subtypes through TIC-specific targets. Vismodegib performs best at slowing the growth of metastases formed by TICs, whereas lapatinib also causes TIC and differentiated tumor cell death. Future studies are intended to evaluate these drugs in dogs post-amputation. Human clinical trials will follow if successful.

DEDICATION

My dissertation is dedicated to those I have lost to cancer while conducting my research.

Juan Carlos Robles-Emanuelli was a kind mentor when we were at Michigan State University and a supportive colleague while we were graduate students at Texas A&M University. He will be missed.

Jim Cetlinski brought so much joy and love to everyone around him. Thank you for giving my friend the true love that she deserved.

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NOMENCLATURE

ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2
AKT1	V-Akt murine thymoma viral oncogene homolog 1
AO/PI	Acridine orange and propidium iodide
CD34	CD34 molecule
CD44	CD44 molecule
CD117	V-Kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CD133	Prominin 1
CSCs	Cancer stem cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FDA	Federal Drug Administration
G_0	G_0 resting phase
GLI	Glioma-associated oncogene family zinc finger transcription factors
GLI1	Glioma-associated oncogene family zinc finger 1
GLI2	Glioma-associated oncogene family zinc finger 2
GLI3	Glioma-associated oncogene family zinc finger 3
H&E	Hematoxylin and eosin
HER2	Erb-B2 receptor tyrosine kinase 2

HH	Hedgehog
Ki-67	Marker of proliferation Ki-67
LC	Lethal concentration
mRNA	Messenger ribonucleic acid
NANOG	Nanog homeobox
OCT4	POU class 5 homeobox 1
P21	Cyclin-dependent kinase inhibitor 1A
P27	Cyclin-dependent kinase inhibitor 1B
P53	Tumor protein P53
PBS	Phosphate buffered saline
PTCH1	Patched 1
qPCR	Quantitative real-time polymerase chain reaction
R10	R10 complete medium
RNA	Ribonucleic acid
RT-qPCR	Reverse transcription real-time polymerase chain reaction
SHH	Sonic hedgehog
SMO	Smoothened
SOX2	SRY (sex determining region Y)-box 2
TICs	Tumor-initiating cells

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INTRODUCTION

Preface

The importance of targeting metastases

For much of cancer research history, scientists and doctors have become successful at developing therapies that destroy primary tumors. Nevertheless, metastatic disease ultimately kills the patient. Over thirteen million people in the United States are currently battling cancer [1]. The American Cancer Society estimates that there will be 1,658,370 new cancer patients diagnosed and 589,430 cancer deaths in 2015 [2]. Cancer is the second leading cause of death in the United States [3].

In veterinary medicine, cancer is even more prevalent in dogs. It is much more difficult to determine how many dogs are diagnosed with cancer annually. The most accurate estimate is four million dogs [4]. This estimate places dogs at a much higher cancer rate than humans given that there are fewer dogs in the United States (5,300/100,000 for dogs versus 500/100,000 for humans). This dissertation uses canine osteosarcoma as a model for metastatic disease. The information gathered from the drugs evaluated is intended to predict treatment response in both human and canine patients, and possibly other forms of metastatic cancer.

Osteosarcoma is a devastating bone cancer with a very poor prognosis [5-7]. The osteosarcoma pathogenesis is identical between dogs and humans, making dogs an excellent spontaneous model for study [8-10]. In both species, the primary tumor can be successfully managed. However, osteosarcoma tumors are known to seed metastases throughout the body early in the disease process, especially to the lungs, which results in death [11-13]. Drugs that prevent metastases from occurring or target metastases for death are highly desirable.

Canine osteosarcoma

Osteosarcoma is the most common primary bone tumor of dogs [8, 14-19]. Large to giant breed dogs are more commonly affected. Affected breeds include greyhounds, rottweilers, German shepherds, doberman pinschers, Scottish deerhounds, great Danes, Afghan hounds, Irish wolfhounds, Borzois, Leonbergers, Irish setters, Saint Bernards, golden retrievers, Labrador retrievers, and mastiffs [12, 20-27]. The American Kennel Club Canine Health Foundation estimates that there are 10,000 new cases of canine osteosarcoma annually [28]. While others estimate up to 75,000 new cases of canine osteosarcoma are diagnosed annually [4]. National monitoring programs do not exist for dogs, so we may never know the exact number. But we can be certain that osteosarcoma is more prevalent and no less deadly in the canine population than in the human population.

With aggressive therapy only 50% of dogs will survive one year from diagnosis and less than 20% of dogs will survive two years from diagnosis [8, 11-13, 29]. Aggressive therapy consists of amputation followed by adjuvant chemotherapy. The cause of death is rarely attributed to the primary tumor because most primary bone tumors are able to be surgically removed. Instead, the high mortality is due to metastases in the lungs, or less likely in other bones or soft tissues (i.e. lymph nodes, kidney, and liver) [8, 12, 30-33].

Human osteosarcoma

Osteosarcoma is also the most common primary bone tumor of humans [34]. About 800 patients are diagnosed with osteosarcoma each year in the United States with half of that number comprised of children and teenagers [35]. Osteosarcoma patients are treated more aggressively and with more modern chemotherapeutic agents compared to dogs. Instead of amputation, they are often candidates for radical limb-sparing surgery [5, 7, 36]. Patients with localized osteosarcoma have a five-year disease-free survival rate of 71% [1]. Similar to dogs, approximately 20% of osteosarcoma patients have metastatic disease at diagnosis [37]. The five-year survival rate for patients that have metastatic disease at the time of diagnosis or patients with recurrent disease is poor [6, 38, 39].

Very few advances have been made in the treatment of osteosarcoma over the last 15-20 years. This is due to a lack of fundamental knowledge

regarding the tumorigenesis of osteosarcoma. It is crucial that we gain a better understanding of the mechanisms of tumor progression and metastasis in order to develop more effective therapies. Useful animal models are necessary to develop such therapies.

Dogs as a spontaneous model of osteosarcoma

Dogs and cats spontaneously develop cancers with the same genetic profiles, histologic diagnoses, and biological behavior as in humans. The tumor types include round cell neoplasms (i.e. non-Hodgkin's lymphoma, leukemia, myeloma), sarcomas (i.e. osteosarcoma, soft tissue sarcomas, melanoma), brain tumors (i.e. meningioma, glioma/glioblastoma), and carcinomas (i.e. mammary/breast tumors, oral/nasal carcinomas, prostate cancer) [40]. This gives the researcher a wide variety of spontaneous tumors to evaluate. The canine spontaneous model for cancer cannot and should not replace rodent models. Murine models are known for being inherently inbred, but this provides accuracy and precision when controlled experimentation is necessary [41]. Less than 8% of treatments tested in rodents are successfully translated to human therapies [42]. This is due to vast differences between humans and rodent models. For example, mice can tolerate much higher doses of drugs [43]. Tumors grown in mouse xenografts may not vascularize properly or grow in the proper niche. Finally, the compromised immune system of murine models does

not mimic what happens in human cancer [44, 45]. Taken together, these things grossly overestimate potential clinical responses in humans.

Unlike laboratory rodent models, our pets share the same environment as humans, and thus share the same environmental risk factors for cancer [46]. Dogs and cats have a heterogeneous genome, an intact immune system, and have been shown to respond to the same therapies used in humans [9, 10, 47]. Another advantage is their large size, which allows for multiple blood samplings, advanced diagnostic imaging, and realistic surgeries [10]. Human and canine genomes have more homology compared to human and murine genomes [48-54]. All of these key features support using spontaneous canine models for evaluating the efficacy of treatments for extrapolation to human use.

Human clinical trials start by using patients in the latest stages of disease and use a low dose of the therapy in question [55]. It takes many years and many cohorts of patients before the therapy can be evaluated for safety and efficacy at the optimal dosage. Dogs, on the other hand, have an accelerated aging process compared to humans and often an accelerated cancer progression [48, 56-58]. One year of a large-breed dog's life is equivalent to six years of human life. This allows the researcher to translate canine one-year survival rates to predict human five-year survival rates in a specific study. Also, the therapeutic end-point results of canine clinical trials are gathered more quickly. Due to the lack of standards of care in veterinary medicine, owners are able and often willing to consent to clinical trials early in the disease process

allowing for treatment of naïve diseases with novel therapies [10]. Veterinarians are allowed to use more realistic doses and dose schedules for the therapy being evaluated in their canine patients. As stated earlier, dogs have a much higher prevalence of osteosarcoma compared to humans, which will allow trials to be adequately filled in a relatively short period of time. All of this adds up to knowing which therapies are most likely to help humans earlier and more accurately than if only experimental rodent models are used [47, 48, 59, 60].

Canine osteosarcoma closely mimics human osteosarcoma. The majority of osteosarcoma in both species develops at the metaphysis of long bones, metastases tend to occur in the lungs, and both species have similar genetic changes within the cancer [9, 10, 26, 48, 59, 61-68]. Dogs have already been used successfully as a model for human osteosarcoma [69]. Research dogs are a common species for toxicology studies, yet companion dogs are relatively new to being used to understand more about human cancer. The spontaneous model of canine osteosarcoma could lead to more effective therapies for dogs and humans after more studies are completed, especially for metastatic-targeting therapies. It is important for veterinarians to work closely with cancer researchers and physicians in this exciting endeavor.

Tumor-Initiating Cells in Cancer

An all too common example of cancer

It is important to briefly summarize the clinical presentation of cancer before discussing intricate details at the cellular level. Patients with late staged cancers involving macrometastases will not be discussed. The goal of this research is to target cells that form micrometastases, by either preventing the cells from proliferating or by eliminating them. This is representative of patients with early staged osteosarcoma.

The initial treatment objective for aggressive cancers is to remove as many cancerous cells as possible. The patient receives some type of surgery, such as a limb amputation or debulking of the tumor. The cancer may eventually return in the form of metastases if no other treatments are performed. Deductive reasoning indicates that cancer cells had to have travelled to other organs before surgery in order for recurrence to take place at a later time distant from the primary tumor. These cells are not detected by modern diagnostic imaging. This implies that these metastatic cells clearly have the ability to remain microscopic and dormant until conditions improve at a later time.

Survival times in patients may be improved with aggressive chemotherapy following surgery. Chemotherapy targets actively dividing cells [70]. As stated earlier, the metastatic cells are lying dormant; therefore, these drugs may not be effective in destroying them [71-77]. The end result is a

temporary remission with a cancer that is much more difficult to treat upon recurrence [78]. Ideally, patients should be treated with drugs that specifically target the dormant metastatic cells at the same time the primary tumor is being treated to prevent recurrence. Currently, no such treatment exists.

The complexity of cancer

Young scholars are taught about clonal evolution and the hallmarks of cancer, but this information is basic when it comes to understanding cancer progression in its entirety [79-85]. Most cancers take years or decades to develop by means of clonal evolution [80]. As an oversimplified explanation, a cell first acquires a neoplastic mutation. This cell with its unique and advantageous phenotype becomes more populated through clonal expansion. Eventually a cell with the unique phenotype will acquire another mutation. Additional cycles of clonal expansion and mutations occur. Some of these mutations are harmless. Other mutations contribute to carcinogenesis, such as inactivation of tumor suppressor genes and oncogene activation [86]. Most tumors require two to eight of such mutations in order to become neoplastic [87]. Carcinogenesis is achieved when the cells have acquired favorable mutations that enhance functional capabilities, designated the hallmarks of cancer [79]. These ten categories include sustaining proliferative signaling, insensitivity to anti-growth signals, tissue invasion/metastasis, limitless replicative potential,

inducing angiogenesis, evading apoptosis, tumor-promoting inflammation, and others (Table 1) [85].

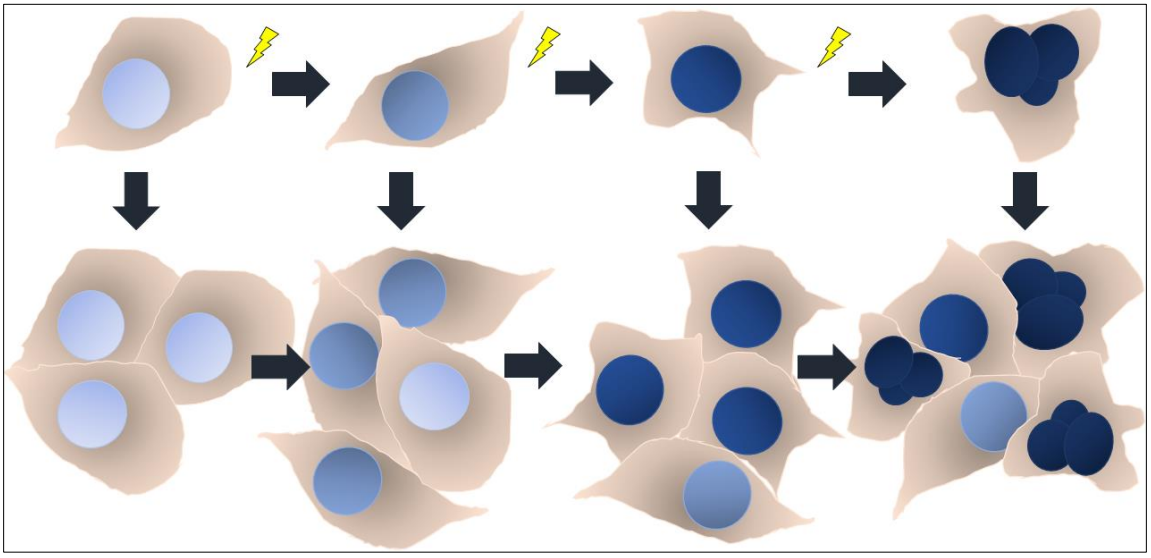

Figure 1. Clonal expansion in cancer depiction. Clonal evolution is composed of a series of mutations (lightning bolt) and expansions over time (top row). As carcinogenesis progresses, the cancer population becomes more heterogeneous (bottom row).

Table 1. The ten hallmarks of cancer adapted from Hanahan and Weinberg. [85]

Acquired Capability	Example
Evading growth suppressors	Dysregulation of tumor suppressor gene retinoblastoma protein
Sustaining proliferative signaling	Overexpression of epidermal growth factor
Deregulating cellular energetics	Increased glucose consumption by upregulating glucose transporter 1
Resisting cell death	Increased expression of the B-Cell CLL/Lymphoma 2 gene
Genome instability and mutation	Breakdown of breast cancer type 1 susceptibility protein
Inducing angiogenesis	Overexpression of vascular endothelial growth factor
Activating invasion and metastasis	Activation of tumor-associated macrophages
Tumor-promoting inflammation	Tumor necrosis factor alpha overexpression
Enabling replicative immortality	Loss of tumor protein 53 (p53)
Avoiding immune destruction	Activation of the indoleamine 2,3-dioxygenase pathway

The last several decades have brought us many treatments that target the functional capabilities of cancer. For example, bevacizumab blocks angiogenesis to starve the tumor and lomustine damages deoxyribonucleic acid (DNA) so that cells cannot replicate. Some oncologists have successfully treated patients with combinations of therapies to inhibit two or more arms of the functional capabilities simultaneously. Sadly, many patients achieve remission, but then later the cancer reemerges with resistance to the previously effective treatments. It was once thought that defeating cancer required targeting the functional capabilities. Certainly, survival rates have increased with new treatment strategies. Still, researchers do not fully understand how cancer reemerges and they have not discovered a successful pre-emptive treatment.

Tumor-initiating cells

The concept of cancer cell heterogeneity was described in the 1800s by Johannes Muller and Rudolf Carl Virchow shortly after they developed the specialty of microscopic pathology [88, 89]. Indeed, if all cancer cells within a tumor are identical, then any one cell would be able to repopulate the entire tumor. This is not the case. Cancer homogeneity was first disproven in 1962 when doctors performed autografts on cancer patients with less than 35% success [90, 91]. Cancers are heterogeneous and only certain cells can repopulate the tumor [72, 85, 92-98]. Yet, traditional cancer therapies treat the cancer as if it the cell population is homogeneous [99]. Development of the

heterogeneous tumor is a complex process that begins with cells that specifically populate the tumor. Understanding these cells is the key to developing new classes of treatments.

In 1875 a student of Virchow, Julius Cohnheim, proposed that cancer cells arise from initiating-type cells similar to what is observed in cellular propagation during embryogenesis [100]. This theory was revisited in the 1960s and 1970s, but fell short due to lack of technology [101-103]. Flow cytometry and immunocompromised mice became readily available in the 1990s. Finally, in 1994 researchers successfully isolated initiating cells of acute myeloid leukemia [104]. This was followed by identification of tumor-initiating cells (TICs) in a variety of cancers [92, 105-116]. One hundred forty years later, it is accepted by almost all oncology researchers that cancers arise from cells that have mutations affording them tumor-initiating abilities. These TICs are a subpopulation of plastic cells within the tumor that have the ability to repopulate the tumor [105, 106, 117-122]. The two strongest theories of tumor-initiation and propagation are the hierarchical model and the stochastic model. They explain the evolution of a mature cancer from the first cell by adding an additional layer of sophistication to the model of clonal evolution.

The hierarchical model of carcinogenesis

In the hierarchical model of carcinogenesis, a stem cell or a progenitor cell follows the path of clonal evolution. The stem cell acquires tumor-initiating

properties after a series of mutations (Figure 2B). Thus, these cells are called cancer stem cells (CSCs) [123-125]. Like stem cells, they retain the ability to self-renew, enter quiescence, but they also lack boundaries on cell proliferation [96, 126]. Unlike embryonic stem cells, CSCs divide symmetrically and asymmetrically, yet retain the ability to divide indefinitely [127].

A large magnitude of divisions is necessary to produce the first neoplastic cell. In adults, stem cells are relatively fewer in numbers and typically remain quiescent. Therefore, it would be mathematically improbable for stem cells to become CSCs in adult cancers [128]. In contrast, most of the common infant cancers originate from regenerative cells. Examples include neuroblastoma, leukemia, retinoblastoma, nephroblastoma, gonadal germ cell tumors, and hepatoblastoma [129, 130]. Therefore, the term “cancer stem cell” is more appropriate for infancy cancers. It is also used in some exceptions of adult cancers, such as teratomas or basal cell carcinomas, which also derive from stem or progenitor cells.

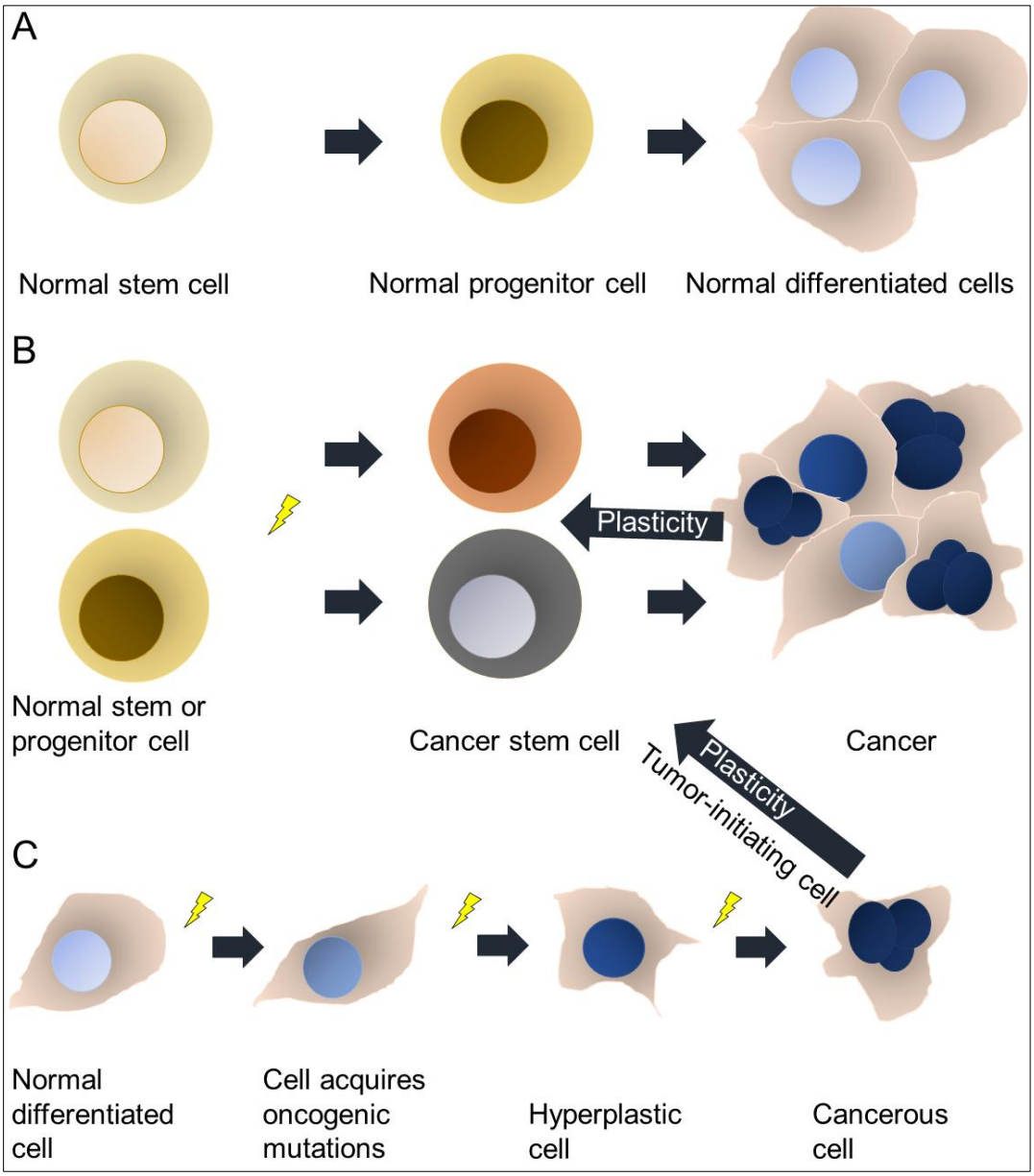

Figure 2. The hierarchical and stochastic models of carcinogenesis. (A) A normal stem cell has the capacity for self-renewal and may also give rise to progenitor cells. Progenitor cells give rise to differentiated cells. (B) If mutations cause normal stem cells or progenitor cells to become neoplastic, then they become cancer stem cells. These cells can populate a cancer. The cancer cells have the ability to dedifferentiate back into cancer stem cells. This is the hierarchical model. (C) In the stochastic model, differentiated cells go through a series of mutations and become neoplastic. The cancerous cell can then dedifferentiate into a tumor-initiating cell, with cancer stem cell properties. Adapted from Plaks et al. [131]

The stochastic model of carcinogenesis

Cell plasticity is the central dogma of the stochastic model of carcinogenesis. Any cell from within the tumor is equally as likely to have been the first cancer cell (Figure 2C) [132-137]. For example, a normal differentiated cell may acquire a series of mutations in key cellular processes through clonal expansion. Eventually, mutations arise that allow dedifferentiation and this new phenotype activates progenitor or stem cell genes. This cell with the stem-like phenotype is a TIC. Tumor-initiating cells are similar to CSCs in that they have the ability to self-renew, enter a quiescence state, and divide indefinitely. Their plasticity allows them to phase between dedifferentiated and differentiated states [131, 133, 138-140].

Adult and childhood cancers, such as osteosarcoma, most likely follow the stochastic model of carcinogenesis. Therefore, the term “tumor-initiating cell” will be used to describe repopulating cells with stem-like properties throughout this document. Other researchers may use TICs and CSCs interchangeably, but the current trend is to differentiate the two [141, 142]. To complicate matters further, it is possible that tumors could contain both CSCs and TICs within the heterogeneous population [143, 144]. Controversy aside, the important thing to note is that drugs targeting TICs would theoretically also target CSCs, and vice versa. Targeting TICs is believed to be the solution to cancer reemergence and treatment resistance [142].

Properties of tumor-initiating cells

A vast amount of information has been learned about TICs. They are resistant or can quickly become resistant to traditional radiation and chemotherapy treatments due to their slow replication rates and enhanced DNA repair mechanisms [142, 145-156]. Tumor-initiating cells heighten the tumor's aggressiveness through angiogenesis, local invasion, and metastases [72, 105, 157, 158]. One way they accomplish this is by activating stem cell genes that should otherwise be inactive in mature cells [157, 159, 160]. These genes can be used to identify or isolate TICs from the differentiated tumor cell population [84, 111, 161]. Expression levels of stem cell genes in cancer may also prognosticate the tumor [162-165]. Examples of stem-cell-specific genes include POU class 5 homeobox 1 (POU5F1 or OCT4), nanog homeobox (NANOG), and (Sex Determining Region Y)-Box 2 (SOX2) [166, 167]. TICs can also be identified by inherently increased cytoplasmic aldehyde dehydrogenase concentrations [168, 169].

As described earlier, TICs are plastic cells with the ability to phase between differentiated and dedifferentiated states [133, 139, 140]. Morphing into a dormant phase can be quite advantageous during times of stress [73-75, 146, 170-172]. For example, chemotherapy that targets rapidly dividing cells would circumvent TICs hibernating in the G_0 resting phase (G_0) of the cell cycle. These unharmed cells could repopulate a heterogeneous tumor at a later time. Another way TICs avoid chemotherapy is through resistance. They have increased

expression of ABC transporters, which allows them to efflux toxic chemicals from the cell [173]. These cells are very efficient at DNA repair allowing them to heal after chemotherapy and radiation exposure [148, 174, 175]. Finally, it was initially thought that there was a very low proportion of TICs in tumors. However, this number can be quite high in some cancer types [73, 92, 120, 176]. Plus, the proportion of TICs can fluctuate as the properties of TICs evolve over time [142].

Genes of interest

As one can imagine, there are a large number of genes involved in cancer and TICs. For the sake of conciseness, the canine osteosarcoma TIC genes of interest are briefly discussed next.

NANOG

An important gene for TIC maintenance and identification is NANOG. In normal embryonic cells, NANOG is a transcription factor that is responsible for undifferentiated embryonic stem cell self-renewal. In other words, it prevents the stem cell from differentiating and losing its important properties. NANOG works with OCT4, SOX2, and Kruppel-Like Factor 4 in order to retain pluripotency [166, 177, 178]. Differentiated cells do not express NANOG and it has detrimental effects when expressed in neoplastic cells. It has been detected in most types of cancers and is associated with tumorigenicity, cell proliferation, tumor invasion, and therapy resistance [179-183]. Just as in embryogenesis,

NANOG requires cooperation with other genes in order to be oncogenic [184-187].

Perhaps the most interesting thing about NANOG is its genetic history and profile [188, 189]. There is a tandem duplication of NANOG in the human genome. The original copy is termed NANOG1, while the shorter duplication is NANOG2. NANOG2 does not seem to be functionally important. On the other hand, there is a functionally important complete duplication of NANOG1 on a separate chromosome, called NANOGP8. This copy lacks introns, which indicates that it is a retrotransposed gene. The NANOG1 and NANOGP8 proteins differ by only one amino acid.

NANOG1 is transcriptionally silenced once it has completed its role in embryogenesis. The data shows that the NANOGP8 paralog is reactivated in most cancers [190-196]. There is only one version of NANOG identified in dogs. Most of the canine genome has been sequenced so there is still a possibility of NANOG paralogs to exist. This may be interpreted that activation of NANOG in human cancer may differ from activation of NANOG in canine cancer.

OCT4

Just like NANOG, OCT4 is also an embryonic transcription factor [197]. It has additional abbreviations, such as OCT3 and OCT3/4, in some reports. OCT4 is the first gene to start the process of embryogenesis, establishing totipotency and pluripotency [198, 199]. OCT4 is found in rare adults cells, such

as basal cells within the skin [200]. In humans, OCT4 can be transcribed three ways, resulting in four protein isoforms [201-203]. The OCT4B transcript is generally exploited in cancer [204]. Pseudogenes of OCT4 have also been identified in cancer [205]. The canine ortholog of OCT4 has not been adequately evaluated, but is predicted to have one transcript. The canine genome sequence also has OCT4 pseudogenes (accession numbers XR_134596 and DQ131480). Expression of OCT4 been detected in numerous types of cancers [160, 166, 180-183, 206-210]. In cancer, OCT4 is associated with initiation and aggressiveness of the tumor [209, 211, 212]. OCT4 is considered a therapeutic target due to its key role in carcinogenesis [200].

p21

Cyclin-dependent kinase inhibitor 1A (p21) is a regulator of the cell cycle. Its major role is to stop progression of the cell cycle at G1 to keep the cell from going into the S phase. When the cell experiences DNA damage, tumor protein P53 (p53) induces p21, which then stops the cell cycle until repairs are complete [213]. In stem cells, p21 expression leads to cell quiescence [214-220]. This is an important ability for stem cells because it protects its valuable DNA [221]. Loss of p21 in stem cells results in a decreased number of stem cells or impaired function [222].

In cancer, the role of p21 is quite controversial and sometimes contradictory [223]. Loss of or mutation of p21 may make tumors more

aggressive [224, 225]. It is believed that p21 may be induced independent of p53 in some cancers [155]. p21 has a dual role of preventing or encouraging apoptosis depending on the specific cancer and the conditions. Apoptosis is inhibited and cells proliferate when p21 is localized in the cytoplasm [223]. When CSCs or TICs overexpress p21 and enter quiescence, the outcome is cells that can avoid traditional therapies and later recur [144, 226-228]. This is clearly an advantage for the cancer, but a disadvantage for the patient. Some researchers believe that therapies targeting p21 will prevent cancer recurrence [118, 144]. Finally, p21 is a good marker to indicate quiescent TICs in experiments.

p27

The cyclin-dependent kinase inhibitor 1B (p27) is another regulator of the cell cycle with a role in cancer [214, 215]. p27 is found in all normal differentiated cells and it plays a role in exiting quiescence. Loss of p27 is associated with cellular proliferation [213]. p27 messenger ribonucleic acid (mRNA) is expressed ubiquitously in cells while the p27 protein is regulated by degradation [229]. Quiescent cells have high levels of p27 protein [230]. Loss of p27 in stem cells results in proliferation of cells, but also loss of the stem cell phenotype [215]. An artificial gain of p27 results in increased numbers of stem cells, but also numerous tumors [231]. In cancer, loss of the p27 gene may result in benign hyperplasia or carcinogenesis progression [231-237].

Evidence for tumor-initiating cells in cancer

The expression of genes such as NANOG and OCT4 can be relatively quantified with reverse transcription real-time polymerase chain reaction (RT-qPCR). A subpopulation of cells with high expression of the stem cell gene, NANOG, is considered the TIC subpopulation, whereas the cells that do not express NANOG are the progenitor or differentiated subpopulation. The TIC subpopulation can also be verified with *in vitro* invasion assays or viability assays. The TIC subpopulation will be more invasive or more resistant, respectively, when compared to the differentiated tumor cells. The presence of TICs is ultimately supported when they grow after being injected into an immunodeficient mouse: a small number of TICs will produce a heterogeneous tumor at the injection site, whereas a much larger number of unsorted or negatively sorted cells are needed to grow the same tumor [142, 158, 238, 239].

There are several limitations to conducting mouse xenograft experiments. First, TICs undergo harsh processing before being implanted [142]. It could take many hours or days before the cells are implanted. The phenotype of the cells could change while being cultured, depending on the plasticity of the cells. It is possible to inject TICs into the mouse but they fail to grow because the processing has killed them or driven them into quiescence. Next, important mouse proteins do not perfectly replicate proteins from other species. Growth factors, such as interleukin 3 and tumor necrosis factor, are not cross-species reactive [240, 241]. This could cause problems with tumor growth when

implanting cells from another species in mice. Lastly, the tumor microenvironment of the mouse may not adequately match the microenvironment from which the tumor derived [92]. It is quite common to inject a cancer of abdominal organ origin into the subcutis of the mouse. Researchers are trying to overcome these limitations with humanized mice [242], but this is not helpful when working with tumors cells from non-human species. Despite their limitations, immunodeficient mice are more commonly used over humanized mice for xenograft research because they are more affordable or because the humanized mouse required has not yet been created.

Limitations of cell culture

As mentioned above, a problem faced by researchers is the change in phenotype of cancer cells in culture. Cell culture is an incredible tool for understanding cancer. But, taking cells from a living organism and growing them on plastic with artificial nutrients and a loss of stroma changes the cell phenotype [243, 244]. This process selects for the most aggressive cancer cells in an expedited evolution process. This is not necessarily a bad outcome because the goal of cancer research is to find treatments for the most aggressive cells. But, this may select for cultured cells having a better adaptation at plasticity and thus increasing the true TIC proportion. The end result is a higher proportion of TICs in cultured cells versus the original tumor. It

is not uncommon to see differences between TICs samples directly from patients and TICs from cultured cancer cells [244].

Some researchers have isolated TICs using antibodies specific to cell surface markers, such as prominin 1 (CD133) [119]. Sadly, cell surface markers are only reliable in a limited number of types of cancers and a limited number of species [245]. Solid tumors, especially sarcomas, lack reliable cell surface markers for TIC isolation [142]. To make matters worse, surface markers are dependent on the specific cell line in use. Variables such as passage number or culture conditions may change the surface proteins that are used to identify TICs [245]. In dogs, TIC surface marker research has been disappointing, especially in osteosarcoma [244, 246]. Sphere assays are also used by some laboratories to enrich for TICs, but this method has many flaws, such as an inability to differentiate between aggregated cells and true TIC spheres [247]. An alternative method to isolating TICs is to exploit their ability to resist chemotherapy. One group first treated the total cell population with epirubicin followed by a colony forming assay [248]. The result is an enriched TIC population, but there is no way to enrich for an epirubicin-susceptible population. In summary, it is possible for laboratories to use different methods for isolating TICs. This is acceptable as long as they use additional methods to validate that the cell fraction does indeed have properties of TICs [245].

Tumor-initiating cells in dogs

The area of TIC research in canine cancers lags behind human cancer research. TICs from many types of cancers have been identified *in vitro* [244, 246, 249]. But, only two types of canine TICs have been injected into mice: glioblastoma and mammary tumors [250-254]. Only one laboratory used negative controls for their canine TIC murine xenografts [252, 253]. Regardless, the *in vitro* work has produced useful information for future studies. As with human cancers, TICs from canine cancers are resistant to chemotherapy and radiation [255]. Unlike human cancers, TICs from canine cancers are not reliable identified with aldehyde dehydrogenase or dye efflux methods to isolate side populations [246]. As stated earlier, surface markers that work to isolate human cancer TICs rarely work with canine cancers [244, 246]. For example, CD44 molecule (CD44) is expressed in almost every canine cancer cell making it impractical for isolating a TIC population [256]. Other markers are present in the solid tumor, but decrease once cultured in the laboratory [244]. Finally, NANOG and OCT4 have been identified in several types of canine cancers, including osteosarcoma [249, 257, 258]. These various reports give a broad but shallow summary of the current knowledge in this area of investigation. There is a clear need for more well-designed experiments in canine TIC research.

Tumor-initiating cells in osteosarcoma

Compared to dogs, there are dozens of human osteosarcoma TIC reports published. Both aldehyde dehydrogenase and dye efflux methods, such as Hoechst, have been unreliable in sorting human osteosarcoma TICs [259-262]. Researchers have shown evidence that TICs can successfully grow in mice using the surface marker CD133 [263] or a combination of V-Kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (CD117) and STRO-1 [158] to isolate TICs. Human osteosarcoma TICs have upregulated embryologic genes such as NANOG, OCT4, and SOX2 [167, 259, 263-266]. They resist chemotherapy through upregulation of membrane transporters, such as ATP-binding cassette, sub-family G (WHITE), member 2 (ABCG2) [158, 259, 264, 267]. Human osteosarcoma TICs are known to have self-renewal properties, have increased DNA repair enzymes, and cause metastases [167]. Human osteosarcoma TICs with a loss of p27 have been shown to be more aggressive [268]. The next goal for researchers is to find treatments that reliable target these cells in hope of ending osteosarcoma metastasis and recurrence.

Smoothened-Inhibition in Osteosarcoma

The hedgehog pathway

The hedgehog (HH) pathway has three ligands, with sonic hedgehog (SHH) being the most essential. Key embryologic events, such as midline

symmetry and dorsoventral organization, are mediated by SHH [269, 270]. This pathway is activated in cancers with detrimental results [271]. The HH pathway has a very interesting history in embryogenesis, cancer, and veterinary pathology.

During normal embryologic development, SHH inhibits the membrane protein patched 1 (PTCH1) (Figure 3A) [272]. When PTCH1 is inhibited, smoothened (SMO) is freed to induce the glioma-associated oncogene family zinc finger transcription factors (GLI1, GLI2, GLI3). GLI activates genes needed for midline symmetry, neural development, limb development, facial morphogenesis, and hair development. Once these structures are generated and the genes are no longer needed, SHH is downregulated and PTCH1 is free to shut down the pathway by inhibiting SMO (Figure 3B) [271].

There are four ways that cancers activate the SHH pathway [271]. The first category is ligand-independent activation of the HH pathway. This may arise by a loss-of-function mutation in PTCH1, aberrant activation of SMO, or aberrant activation of GLI (Figure 3C). The second category is ligand-dependent autocrine signaling. In other words, the cell overexpresses and secretes SHH, which in turn activates the cell. The third category is ligand-dependent paracrine signaling [273]. The secreted SHH activates adjacent cells in the supporting stroma. The fourth category is ligand-dependent reverse paracrine signaling, where stromal cells secrete SHH and activate the HH pathway in adjacent tumor cells. It is important to note that these differences play an important part in

treatment [271]. For instance, a SMO-inhibiting drug might not work on a cancer with aberrant GLI activation.

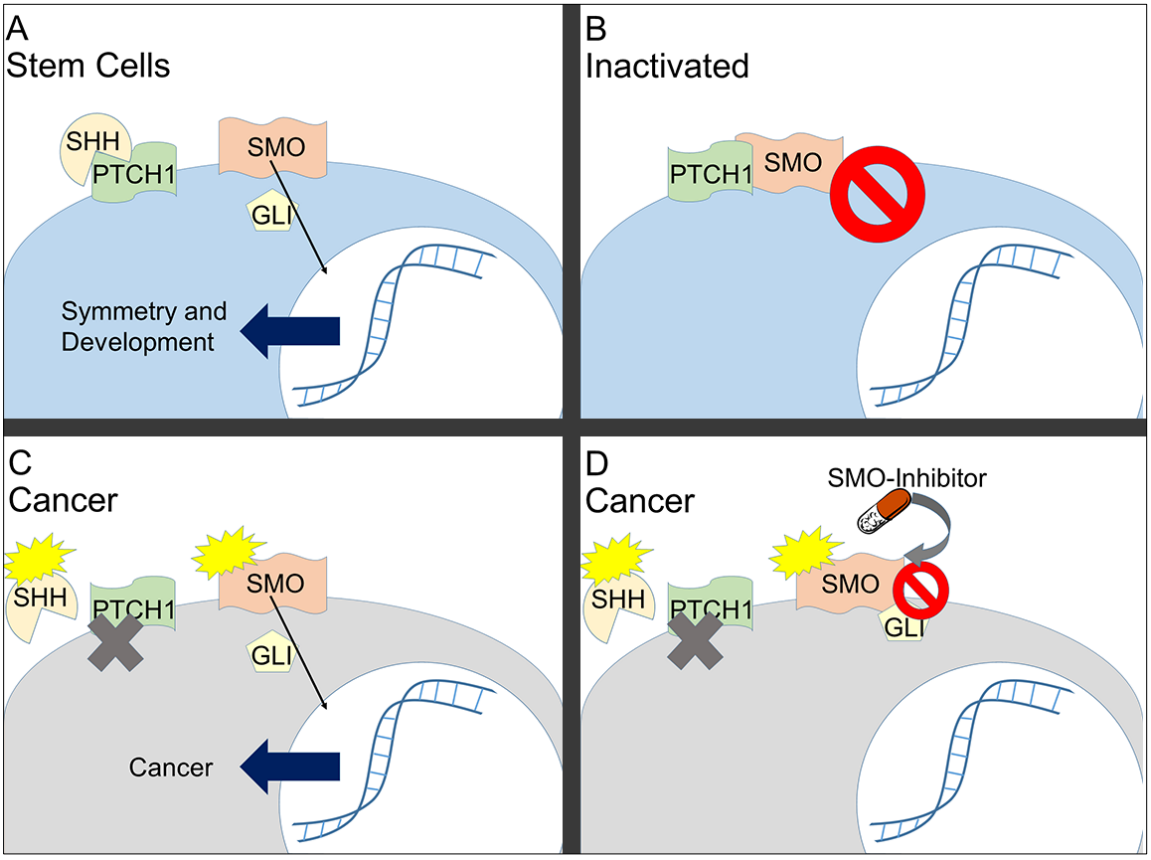

Figure 3. The hedgehog pathway. (A) During normal embryologic development, SHH is secreted and inhibits PTCH1. This frees SMO to activate the GLI transcription factors for development. (B) Once complete, SHH is no longer expressed. The pathway becomes inactivated when PTCH1 inhibits SMO. (C) Cancer mutations in the HH pathway include: uncontrolled secretion of SHH, loss-of-function mutation in PTCH1, or aberrant activation of SMO. (D) SMO-inhibiting drugs can return the cell back to the inactivated state by inhibiting SMO.

Regardless of how the HH pathway is activated in cancer, the result is similar. The GLI transcription factors activate genes for tumor-initiation,

proliferation, angiogenesis, apoptosis inhibition, metastases, and treatment resistance [271, 274-276]. The HH pathway also promotes stemness by regulating NANOG through a positive feedback loop [277, 278].

Drugs that target the HH pathway have shown promising results in cancer research [279]. They function in a symbiotic relationship with other treatments: once the hedgehog pathway is inhibited then the cells become more vulnerable to traditional cytotoxic drugs. The HH pathway is a remarkable target because drugs that block the HH pathway ignore healthy stem cells; however, these drugs are obvious teratogens and should not be used during pregnancy. Upregulation of the HH pathway has been identified in a wide variety of cancers, making it a promising fundamental pathway for all carcinogenesis [280-289]. In addition, HH upregulation has been specifically identified in various cancer TICs [289-293]. The HH pathway has been shown to be upregulated in canine osteosarcoma [294].

Smoothened-inhibitors

SMO-inhibitors block the SHH pathway by inhibiting SMO (Figure 3D) [295]. This essentially shuts down the rest of the pathway. Cancer biologists have been evaluating several types of small molecules that target SMO as potential cancer treatments [280]. Two SMO-inhibitors, cyclopamine and vismodegib, are chosen for evaluation in this research.

Cyclopamine

Cyclopamine is best known in veterinary medicine as the teratogen that causes cyclopia in fetuses of grazing animals [269, 296]. Much of what is known about the HH pathway was learned from cyclopamine toxicity. It is a steroidal jerveratrum alkaloid naturally found in corn lilies (*Veratrum californicum*) [297]. Cyclopamine very specifically targets and destroys stem cells by blocking the HH signaling pathway via SMO binding [298, 299]. In the pregnant animal, the result is lethal for the fetus with the dam remaining unaffected.

Cyclopamine is easily accessed and inexpensive, making it a great chemical for *in vitro* studies. Unfortunately, it has poor bioavailability and causes unwanted side effects in mice [300, 301]. Cyclopamine treatment successfully transitioned drug resistant cell lines to become sensitive to chemotherapy in leukemia, prostate, and pancreatic cancers [276, 302-304]. In other cancers, cyclopamine inhibits self-renewal, slowed replication, enhanced apoptosis and targeted TICs [290, 293, 303-305]. Studies show a decrease in GLI expression in cells after cyclopamine treatment [305-307]. Cyclopamine is most effective when used in combination with other drugs [293]. Cyclopamine successfully inhibited human osteosarcoma cell lines [300] and canine transitional cell carcinoma cell lines [308]. It has not been evaluated on canine osteosarcoma.

Vismodegib

Genentech developed Vismodegib as another drug that targets SMO, but it has better bioavailability and less side effects compared to cyclopamine [309]. It is the first Federal Drug Administration (FDA)-approved HH inhibiting drug, labeled for treatment of the hedgehog-predominant cancer basal cell carcinoma. Vismodegib treatment results in significant shrinkage of basal cell carcinomas [309]. Vismodegib has also shown promising results in other forms of cancer, like medulloblastoma and mesothelioma [310, 311]. Vismodegib specifically targets TICs in gastric cancer by making the cells less resistant to other chemotherapies [293]. Just this year, vismodegib has been shown to inhibit osteosarcoma metastasis in a mouse model [312]. Canine osteosarcoma has not been evaluated with vismodegib. As with cyclopamine, vismodegib also works symbiotically with other chemotherapies, and even other HH pathway inhibitors [312].

HER2-Inhibition in Osteosarcoma

A brief history of HER2 in cancer

Erb-B2 receptor tyrosine kinase 2 (HER2) is an epidermal growth factor. It is also called neu or ErbB2. The role of HER2 is to promote cellular proliferation and to oppose apoptosis by activating various signaling pathways [313]. The HER2 protein was discovered in the 1980's from neuroblastomas of rats [314].

Not long after, HER2 overexpression was revealed to be an important negative prognosticator identified in a fraction of all breast cancer biopsies [315]. In 1997, the first therapy targeting HER2 was approved by the FDA, called trastuzumab. The American Society of Clinical Oncology immediately updated their recommendations to check breast cancer biopsies for HER2 overexpression in order to target the HER2-positive fraction of patients appropriately [316]. This resulted in a ten-year survival improvement from 75% to 84% for HER2-positive breast cancer patients [317]. Since then, additional therapies have been created that target HER2.

HER2 is expressed in low amounts in few healthy adult tissues, such as the breast and proliferating hematopoietic cells, whereas HER2 is generally widely overexpressed in HER2-positive cancers [318, 319]. Other cancers have been discovered to overexpress this protein, such as gastric, esophageal, ovarian, and uterine cancers [320-324]. HER2 has also been identified in human osteosarcoma [325]. In breast cancer, HER2 regulates TICs resulting in tumorigenesis, invasion, and radiation resistance [326, 327].

HER2 in osteosarcoma

In humans, the prognostication of HER2 in osteosarcoma is controversial. The majority of reports in osteosarcoma have shown that HER2 overexpression is associated with a poorer prognosis or metastasis [325, 328-334]. One study found patients with HER2-positive osteosarcoma have a better prognosis [335].

Yet others claim that HER2 does not prognosticate osteosarcoma [336-340]. Perhaps the confusion in HER2 prognostication reports stems from the difference in HER2 expression and location between breast and bone tissues. Breast cancers that have multiple copies of the ERBB2 gene subsequently overexpress HER2. HER2 is located in the membrane of breast cancer resulting in intense membranous staining seen on immunohistochemistry [315]. In contrast, HER2 is generally diffuse throughout the cytoplasm in osteosarcoma [328, 332, 341]. Criteria outlined for breast cancer immunohistopathology would result in negative scoring of osteosarcoma biopsies. In addition, breast samples are evaluated from frozen sections, which have much more vivid staining compared to fixed samples used in retrospective studies [320]. Evidence suggests that HER2 does not need to be located in the membrane of osteosarcoma nor does the gene need to be duplicated in order to encourage carcinogenesis [342]. In other words, the mere presence of HER2 in osteosarcoma is enough evidence to warrant evaluation of HER2-targeting drugs.

Lapatinib

Trastuzumab is being used in human osteosarcoma studies [343], but it is a humanized monoclonal antibody. The future goal of this research is to use the dog in clinical trials. A human antibody would likely cause anaphylaxis in the dog with repeated dosing and would likely be less effective against canine HER2.

Instead, the drug lapatinib will be evaluated. Lapatinib was developed by GlaxoSmithKline as an alternative therapy for patients that become resistant to trastuzumab. Trastuzumab targets HER2 from the extracellular matrix, whereas lapatinib enters the cell and targets HER2 from inside the cytoplasm (Figure 4). This makes lapatinib a more attractive therapy for osteosarcoma. Lapatinib has the affinity to block both HER2 and its homolog, HER1 [344]. Lapatinib is cytotoxic to HER2-positive cancers by preventing activation of the pathways downstream to HER2 [345]. Lapatinib has not yet been evaluated as a treatment for osteosarcoma.

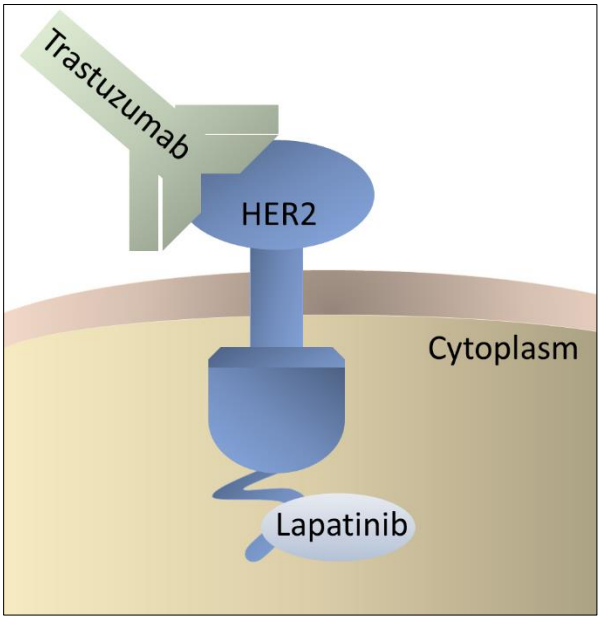

Figure 4. HER2 binding sites. The membrane receptor, HER2, can be targeted extracellularly (trastuzumab) or on the cytoplasmic side (lapatinib). Adapted from Vogel et al. [346]

Efficacy of lapatinib is evaluated by examining the expression of HER2 or by examining the expression of other genes downstream. V-Akt Murine Thymoma Viral Oncogene Homolog 1 (AKT1) is a member of the phosphatidylinositol 3-kinase pathway, an effector of HER2 mediation. AKT1 is involved with almost every category within the six hallmarks of cancer [347]. It is also associated with TIC self-renewal [348]. AKT1 will also be used for evaluation of HER2 inhibition by lapatinib for this study.

HER2 has been identified in a range of canine cancers: mammary tumors, gastric carcinoma, astrocytoma, and melanoma [349-352]. HER2 is overexpressed in 86% of canine osteosarcoma cell lines and 40% of biopsies [353]. This suggests that lapatinib is ideal for evaluation as a treatment for canine osteosarcoma.

Goals of Dissertation

The goals of this dissertation are as follows:

1. Develop a method to isolate TICs from osteosarcoma
2. Determine the phenotypic differences in canine osteosarcoma TICs compared to differentiated cancer cells
3. Evaluate two classes of drugs that specifically target TICs by taking advantage of their unique phenotypes *in vitro* and *in vivo*

MATERIALS AND METHODS

Cell Lines and Management

Cell lines

Three immortalized canine osteosarcoma cell lines were used for the subsequent experiments: Abrams, NIH KOS-003 (MCKOS), and UWOS2. Abrams and UWOS2 were kindly provided by Dr. David Vail from the University of Wisconsin. The MCKOS cell line was a gift from the Comparative Oncology Trials Consortium at the National Institutes of Health National Cancer Institute.

Cell culture

Cells were cultured in 75 cm^2 flasks containing R10 complete medium (R10) made of RPMI-1640 supplemented with 10% fetal bovine serum, 1% Penicillin/Streptomycin, and 0.2% Plasmocin™ (InvivoGen, California, USA). The cells were maintained at 37°C in 5% carbon dioxide.

TIC enrichment

For TIC enrichment, osteosarcoma cells were grown as described above to 90% confluency. The medium was removed and replaced with R10 containing 0.2 µg/ml of epirubicin (Areva, North Carolina, USA). The cells were incubated for 24 hours at 37°C in 5% carbon dioxide. The medium containing the dead

differentiated cells was decanted and more dead cells were washed away with 1x phosphate buffered saline (PBS). The remaining cells were trypsinized, centrifuged, and returned to a clean flask containing R10. The R10 was changed daily in flasks containing TICs until used in subsequent experiments. In two experiments where noted, TICs were enriched using R10 containing 2 µg/ml of epirubicin for 48 hours.

Cell Culture Viability

Calcein AM assay

Cells were seeded onto black 96-well plates and allowed to attach to the bottom of the wells. Drugs were diluted in R10 and added to the wells in triplicates as follows: 0, 10, 20, 40, 60, and 80 µg/ml for epirubicin; 10, 30, 50, 70, 90, 125, and 150 µM for cyclopamine (C4116, Sigma-Aldrich, Missouri, USA); 1, 5, 10, 50, 100, and 500 µM for vismodegib (kindly provided by Genentech for viability analyses, California, USA); and 5, 10, 20, 40, 60, 80, and 100 µM for lapatinib (L-4804, LC Laboratories, Massachusetts, USA). Negative controls containing only R10 were used for epirubicin and vismodegib curves. The negative control for cyclopamine and lapatinib was R10 with 5% dimethyl sulfoxide (DMSO). The cells were incubated for 48 hours at 37°C in 5% carbon dioxide. Three wells were treated with PBS containing 0.2% Triton X-100 for 15 minutes to serve as a positive control for complete cell death. All wells were

decanted and washed with PBS. Next, 8µM Calcein AM (C0399, Life Technologies, California, USA) in PBS was added to all wells and incubated for 30 minutes. The fluorescence was measured at 485 nm for excitation and 520 nm for emission. The results were plotted on a curve and the equation of the curve was used to determine the lethal concentration (LC).

Epirubicin viability assay

Cells were seeded in flasks and grown to 70% confluency. The cells were trypsinized and resuspended in serum-free N2 medium, composed of Dulbecco's Modified Eagle's Medium (D8900-10X1L, Sigma-Aldrich, Missouri, USA) with 1% N-2 supplement (17502-048, ThermoFisher Scientific, California, USA), 1% Penicillin/Streptomycin, 0.2% Plasmocin™, 0.01% epidermal growth factor, and 0.01% fibroblast growth factor. The cells were added at a density of 3 x 10^5 cells/ml using low attachment 6-well plates. Epirubicin was added to the treatment groups at a concentration of 2 µg/ml. The cells were incubated for 48 hours at 37°C in 5% carbon dioxide. The cells were differentially centrifuged. First, the cells were pelleted at 300 g for ten minutes. Next, the supernatant was removed and centrifuged at full speed for 5 minutes to collect any remaining cells. The two pellets were combined in 10µl of Trypsin-Versene® (Lonza, Basel, Switzerland). Viability was determined by counting the number of live and dead cells using the fluorescent acridine orange and propidium iodide (AO/PI) Assay (Nexcelom, Massachusetts, USA). Each cell line and variable was repeated in

triplicate. A repeated measures analysis-of-variance was used to determine if there was a statistical difference between the proportions of viable cells in the treated group.

Viability assays for TICs

TICs were prepared as described in the section above on TIC enrichment. The R10 was changed for three days. Next, the TICs were washed with 1x PBS and trypsinized. The TICs were counted using AO/PI solution in the Cellometer Auto 2000. The TICs were added to a 6-well low attachment plate suspended in N2 in triplicates at a density of 2×10^5 cells per well. Three wells were incubated with 15 µM and 50 µM vismodegib for 48 hours. Three wells were incubated in untreated N2 for negative controls. Three wells were incubated with 15 µM lapatinib for 24 hours, using equal volume DMSO for their three control wells. The cells were collected using differential centrifugation method described above. A repeated measures analysis-of-variance was used to determine if there was a statistical difference between the proportions of viable TICs in the treated group.

Immunocytochemistry and Immunohistochemistry

Immunocytochemistry

TICs were prepared as described in the section above on TIC enrichment. Unenriched cells and TICs were trypsinized and seeded onto separate chambered glass slides in R10. Once the cells attached to the glass, the medium was decanted and the slides were washed in 1x PBS and air dried. Antigen retrieval was conducted using Retrieval Buffer in a Decloaking Chamber (BioCare Medical, California, USA). The slides were washed with Tris buffer followed by an incubation with 3% hydrogen peroxide. Background Sniper was used as a blocking agent (BioCare Medical, California, USA). Anti-marker of proliferation Ki-67 (Ki-67) antibody was used at 1:100 dilution for one hour (275R-15, SP6; Cell Marque, California, USA). Anti-HER2/neu antibody was used at 1:2,000 dilution for one hour (sc-284; Santa Cruz, Texas, USA). The primary antibody was replaced with rabbit nonimmune sera for the negative controls (NP001, C-18, Dako, Glostrup, Denmark). The secondary antibody was applied for one hour (MACH2 anti-rabbit secondary antibody; BioCare Medical). Diaminobenzedine was used for antibody visualization, counterstained with hematoxylin. Finally, the slides were dehydrated through a series of ethanol and xylene and were coverslipped. A normal piece of canine bone was used as a tissue negative control. A piece of canine intestine with active cell division and a

HER2-positive human breast cancer tumor from a mouse xenograft were used as positive controls.

Immunohistochemistry

Immunohistochemistry was performed on formalin fixed paraffin embedded sections as described in the section on immunocytochemistry with the following addition. Before antigen retrieval, the slides were deparaffinized and rehydrated with a series of xylene and ethanol.

Proportions of positive cells

Multiple images of the slides were captured at 200x magnification (Microcast HD 3CCD 1080p Microscope Video Camera, Optronics, California, USA). The images were imported into ImageJ (National Institutes of Health, Maryland, USA). A script was designed to count the number of positive cells and the total number of cells (Figure 5). Images with large numbers of dead cells were counted manually. For Ki-67, the mitotic fraction was calculated by dividing the number of KI-67-positive cells by the total number of cells. The Wilcoxon rank sum test was used to determine if there was a statistical difference between the proportions of TIC-enriched cells versus the unenriched cells.

Batch 1

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mean-2.7*std;run("RGB Stack");run("Stack to
Images");selectWindow("Red");close();selectWindow
("Green");close();selectWindow("Blue");rename
(name);setThreshold(0,newmax);run("Convert to
Mask");run("Make Binary");run("Analyze Particles...",
"size=75-5000 circularity=0.00-1.00 show=Nothing
clear summarize");selectWindow(name);close();
setBatchMode(false);
```

Batch 2

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circularity=0.00-1.00 show=Nothing clear summarize");
```

Figure 5. Scripts used for ImageJ analysis. Batch 1 counts only cells with DAB staining and Batch 2 counts the total number of cells.

Evaluation of archived specimens from canine osteosarcoma patients

The records of fifty dogs diagnosed with canine osteosarcoma from 2005-2010 were abstracted for signalment, clinical findings, treatment, and date of death. Dogs that died from causes other than osteosarcoma were excluded. For each dog, hematoxylin and eosin (H&E) and HER2-stained slides were evaluated for osteosarcoma subtype, osteoid production, necrosis, HER2 overexpression, mitotic score, and the average number of mitoses in five fields at 400x magnification. The Kaplan-Meier method was used to generate survival curves and the Cox proportional hazards model was used to evaluate these

variables. Dogs that were euthanized at diagnosis were not included in the analysis.

Gene Expression

Gene expression of canine osteosarcoma TICs

TICs were prepared as described in the section above on TIC enrichment. The R10 medium was changed daily for seven days and each day one set of triplicates was frozen for ribonucleic acid (RNA) purification as described below. Triplicates of unenriched canine osteosarcoma cells and enriched TICs were compared over one week for the following genes: NANOG, OCT4, p21, and p27.

Gene expression of canine osteosarcoma TICs following smoothened-inhibition

TICs were prepared as described in the section above on TIC enrichment. Plated triplicates of TIC-enriched cells were treated with 25 μM of cyclopamine in R10 for 48 hours. Control TICs were incubated in R10 containing an equal volume of DMSO. Plated triplicates of TIC-enriched cells were also treated with 0 or 15 μM of vismodegib for 48 hours. Three sets of GLI primers were used to determine if the hedgehog pathway was downregulated by these smoothened-inhibiting drugs using RT-qPCR as described below.

Gene expression of canine osteosarcoma TICs following HER2-inhibition

TICs were prepared as described in the section above on TIC enrichment. Triplicates of plated TIC-enriched cells were treated with 15 µM of cyclopamine in R10 medium for 48 hours. Control TICs were incubated in R10 containing an equal volume of DMSO. AKT1 and HER2 primers were evaluated for suppression in the treated samples versus the untreated samples, as described below.

Relative RT-qPCR

For the experiments listed above, RNA was purified according to the manufacturer's instructions (Isolate II RNA Mini Kit #BIO-52072, BioLine, London, UK). Equal amounts of RNA was used to generate cDNA (iScript™ Reverse Transcription Supermix for RT-qPCR #1708841, BioRad, California, USA). Relative RT-qPCR was performed with biological and technical triplicates (SsoAdvanced™ Universal SYBR® Green Supermix #1725271 and CFX Connect™ Real-Time PCR Detection System #1855201, BioRad, California, USA). Two normalization genes were used: GAPDH and RPS19. All of the primers are listed in Table 1. Primers for GLI2 were created using GenBank sequence XM_003432472.2. Primers for GLI3 were created using GenBank sequence XM_005630926.1. The cycling conditions were 30 seconds at 95ºC, followed by 40 cycles of 10 seconds at 95ºC and 5 seconds at 62ºC. The Delta-Delta-C_T method was used to determine the relative fold change between

unenriched and TIC-enriched cells. The standard deviation was calculated based on the C_T values and converted with the fold change analysis. Significance was determined by calculating a Student's t-test on the difference between C_T values of the normalizing gene and gene of interest and between the control and target groups.

Table 2. Canine specific primer pairs used in RT-qPCR.

Primer	Sequence 5' to 3'	Amplicon Size	Citation
GAPDH Forward	TATCAGTTGTGGATCTGACCTG	172 bp	[354]
GAPDH Reverse	GCGTCGAAGGTGGAAGAGT		
RPS19 Forward	CCTTCCTCAAAAA/GTCTGGG	95 bp	[355]
RPS19 Reverse	GTTCTCATCGTAGGGAGCAAG		
NANOG Forward	CCTGCATCCTTGCCAATGTC	98 bp	[354]
NANOG Reverse	TCCGGGCTGTCCTGAGTAAG		
OCT4 Forward	GAGTGAGAGGCAACCTGGAG	274 bp	[356]
OCT4 Reverse	GTGAAGTGAGGGCTCCCATA		
p21 Forward	ACCTCTCAGGGCCGAAAAC	88 bp	[357]
p21 Reverse	TAGGGCTTCCTCTTGGAGAA		
p27 Forward	CAGAGGACACACACTTGTAGA	124 bp	[357]
P27 Reverse	TCTTTTGTTTTGAGGAGAGGAA		
GLI1 Forward	ACCTCCATGATAGGCAGTGG	217 bp	[253]
GLI1 Reverse	ACTCACCCCATGGTTCAGAG		
GLI2 Forward	GGTGGTACACATGCGCCGA	107 bp	N/A
GLI2 Reverse	ACCGCAGGTGTGTCTTCAGG		
GLI3 Forward	CCACGGGGCCAGCAGGAACA	128 bp	N/A
GLI3 Reverse	TCCCGCGTGCAACCTTCCCA		
AKT1 Forward	CACCGTGTGACCATGAATGAG	83 bp	[358]
AKT1 Reverse	TTCTCCTTGACCAGGATCACC		
HER2 Forward	AGCAGAGAGCCAGCCCTGTGACATC	137 bp	[359]
HER2 Reverse	GCAGCCTCCGCATAGTGTACTTCCG		

Murine Studies

Institutional Animal Care and Use Committee approval

All animal studies were approved by the Texas A&M University Institutional Animal Care and Use Committee. The Animal Use Protocol numbers for the following studies were IACUC 2014-0079 and IACUC 2014-0329. Both Abrams and MCKOS cell lines were tested for murine pathogens before starting the following studies (Mouse Essential Panel. Charles River, Massachusetts, USA; MycoAlert™ PLUS Mycoplasma Detection Kit, Lonza, Basel, Switzerland).

Limiting dilution assay - Abrams

The TICs were enriched using the more aggressive method of 2 µg/ml of epirubicin for 48 hours. Unenriched cells and TIC-enriched cells at concentrations of 100, 1,000, and 10,000 cells were subcutaneously injected suspended in Matrigel® (#356230, Corning®, New York, USA). Eight athymic nude mice (Strain 490, Charles River, Massachusetts, USA) were used for each group, with some mice receiving bilateral injections. Eight control mice were injected with Matrigel only to serve as negative controls. The tumor size and weight of each mouse was recorded twice per week. The tumors were allowed to form and grow for seven weeks. The mice in the 100 unenriched cells group and all three TIC groups were euthanized at seven weeks. The Matrigel control

mice were euthanized at four weeks. The remaining mice were used in subsequent studies. The subcutaneous tissues from all mice were processed to make routine H&E microscope slides and were evaluated for tumor growth.

Oral chemotherapy

The chemicals were purchased in a powdered form and suspended in a vehicle as follows. For vismodegib (V-4050, LC Laboratories, Massachusetts, USA), the drug was suspended in 0.5% methylcellulose with 0.2% Tween 80. For lapatinib, the drug was suspended in 0.1% Tween 80. A mixture of the vehicle for each treatment was prepared without the drug additive to serve as a placebo for the control mice.

Twelve mice with subcutaneous tumors from the Abrams limiting dilution assay above were treated with oral chemotherapy once the tumor reached a volume 0.3-0.6 cm^3. Three mice that received injections of 1,000 unenriched Abrams cells were gavaged with vismodegib at 75 mg/kg twice daily for two weeks. Three additional mice were gavaged with an equal volume of vehicle to serve as controls. Three mice that received injections of 10,000 unenriched Abrams cells were gavaged with lapatinib at 75 mg/kg daily for three weeks. Three additional mice were gavaged with an equal volume of vehicle. The tumor size and weight of each mouse was recorded twice per week. Any mouse with a tumor exceeding 1 cm^3 was immediately euthanized. All mice were euthanized at the end of the treatment period. Sections of the tumor were flash frozen in

liquid nitrogen and a section of each tumor was saved for histology. The lungs were also saved for histology.

Limiting dilution assay - MCKOS

The MCKOS TICs were enriched using the gentle procedure described earlier, using 0.2 µg/ml of epirubicin for 24 hours. The TICs were trypsinized and placed in a clean flask with fresh R10. The R10 was changed daily for one week and used for injection once the TICs began replicating. Unenriched cells and TIC-enriched MCKOS cells suspended in Matrigel were bilaterally injected subcutaneously in groups of four athymic nude mice. The concentrations used were 100, 1,000, and 10,000 cells. Four control mice were injected with only Matrigel to serve as negative controls. The cells were allowed to grow for four weeks. Mouse weight and tumor size was recorded twice weekly. The mice were necropsied at the end of the study. Sections of each tumor and the lungs were saved for routine H&E histology.

Pulmonary metastasis model for vismodegib evaluation

One million unenriched cells were retro-orbitally injected into eight athymic nude mice. One day later, four mice began vismodegib treatment at 75 mg/kg twice daily for up to three weeks. The vismodegib was suspended in corn oil for this treatment study. The other four mice were gavaged with an equal volume of corn oil to serve as controls. Exophthalmos and more than 20%

weight loss were used as endpoints for this study. The mice were necropsied and the lungs were saved for routine H&E histology.

The lungs were sectioned for microscope slides so that five non-serial sections of lungs were available for evaluation from each mouse. The number of metastatic MCKOS colonies was counted in each of the five sections. The slide with the highest number of colonies from each mouse was selected for imaging. Microscopic images of the lungs were taken at 100x magnification. The area of each pulmonary metastasis was measured using ImageJ. The formalin fixed paraffin embedded blocks were then used for quantitative DNA analysis.

Quantitative DNA analysis of lungs

The formalin fixed paraffin embedded blocks from the mice of the vismodegib pulmonary metastases treatment study were evaluated for the quantity of canine DNA in the lungs. Lungs from mice that received only Matrigel injections were used as negative controls. DNA was purified from ten 5 µm wide sections of lungs according to the manufacturer's instruction (QIAamp DNA FFPE Tissue Kit, Qiagen, Limburg, Netherlands). Each sample was run in triplicate using 45 ng of template per well (SsoAdvanced™ Universal SYBR® Green Supermix #1725271 and CFX Connect™ Real-Time PCR Detection System #1855201, BioRad, California, USA). Canine specific primers that target a short interspersed element, or SINE, were used to target canine DNA within the mouse lung DNA samples (forward: 5'-AGGGCGCGATCCTGGAGAC-3',

reverse: 5’- AGACACAGGCAGAGGGAGAA-3’) [360]. A standard curve was created by mixing known quantities of DNA from a canine papilloma biopsy with known quantities of DNA from a mouse lung, ranging from 45 fg to 45 ng per reaction. The cycling conditions were three minutes at 98ºC, followed by 40 cycles of 15 seconds at 98ºC, 30 seconds at 55ºC, 20 seconds at 60ºC, and 15 seconds at 72ºC.

RESULTS AND INTERPRETATION

Tumor-Initiating Cells

Determination of the lethal concentration of epirubicin

A Calcein AM assay was used to determine the lethal concentration of epirubicin. The 95%, 75% and 50% lethal concentrations were estimated from the equation of the logarithmic curve (Figure 6: LC_{95}, LC_{75}, and LC_{50}). The LC_{50} cannot be reliable from this data set because the concentrations of epirubicin were not low enough. Based on these results, concentrations at 2 µg/ml or less were used for subsequent experiments.

Tumor-initiating cell viability assay

An AO/PI viability assay was performed to determine the proportion of cells killed by epirubicin in stressed cell culture conditions. Approximately 22% of unenriched cells died once suspended in serum-free medium (Figure 7). An additional 27% of cells were killed when 2 µg/ml epirubicin was added to the medium, for a final viability of 49%. This difference of means was statistically significant (0.78 for untreated cells, 0.49 for treated cells, $p = 0.004$). Cells in both the untreated wells and the treated wells gathered similar to what is seen in sarcosphere culture, but in this case it was clearly clumping. A higher number of cells was predicted to die in the treatment group. This may indicate that the

harsh conditions drove cells to change phenotypes to increase TIC proportion. Alternatively, the cells that were killed early on in the experiment became lysed and undetectable, artificially lowering the dead fraction.

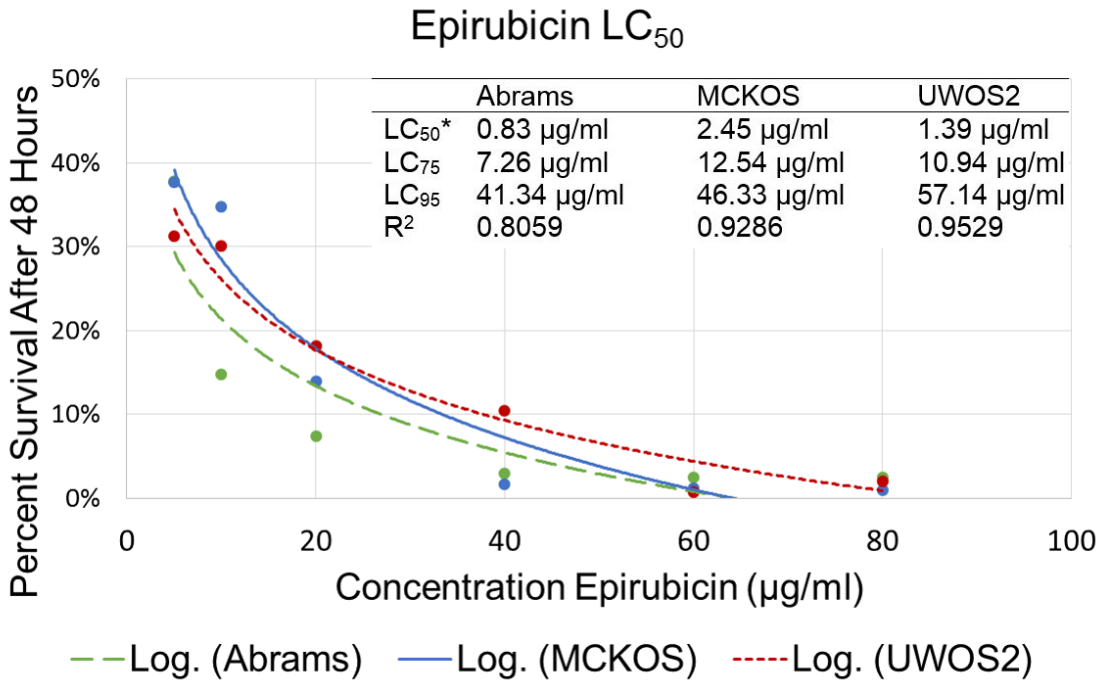

Figure 6. Percent survival of canine osteosarcoma cell lines after 48 hours of epirubicin treatment. The curves shown are the proportions of viable cells after treatments of various concentrations of epirubicin. The logarithmic curve was used to calculate the 95%, 75%, and 50% lethal concentrations. The lethal concentrations are summarized in the inset. The associated R^2 values of the curves are also listed. * The LC_{50} is not accurately predicted because very low concentrations of epirubicin were not used and these values should be interpreted with care.

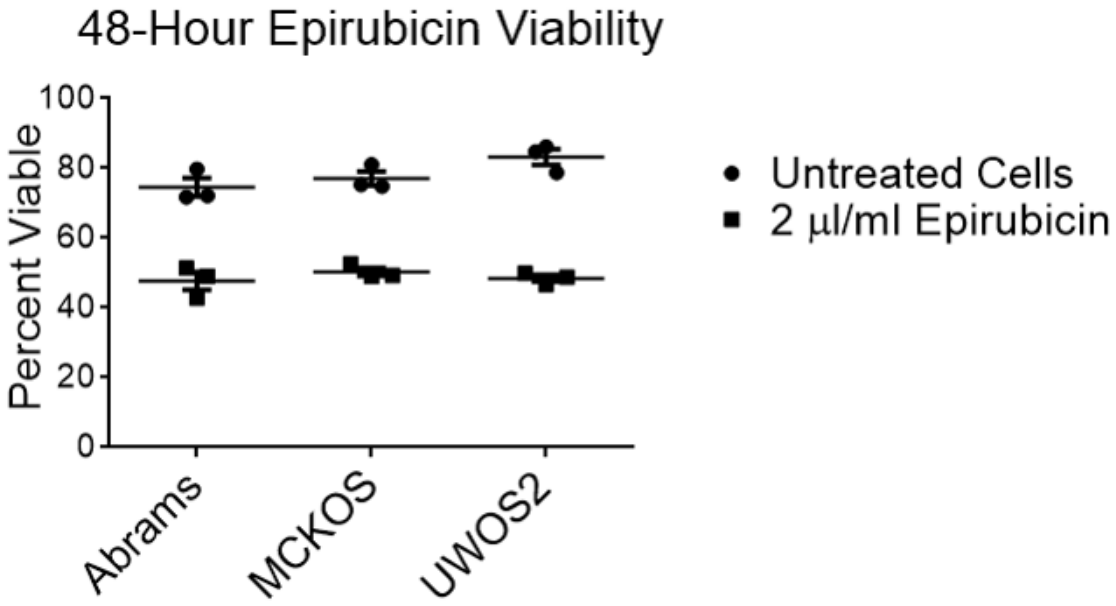

Figure 7. Percent viability of canine osteosarcoma cells after 48 hours of epirubicin treatment in stressful conditions. The experiments were carried out in triplicates as represented by the clusters on the graph. The wide bar depicts the mean and the shorter bars are standard error of the mean. The mean proportion viable differed significantly for the treatment group (0.78 for untreated cells, 0.49 for treated cells, $p = 0.004$).

Properties of tumor-initiating cells in culture

Numerous observations were made regarding TIC morphology and behavior changes during the proceeding experiments. Cells from the TIC-enriched population were generally much larger than the differentiated osteosarcoma cells, with some multinucleated cells (Figure 8). The TICs had more membrane projections and the nuclei were more distinct. When enriched with 2 µg/ml of epirubicin, the cells did not repopulate the flasks for eight weeks. At eight weeks, the cells began replicating and the flask reached confluency in another week. When the more gentle enrichment of 0.2 µg/ml was used, the

cells remained dormant for a much shorter period of time. The first cell replication was observed on the fifth day. These observations indicate a quiescent cell population instigated by the harshness of the environment.

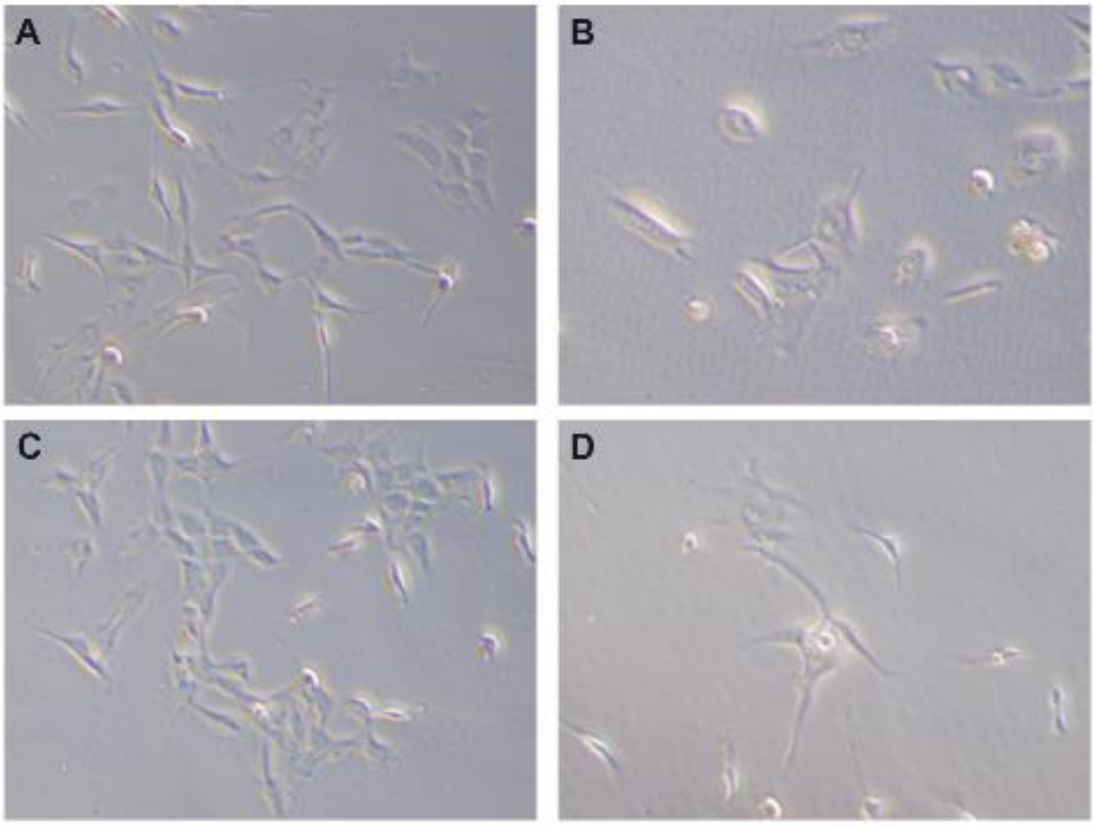

Figure 8. Abrams and MCKOS culture. (A) Unenriched Abrams cells and (C) unenriched MCKOS cells displayed typical osteosarcoma morphology in culture. (B) Abrams TICs and (D) MCKOS TICs had pronounced nuclei and membranous projections. 400x magnification.

Ki-67 immunocytochemistry for evaluation of cell growth fraction

In order to verify that the TICs were quiescent, the cells were labeled with Ki-67 (Figure 9). This antibody selects for cells that are actively within the cell

cycle, and neglect to adhere to cells in G_0. For unenriched Abrams, 53.5% of the cells were in G_0 (Figure 10). For TIC-enriched Abrams, 93.8% of the cells were in G_0. For unenriched MCKOS, 56.3% of the cells were in G_0. For TIC-enriched MCKOS, 96.8% of the cells were in G_0. These results indicate that the majority of TICs enter quiescence with exposure to epirubicin.

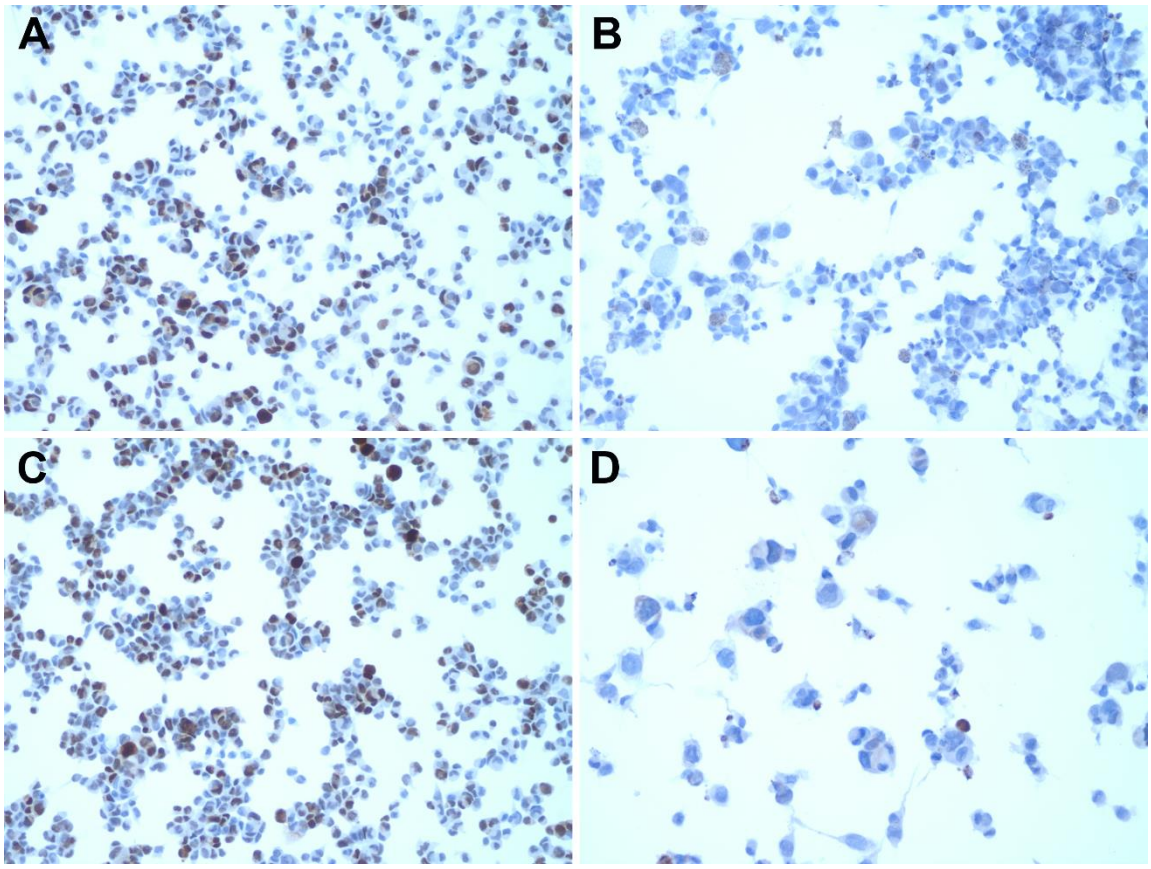

Figure 9. Abrams and MCKOS labeled with Ki-67. Actively dividing cells are labeled with Ki-67. The proportion was higher in (A) unenriched Abrams and (C) unenriched MCKOS compared to their TIC-enriched counterparts. (B) Abrams TICs and (D) MCKOS TICs had few labeled cells. 200x magnification.

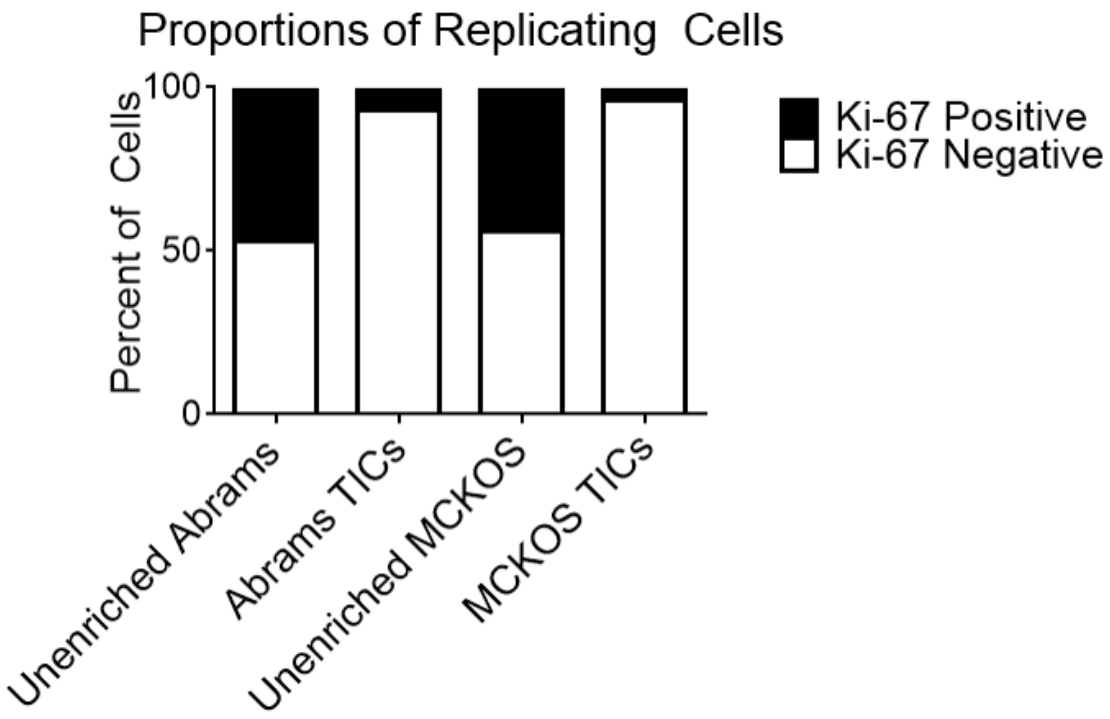

Figure 10. Cell growth fraction. The cells labeled with Ki-76 were counted and the unenriched cells had considerably higher growth fractions. The non-dividing proportions were as follows: 53.5% for unenriched Abrams, 93.8% for Abrams TICs, 56.3% for unenriched MCKOS, and 96.8% for MCKOS TICs.

Tumor-initiating cell gene expression

To validate that the enriched population was composed of TICs, RT-qPCR was performed for the stem cell specific genes, NANOG and OCT4. Enriched cells were collected for one week post-epirubicin treatment. The relative fold change is presented in Figure 11. Both cell lines had an increased expression in NANOG in the TIC-enriched population compared to the untreated cells, which had no detectable NANOG. OCT4 expression was not as expected, with only a small upregulation at the beginning of the week. This may be a product of the difficulty in designing OCT4 primers that disregard the pseudogene. Regardless, the presence of NANOG in the TIC population is enough evidence to support stemness. Next, the cells were evaluated for quiescence through expression of p21 and p27. The level of p21 was increased at the beginning of the week when the cells were not dividing and the level of p27 dropped as cells began repopulating. Thus, canine osteosarcoma cells treated with epirubicin successfully produced a quiescent TIC-enriched subpopulation.

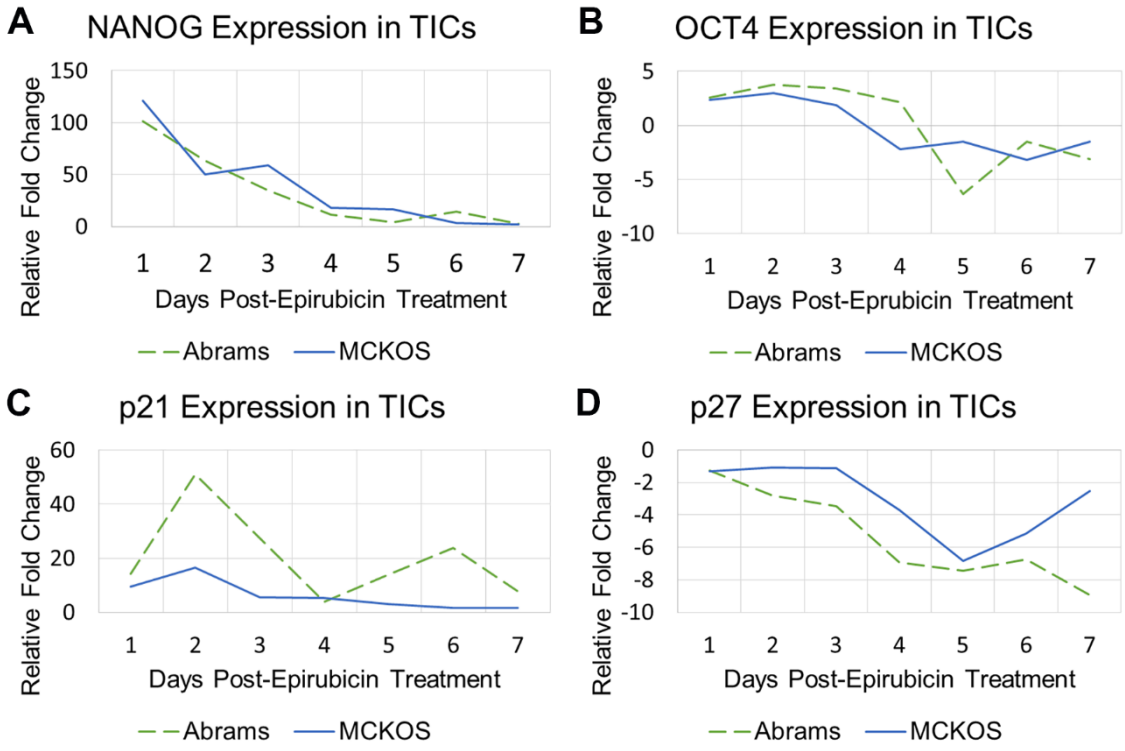

Figure 11. Gene expression in TICs over one week. (A) NANOG levels are high in the TIC population, but once the cells begin replicating it was diluted out. (B) OCT4 expression was lower than expected with only a small increase at the beginning of the week. (C) The levels of p21 were increased when the cells were not dividing, as expected. (D) On day 5, the level of p27 dropped when the cells began dividing.

Demonstration of quiescence in a murine xenograft model

Abrams TICs were ectopically transplanted into athymic nude mice to evaluate quiescence *in vivo*. Compared to mock injections, viable cells were identified in the tissue site six weeks post injection (Figure 12). This indicates that TICs remain quiescent despite a change in microenvironment and that TICs exiting G_0 must be used to determine if TICs can repopulated a tumor *in vivo*.

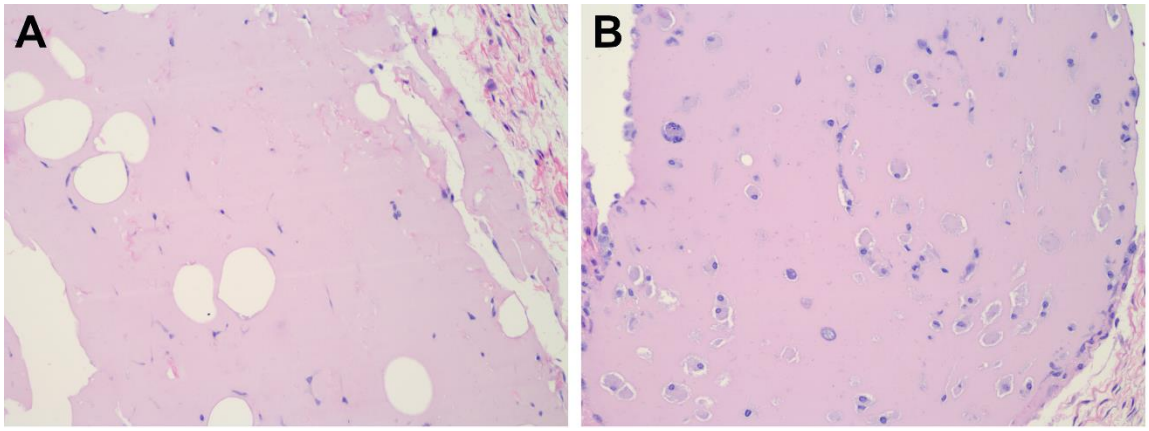

Figure 12. H&E sections of Matrigel. (A) The control mice were injected with only Matrigel. Sections from these mice all had similar features of fibrocytes and fatty infiltration. (B) Most of the mice injected with TICs had numerous cells with large cytoplasm throughout the Matrigel. Many cells were binucleated. 200x magnification.

Limiting dilution assay in a murine xenograft model

In order to determine the lowest number of cells needed to repopulate the tumor, a limiting assay was performed using athymic nude mice. MCKOS TICs recently exiting G_0 were used. It was expected that tumors would grow in the higher groups of unenriched cells, but not the injections of 100 cells. The tumors were confirmed microscopically (Figure 13). Regrettably, tumors grew in quantities of 10,000, 1,000, and 100 cells for the unenriched populations. Some of the Matrigel pellets were unable to be recovered. The results from pellets that were recovered are summarized in Table 3. This could indicate that the TIC subpopulation was not properly identified in these cells. Or, more likely, it means that the MCKOS cell line has a high population of plastic cells allowing for increased numbers of TICs in the new microenvironment.

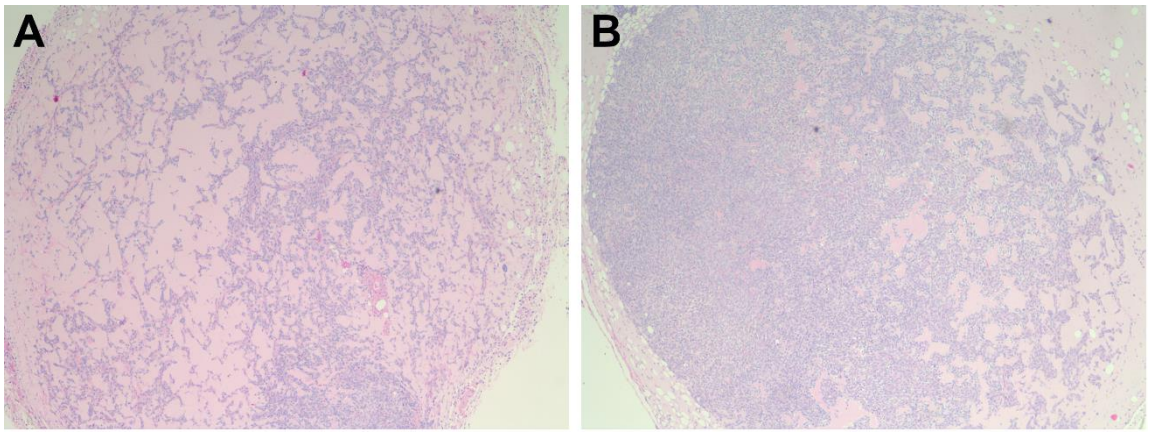

Figure 13. H&E sections of MCKOS tumors from murine xenografts. (A) Unsorted MCKOS cells and (B) MCKOS TIC derived cells invading Matrigel. 200x magnification.

Table 3. Limiting dilution assay of MCKOS cells in athymic nude mice.

Number of Cells	Unenriched	TICs	Matrigel
10,000	8/8	8/8	0/8
1,000	7/7	8/8	
100	8/8	4/4	

Smoothened-Inhibition

Determination of the lethal concentration of cyclopamine and vismodegib

A Calcein AM assay was performed for both cyclopamine and vismodegib. The assay did not give a correlative lethal dose curve because it appears that these drugs do not kill canine osteosarcoma cells in a dose-dependent manner. An AO/PI viability assay was performed on Abrams and MCKOS TICs using vismodegib (Figure 14). After 48 hours, there was no

difference in reduction of TICs treated with vismodegib. This means that smoothened-inhibition does not appear to kill canine osteosarcoma cells. This is not a dire situation because these drugs may have a cytostatic effect on tumor cells through inhibition of invasion or TIC-plasticity.

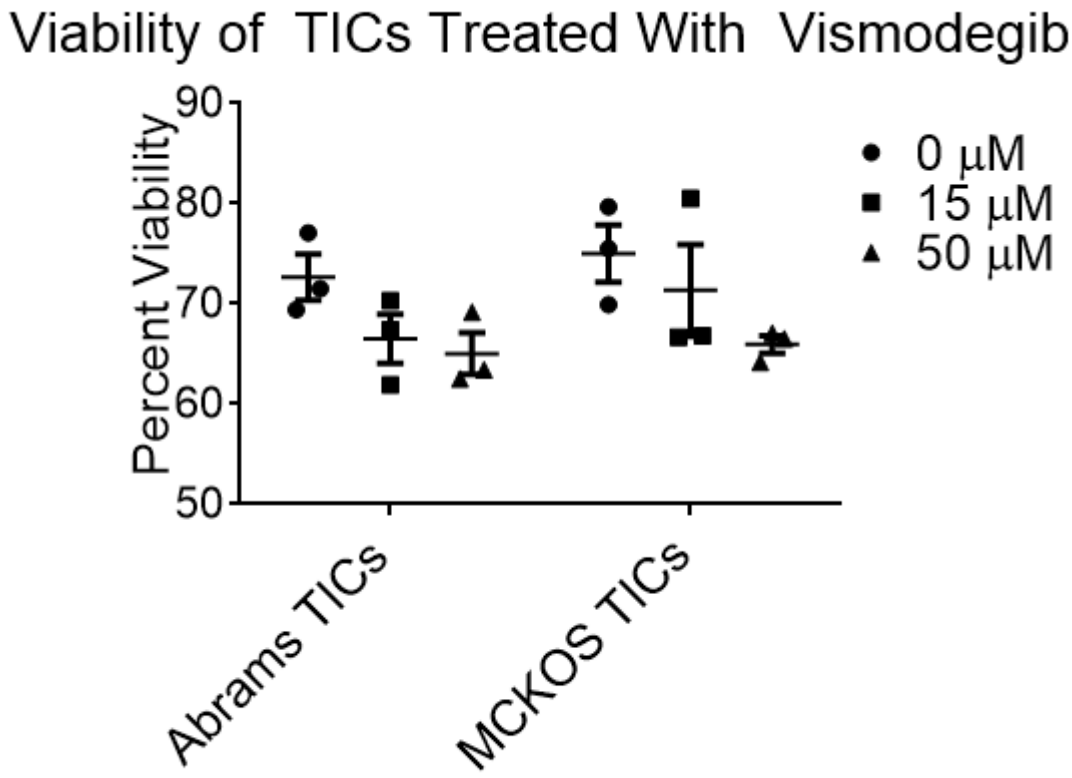

Figure 14. Vismodegib viability assay. The experiments were carried out in triplicates as represented by the clusters on the graph. The wide bar depicts the mean and the shorter bars are standard error of the mean. The mean proportion viable did not significantly differ for the treatment groups indicating that vismodegib did not kill cells off TICs ($p = 0.3$ for 15 µM; $p = 0.1$ for 50 µM).

Gene expression after smoothened-inhibition

Expression levels of the GLI transcription factors was determined in order to verify that smoothened-inhibiting drugs properly target the HH pathway in canine osteosarcoma. Control TICs were compared to treated TICs. Cyclopamine had a much better effect on the HH pathway compared to vismodegib, as shown by the larger decrease in GLI expression (Figure 15). This indicates that cyclopamine and vismodegib do target the HH pathway of canine osteosarcoma TICs.

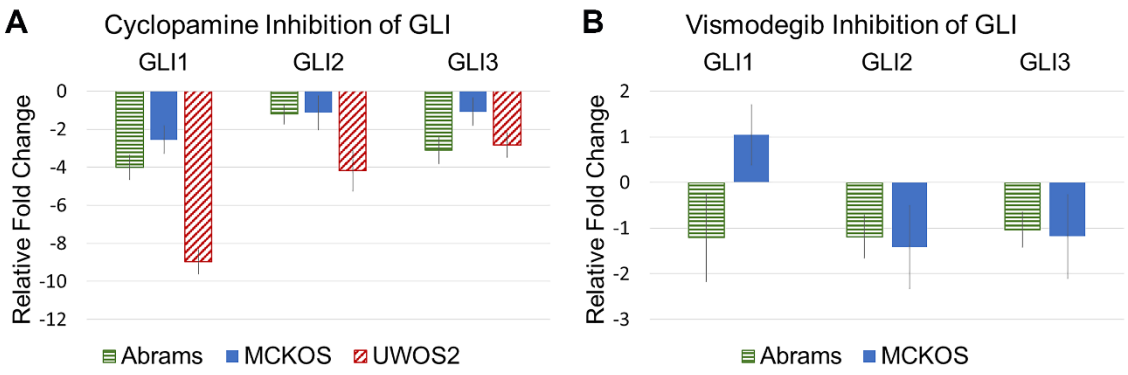

Figure 15. Smoothened-inhibition of GLI. TICs were treated with cyclopamine (A) and vismodegib (B) to evaluate if the HH pathway was inhibited in canine osteosarcoma. The bars represent the standard deviation.

Vismodegib treatment for solid tumors

Next, the efficacy of vismodegib was evaluated for treatment of solid tumors in mice. Tumor size was measured over two weeks with no difference between the treated and untreated groups of mice in size (Figure 16) or percent growth (Figure 17). Frozen tissues for one treated mouse was available.

Compared to three untreated mice, the treated mouse had inhibition of the HH pathway, demonstrated by decreased GLI (Figure 18). These results indicate that vismodegib is probably not adequate for treating primary tumors of clinical patients. Yet, the inhibition of the HH pathway supports that it might work better for preventing or inhibiting micrometastases.

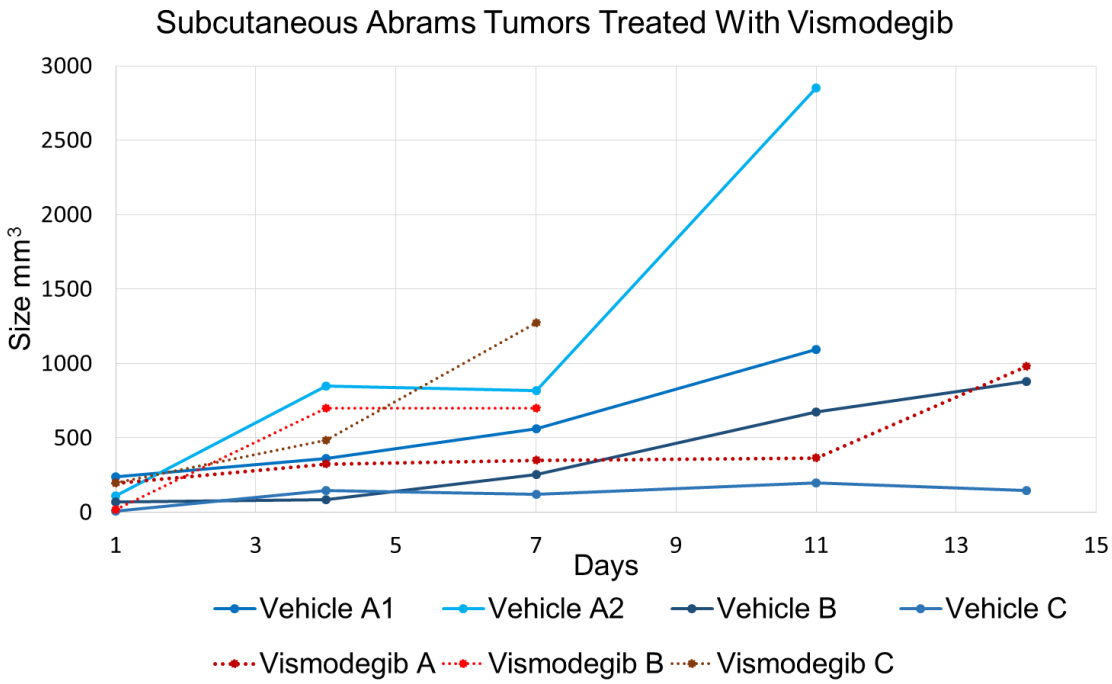

Figure 16. Tumor measurements from mice treated with vismodegib. Tumors were measured for two weeks and vismodegib did not slow the growth of canine osteosarcoma tumors.

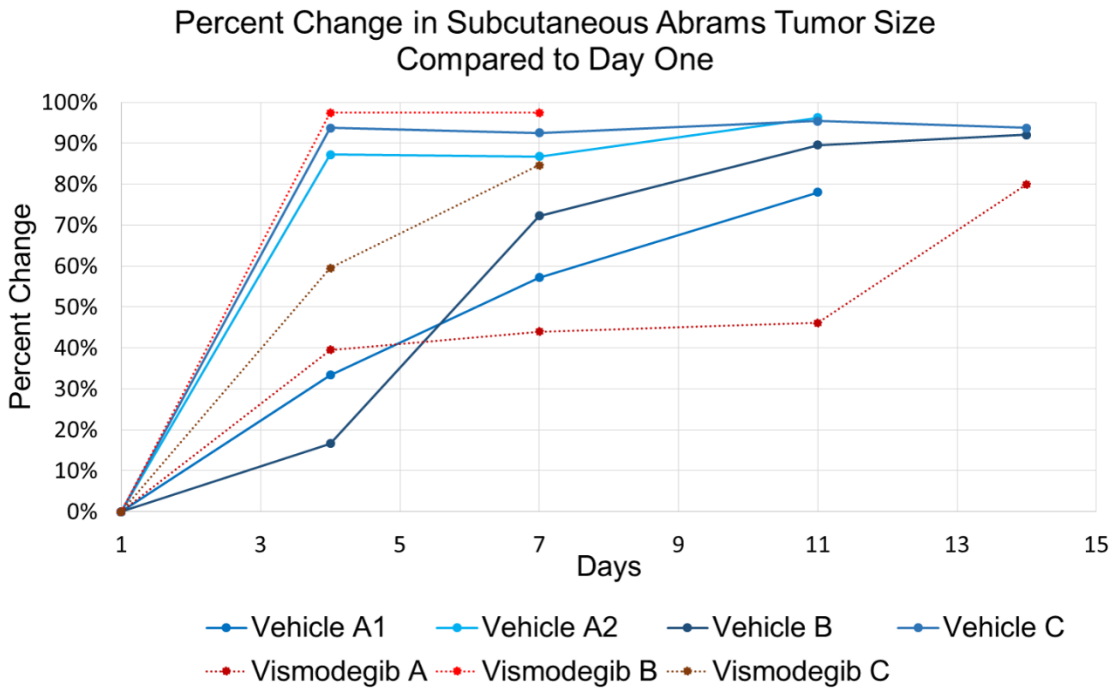

Figure 17. Percent change in tumor growth from mice treated with vismodegib. Tumors were measured for two weeks and vismodegib did not slow the growth of canine osteosarcoma tumors.

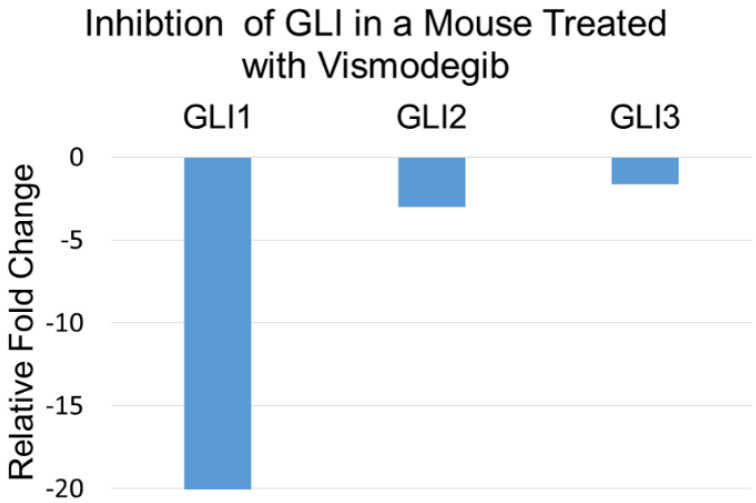

Figure 18. Vismodegib inhibition of GLI. One mouse from the treated group was available for GLI expression analysis. GLI1 showed the most inhibition *in vivo* when compared to the untreated mice.

Vismodegib treatment for pulmonary metastasis

To establish if vismodegib prevented pulmonary metastasis, unenriched cells were intravenously injected into athymic nude mice to represent metastases. MCKOS has a high subpopulation of TICs, which allows for seeding and invasion of the lungs. Vismodegib or placebo treatments were started the following day and the lungs were evaluated for metastases upon completion of the study. One mouse from each group had to be euthanized early due to aspiration pneumonia. Of the remaining six mice, two control mice and one treated mouse had to be euthanized early due to exophthalmos (Table 4).

Table 4. Number of days until mice were euthanized.

Mouse	Vehicle	Vismodegib
D	20 days	20 days
E	15 days	15 days
F	15 days	20 days

The results were recorded two novel ways and analyzed three ways. First, digital measurements were taken of the lungs to determine metastatic area (Figure 19). Next, the digital measurements were reported as a portion of lung area to report the total tumor burden (Figure 20). Finally, quantitative real-time polymerase chain reaction (qPCR) was used to calculate the amount of canine DNA within the mouse DNA from the lungs (Figure 21). All three methods gave similar results with one untreated mouse having a very high tumor burden, and all three treated mice having a low tumor burden. This is just a small pilot study,

but these data support the use of vismodegib as an adjuvant therapy for canine osteosarcoma to prevent the growth and progression of micrometastases after primary tumor removal.

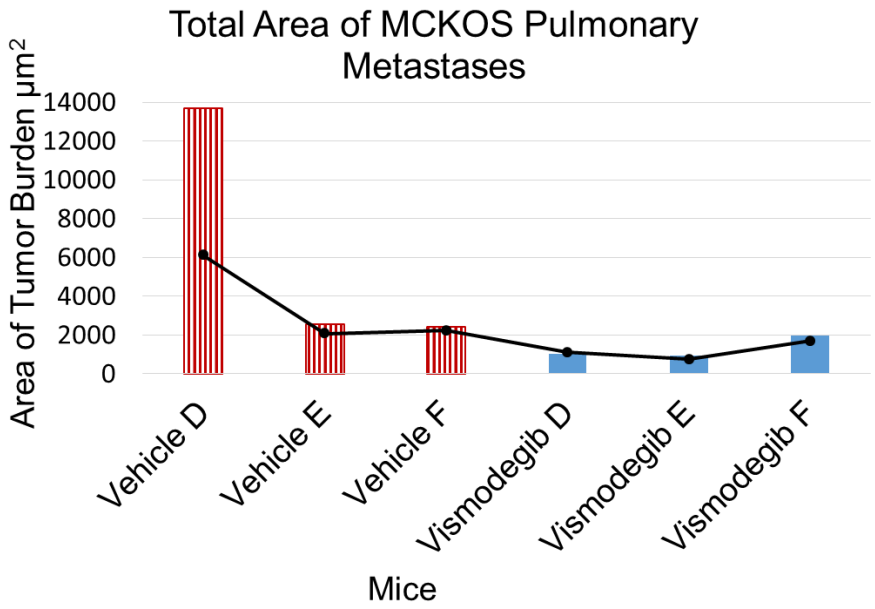

Figure 19. Area of pulmonary metastases. The total area of tumor cells was measured for each mouse. The bars represent the mean area and the line graph represents the median for each mouse.

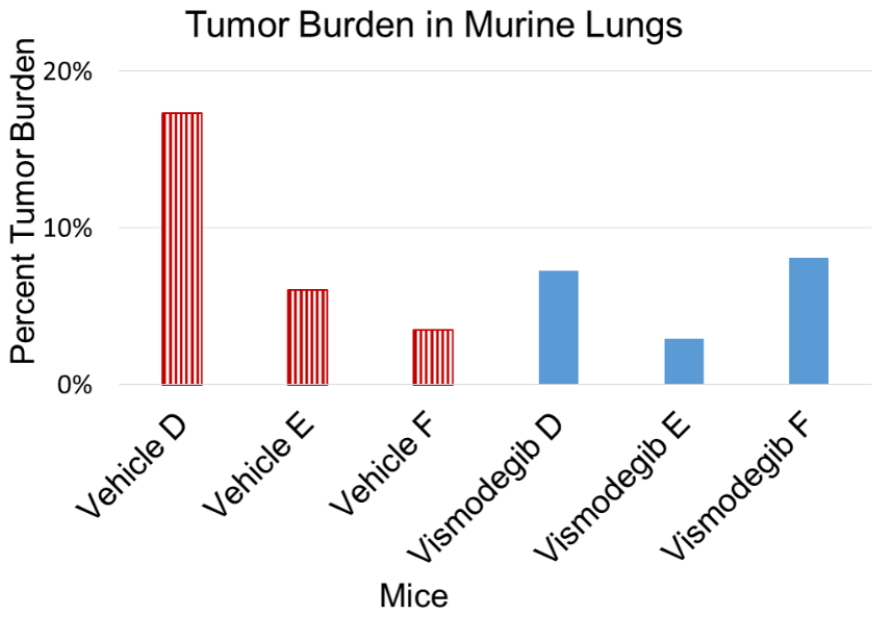

Figure 20. Tumor burden on lungs. The percent tumor burden was calculated by dividing the area of mouse tumor by the total area of lung for each mouse.

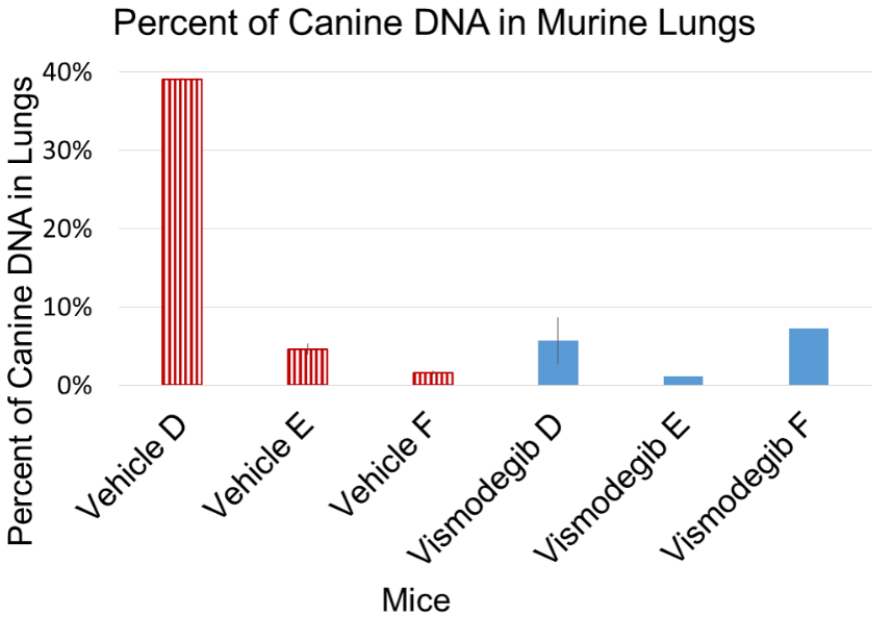

Figure 21. Percent of canine DNA in murine lungs. DNA from the lungs of each mouse was purified. Quantitative DNA analysis revealed similar results to the image analysis. The bars represent the standard deviation.

HER2-Inhibition

HER2 in spontaneous canine osteosarcoma

To determine if HER2 is prognostic in a canine osteosarcoma population, 47 cases were evaluated. The ages of the dogs at diagnosis ranged from one to 17 years. The average age of diagnosis was 7.4 years. Some dogs were euthanized at diagnosis and were not included in the survival analysis. The shortest survival time was 21 days. The dog with the longest survival was 2,533 days. The median survival time as determined by Kaplan-Meier analysis was 159 days. Seventeen different breeds of dogs and six mixed breeds were included in the population. Five purebred breeds were overrepresented: nine rottweilers, nine Labrador retrievers, four golden retrievers, three greyhounds, and three German shepherd dogs. Rottweilers and retrievers had a poorer survival time than the other breeds of dogs within this population ($p = 0.0005$ and $p = 0.07$, respectively). The population was roughly half male (48.9%) and half female (51.1%). Six dogs were intact, whereas 42 dogs were surgically sterilized. Dogs that received surgery had a better survival than dogs that did not ($p = 0.001$).

The seven subtypes observed in this population are represented in Figure 22 (A-G). The HER2 labeling was diffuse and cytoplasmic, as observed in human samples (Figure 22H-I). Tumors were considered positive if the staining intensity was stronger than what is seen in healthy bone. Within this population

85.1% of the canine osteosarcomas overexpressed HER2. Survival was compared to HER2 overexpression, osteosarcoma subtype, mitotic rate, and other variables. HER2 overexpression did not prognosticate survival (Figure 23). The population was not large enough to determine if histologic subtype was associated with survival or HER2 overexpression. Other histologic features did not predict survival nor HER2 overexpression within this population. A larger sample size may have powered this study better in order to determine more predictive variables. Even though HER2 overexpression was not a good prognostic indicator in canine osteosarcoma, the mere presence of HER2 in canine osteosarcoma supports investigation of drugs that target this protein.

HER2 in canine osteosarcoma cell lines

Next, HER2 was identified in Abrams and MCKOS cell lines using immunocytochemistry (Figure 24). Both unenriched cells and TIC-enriched cells had strong and uniform HER2 labelling. Additional cells lines should be evaluated to determine if some canine osteosarcomas exhibit a difference in HER2 expression in the TIC subpopulation.

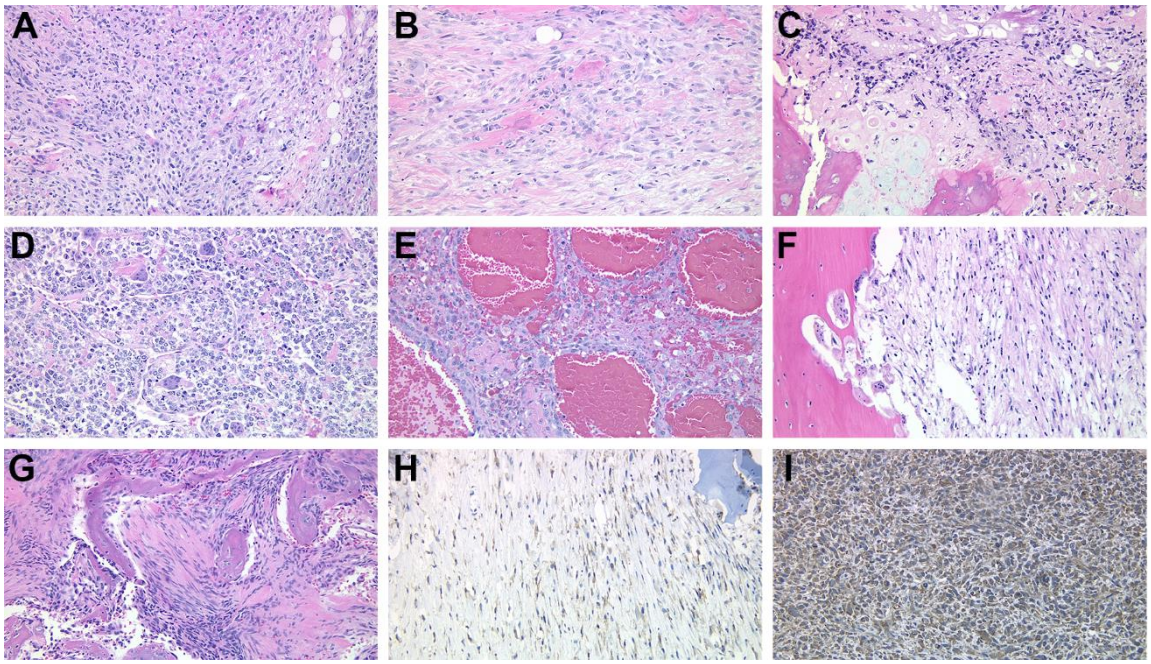

Figure 22. Spontaneous canine osteosarcoma histology. Images of H&E stained tumors represent the different osteosarcoma subtypes identified in this population. They consist of (A) osteoblastic, (B) fibroblastic, (C) chrondroblastic, (D) giant cell, (E) telangiectatic, (F) sclerotic, and (G) anaplastic. An osteosarcoma with normal (H) and overexpressed (I) HER2 labeling demonstrate the difference in staining intensity between groups. 200x magnification.

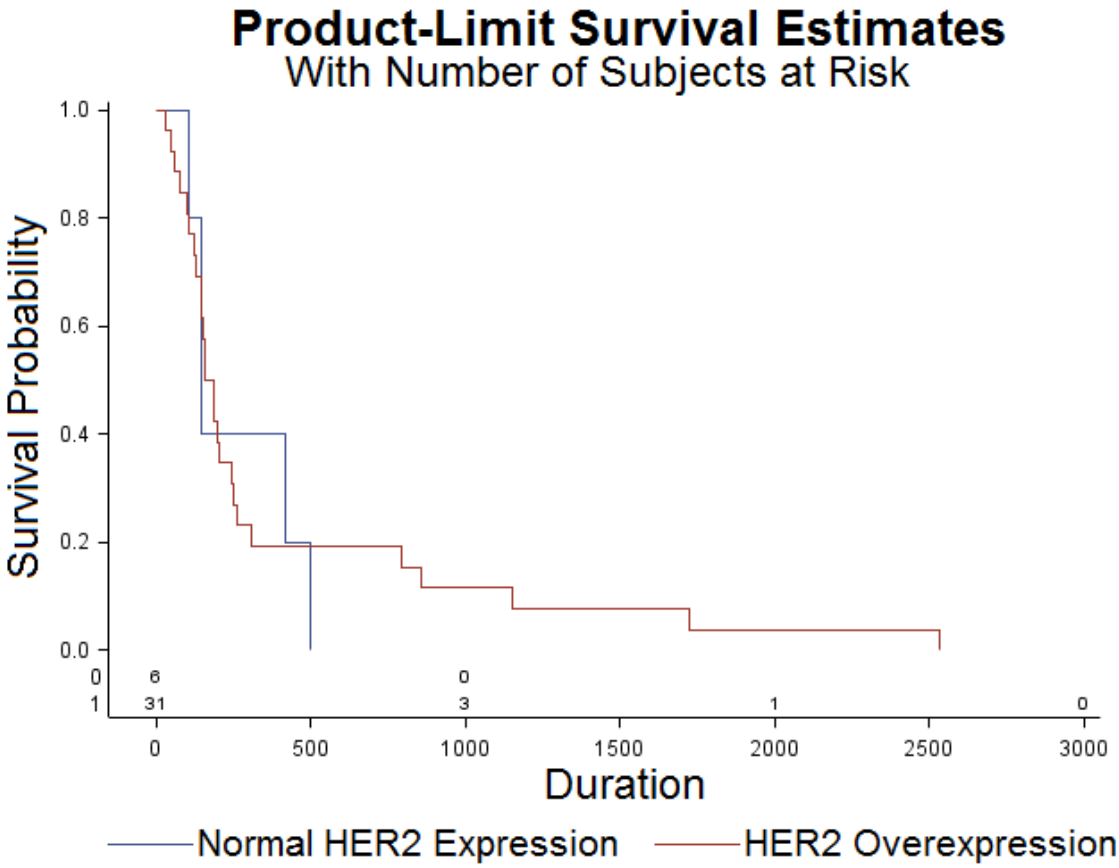

Figure 23. Kaplan-Meier survival curves. HER2 overexpression in spontaneous canine osteosarcomas (red) was not prognostic when compared to osteosarcomas with staining intensity similar to bone (blue).

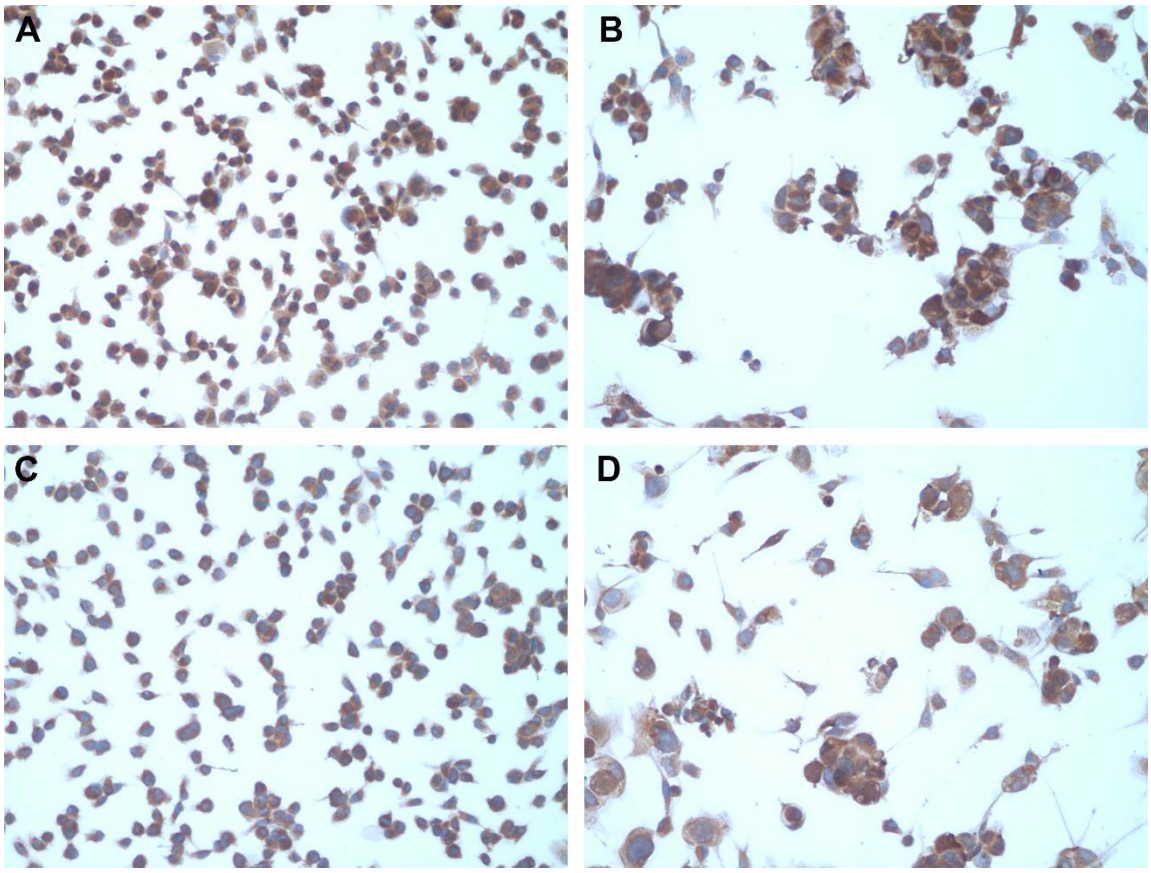

Figure 24. HER2 immunocytochemistry. (A) Unenriched Abrams cells, (B) TIC-enriched Abrams, (C) unenriched MCKOS, and (D) TIC-enriched MCKOS all exhibit strong HER2 labeling. 200x magnification.

Determination of the lethal concentration of lapatinib

A Calcein AM assay was used to determine the lethal concentration of lapatinib. The 95%, 75% and 50% lethal concentrations were estimated from the equation of the logarithmic curve (Figure 25: $LC_{95,}$ LC_{75}, and LC_{50}). These results are consistent with reports from other laboratories for other human cancer cell lines. An AO/PI viability assay was performed on Abrams and MCKOS TICs using lapatinib (Figure 26). After 24 hours, there was a significant reduction in

TICs (0.63 for DMSO-treated control TICs, 0.41 for lapatinib-treated TICs, p = 0.04). This indicates that lapatinib targets HER2 in canine osteosarcoma TICs with lethal consequences.

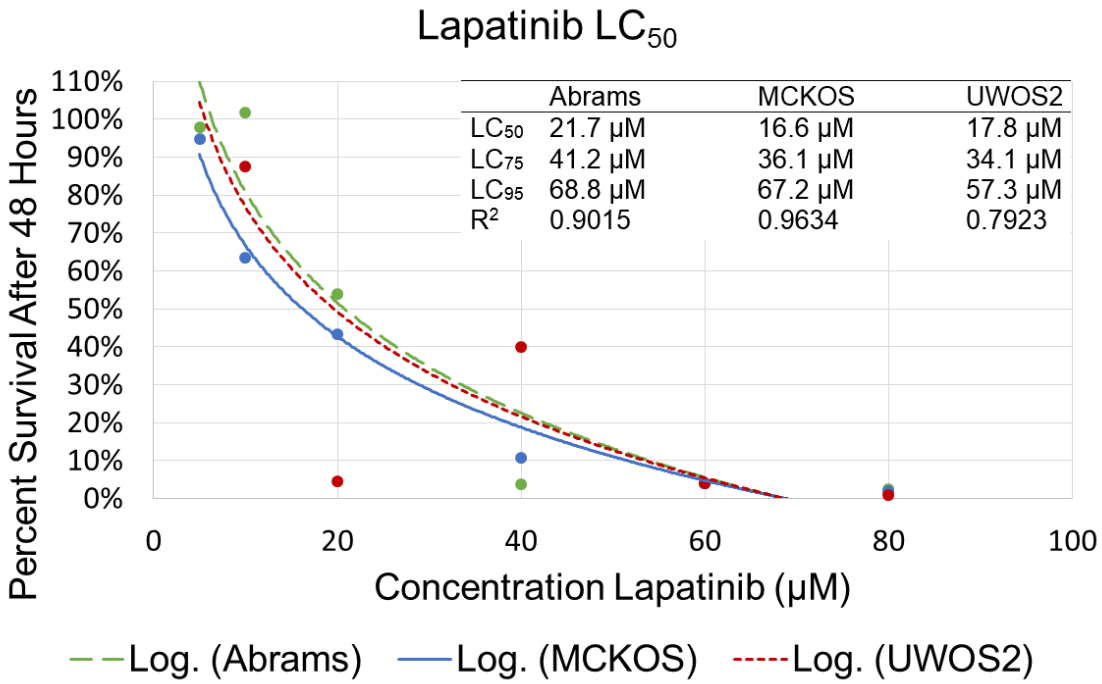

Figure 25. Percent survival of canine osteosarcoma cell lines after 48 hours of lapatinib treatment. The curves shown are the proportions of viable cells after treatments of various concentrations of lapatinib. The logarithmic curve was used to calculate the 95%, 75%, and 50% lethal concentrations. The lethal concentrations are summarized in the inset. The associated R^2 values of the curves are also listed.

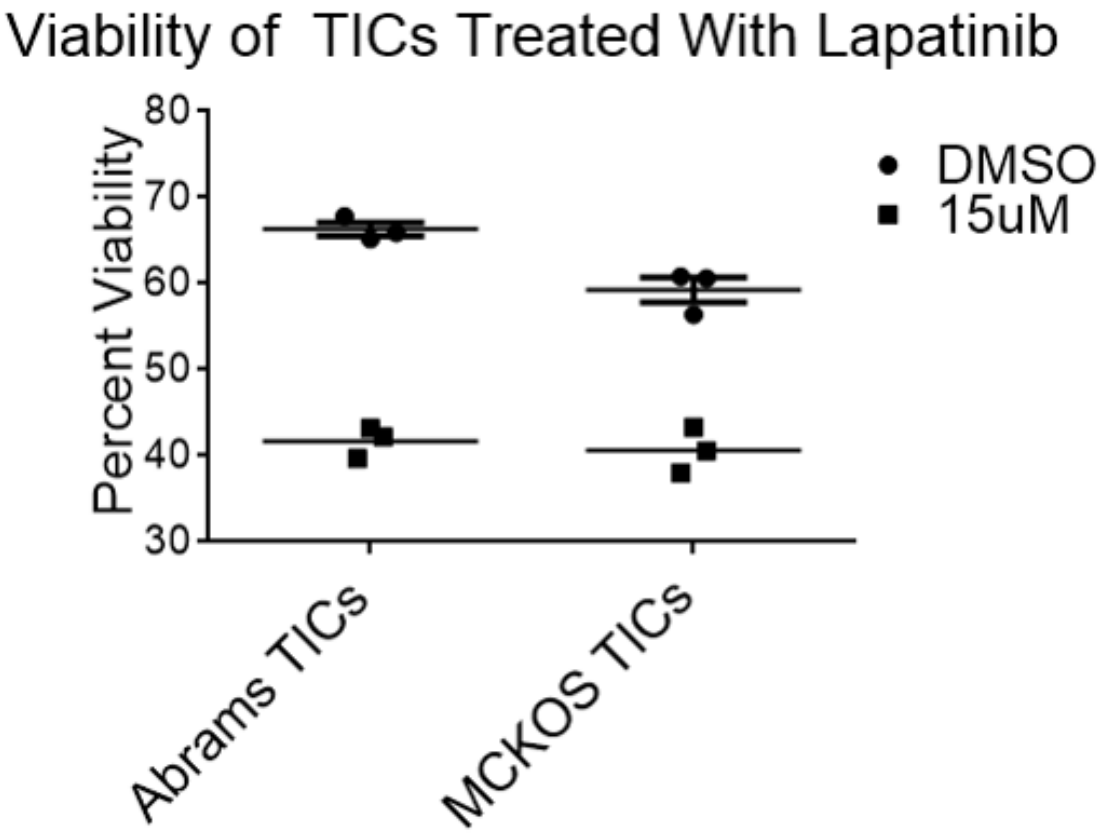

Figure 26. Lapatinib viability assay. The experiments were carried out in triplicates as represented by the clusters on the graph. The wide bar depicts the mean and the shorter bars are standard error of the mean. The mean proportion viable differed significantly for the treatment group (0.63 for DMSO-treated control TICs, 0.41 for lapatinib-treated TICs, $p = 0.04$).

Gene expression following lapatinib treatment

Canine osteosarcoma cells were evaluated *in vitro* to establish if lapatinib treatment inhibits HER2 (Figure 27) and its downstream mediator, AKT1 (Figure 28). First, unenriched cells were evaluated at three concentrations: 7.5, 15, and 22.5 µm. Both genes were downregulated with almost all concentrations. Next, TICs from both cell lines were evaluated after lapatinib treatment. Both AKT1 and HER2 were downregulated in the treated TICs (Figure 29). These results indicate that lapatinib targets HER2 in canine osteosarcoma differentiated cells and TICs.

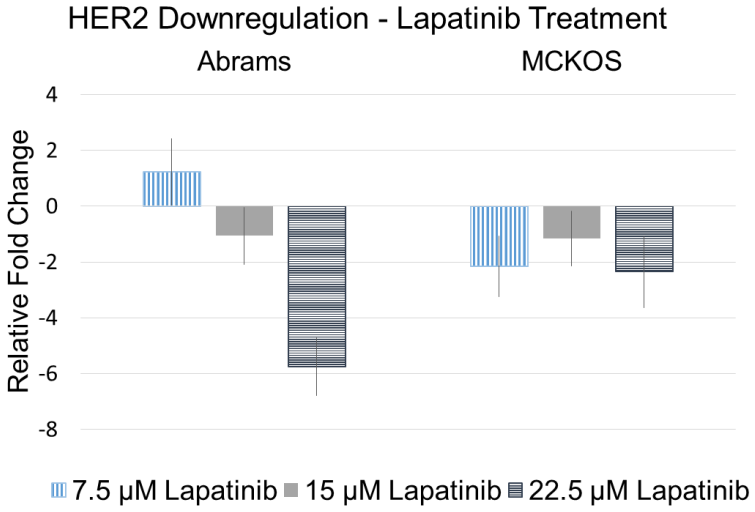

Figure 27. HER2 expression after lapatinib treatment. HER2 was reduced in both Abrams and MCKOS cell lines at higher concentrations of lapatinib. The bars represent the standard deviation.

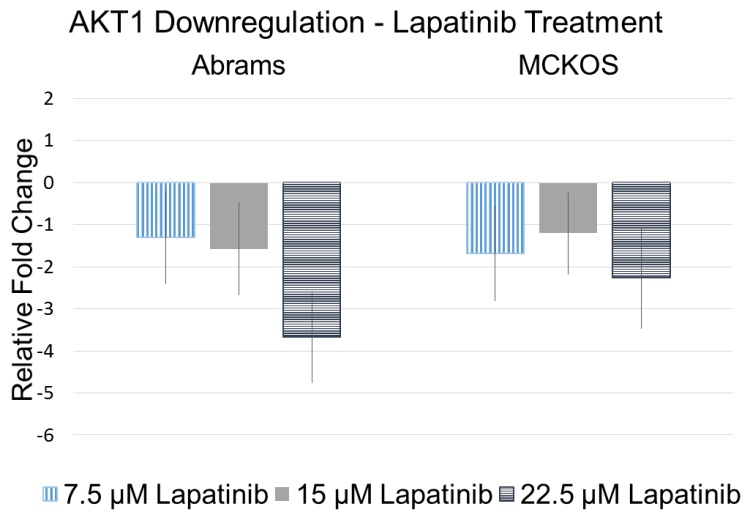

Figure 28. AKT1 expression after lapatinib treatment. AKT1 was reduced in both Abrams and MCKOS cell lines at all concentrations of lapatinib. The bars represent the standard deviation.

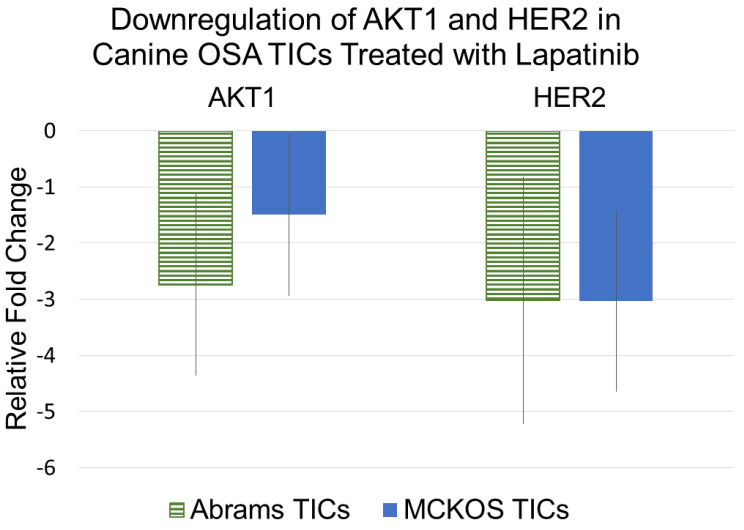

Figure 29. AKT1 and HER2 expression in TICs treated with lapatinib. AKT1 and HER2 were both downregulated in TICs from Abrams and MCKOS. The bars represent the standard deviation.

Lapatinib treatment for solid tumors

Finally, to determine the efficacy of lapatinib, mice with solid Abrams tumors were treated. Half of the tumors from treated mice had slower growth compared to the untreated mice receiving vehicle only (Figure 30 and 31). The tumors from the treated group had depressions indicating necrosis and these findings corresponded with the histologic findings (Figure 32A-B). Four out of four tumors from treated mice had necrosis whereas only two of the four control tumors did. HER2 was downregulated in the tumors from the treated mice, but surprisingly, AKT1 was not (Figure 32C). The mitotic rate was 3% higher in the treated tumors (Figure 32D). These results are inconclusive for a pilot study, but supports investigating lapatinib with larger samples and in other species.

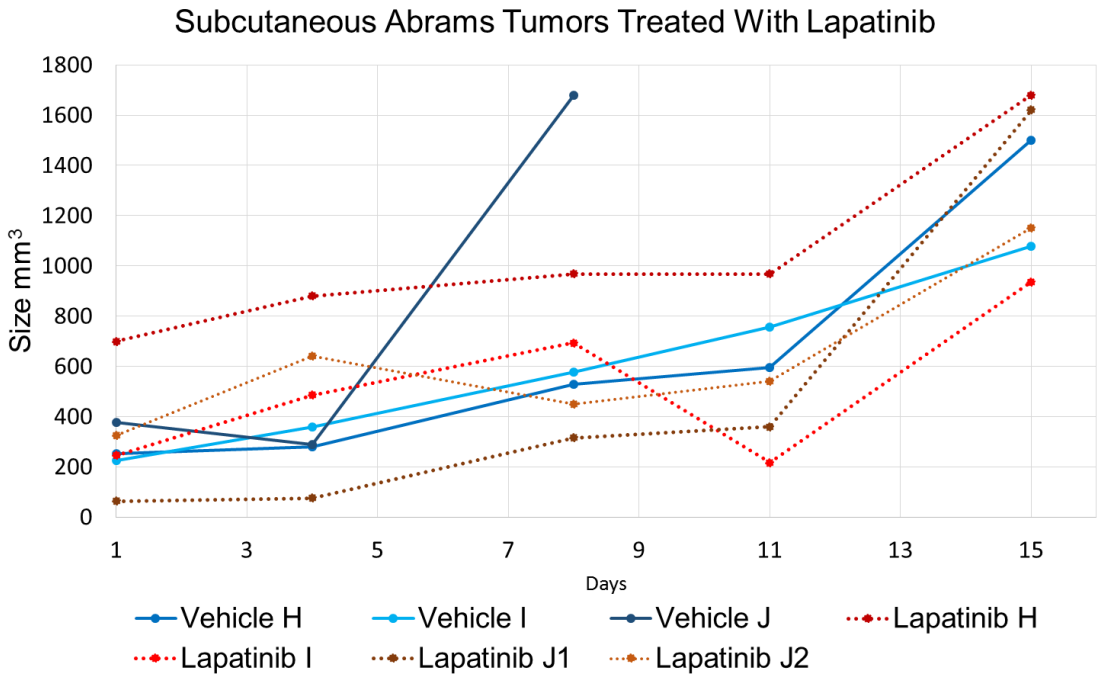

Figure 30. Tumor measurements from mice treated with lapatinib. Tumors were measured over 15 days with approximately half of the tumors slowed with lapatinib treatment.

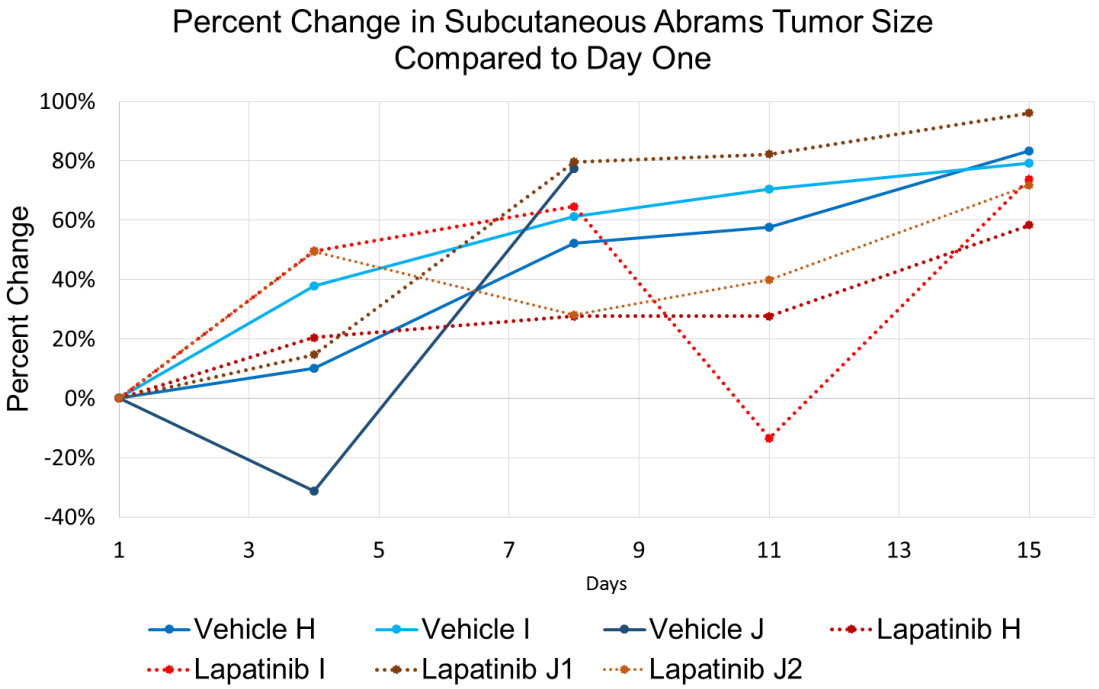

Figure 31. Percent change in tumor growth from mice treated with lapatinib. Tumors were measured for 15 days and the change from the first day was plotted with approximately half of the tumors slowed with lapatinib treatment.

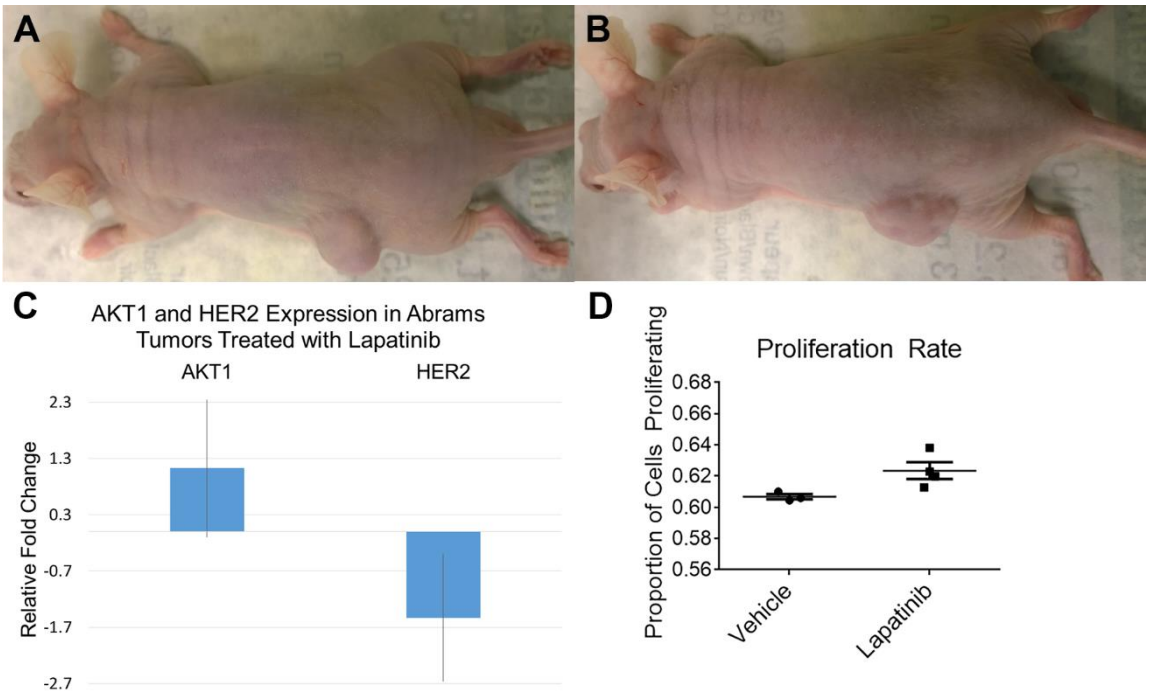

Figure 32. Mice treated with lapatinib. (A) Untreated mice had plump tumors, whereas lapatinib-treated mice (B) had bumpy tumors with areas of collapse from necrosis. (C) HER2 expression was slightly decreased in treated mice, but AKT1 was not. (D) The proliferation rate was determined using Ki-67 immunocytochemistry. The tumors from the lapatinib treated mice had an increase of 3%.

CONCLUSION

Summary and Discussion

In this study, a reliable and reproducible method for creating an enriched TIC population for canine osteosarcoma was developed. It was demonstrated that these TICs can be targeted with vismodegib and lapatinib. Most importantly, these results support beginning clinical trials in dogs with spontaneous osteosarcoma to determine if smoothened-inhibiting or HER2-targeting drugs will have an effect on metastases.

Tumor-initiation cells

Canine osteosarcoma is not only an ideal model for human osteosarcoma, but possibly for general TIC pathogenesis as well. Osteosarcoma can be difficult to diagnose due to its wide range in osteoid production and cellular morphology. In addition, the cancerous cells may redifferentiate to begin producing chondroid or fibrous looking cells. It is purely speculation, but the morphology of increasingly aggressive osteosarcomas follows the same pattern of what is known about TIC progression. It would be interesting to determine the TIC profiles of clearly differentiated osteosarcomas versus osteosarcomas with two or more mesenchymal cell morphologies versus aggressive anaplastic

osteosarcomas. Tumor features may direct treatment in osteosarcoma patients in the future, as is the case with non-Hodgkin's lymphoma and breast cancer.

Cancers from humans have more reliable surface markers for TIC isolation. In addition, human cancers have reliable side populations when processed with aldehyde dehydrogenase or Hoechst methods. In contrast, canine cancers are more limited for TIC isolation procedures. Before optimizing the epirubicin protocol, other methods were used on the canine osteosarcoma cell lines. The surface markers CD34 molecule (CD34), CD44, CD133, and ABCG2 were used to attempt to isolate TICs through magnetic bead sorting and flow cytometry. None of these surface markers reliably separated out the TIC population. Sarcosphere culture was also attempted, but despite using very low seeding concentrations the cells would aggregate. This confounded the results. The aldehyde dehydrogenase assay was also used to attempt to identify the TIC subpopulation, but as other have reported, the canine cells did not work well with the Aldefluor kit [246]. Epirubicin treatment was the only method that reliably produced a TIC-enriched population for study.

Some would argue that an "enriched population" is not appropriate for study and that it is necessary to produce a uniform population of TICs. If the goal is to identify incredibly delicate facts about TICs, such as genome differences from other cells within the tumor, then yes, a clean population is needed. In this case, the goal was to find drugs that target these cells for death. A slightly mixed population of cells still produces data similar to what would be seen in a pure

population. This is enough information to move forward towards getting more treatment options to patients, instead of getting hung up in the details.

With epirubicin treatment, there was a slight difference between the number of cells killed off in the Calcein AM assay versus the AO/PI viability assay. The Calcein AM assay predicted that around 50% of the cells would be killed at 2 µg/ml. This number was expected to be lower when treated in serum-free medium in an anchorage-independent manner. This may indicate that harsh culture conditions activated mechanisms for cell plasticity leading to increased survival. The switch from undetectable NANOG to a higher proportion of cells expressing NANOG also supports this notion.

There was considerable fluctuations in gene expression when the TICs were analyzed over one week. This further demonstrates the plasticity of these cells and reminds scientists to analyze their data spatially. Based on the results of NANOG, these osteosarcoma cell lines have a plastic population of TICs. OCT4 was barely detected in the unenriched cell populations, and NANOG was not detected. It was incredibly difficult to design primer pairs that were specific to OCT4 in the dog. Numerous pairs of published primers were evaluated, but once the product was sequenced it was revealed that the OCT4 pseudogene was targeted instead. It appears that the predicted sequences for OCT4 and the OCT4 pseudogene in the National Center for Biotechnology Information database may be incorrect. Personally designed primers using the OCT4 sequence from the database also resulted in targeting the pseudogene. It is

important for the OCT4 gene and any paralogs to be properly sequenced before OCT4 can be reliably used as a marker for stemness in dogs. There are alternative theories where the gene database is correct. In humans, a NANOG paralog is responsible for most cancers [190-196]. A NANOG pseudogene has not been identified in dogs, yet dogs have a higher cancer rate compared to humans. OCT4 is the first upstream gene responsible embryogenesis, so in cancer it should play a more aggressive role than NANOG. Perhaps the gene database is correct, but canine cancers upregulate the OCT4 psudeogene similar to human cancers utilizing the NANOG pseudogene. If this is the case, it could explain why dogs have a higher cancer rate than humans. A second alternative reason for the primers not working properly is cell culture alteration. Possibly over years of passages OCT4 has mutated in canine osteosarcoma cell lines.

Perhaps the most exciting information about this canine model of osteosarcoma is the evidence of quiescence. In both human and canine osteosarcoma the goal is to target cells that have metastasized and remained stealthed. Other *in vitro* models of osteosarcoma isolated TICs and then immediately demonstrated aggressive replication. The canine osteosarcoma model with epirubicin selection pushed cells into quiescence and this mimics what is seen clinically. The cells went into quiescence to protect themselves, with evidence of p21 upregulation. Conditions were improved with daily medium changes. Three to five days later, the cells exited quiescence as shown by

decreased p27. The amount of NAONG was diluted as the cells began repopulating the differentiated tumor cell population. This is an accelerated model of osteosarcoma TIC plasticity and quiescence, which is what is presumed to take place during metastasis. Others have suggested a treatment strategy of driving the cells out of quiescence so that cytotoxic drugs will affect them [144]. Lapatinib increased the mitotic rate within solid tumors. This should be explored further to determine if targeting HER2 drives TICs out of quiescence, making them subsequently more susceptible to other more traditional therapies.

Other experiments were attempted, but did not properly work on canine TICs. Western blots were attempted with mixed results. The antibodies that did not work with TIC isolation assays gave too much non-specific signal on western blot analysis. Antibodies to intracellular targets, such as NANOG, were also disappointing. Invasion assays were also attempted to determine the drug potential to inhibit metastasis. The canine osteosarcoma cell lines were far too aggressive, which confounded the data. Finally, cells and TICs were irradiated to determine if TICs could repair DNA faster. A fluorescent antibody, anti-H2A histone family, member X was used to monitor DNA repair. The difference between groups was negligible, again possibly due to the aggressive nature of the cell lines used. This area of canine osteosarcoma TIC research would benefit from a comparison between established and early passaged cell lines.

Smoothened-inhibition

Vismodegib showed the greatest potential as an inhibitor of osteosarcoma metastases. Future clinical studies should be designed so that the patient begins taking vismodegib at diagnosis and in combination with other recommended treatments. The mice were treated for three weeks or less using an accelerated model of pulmonary metastases. Long term vismodegib treatment in a human or dog, with a realistic rate of metastases, might result in better performance of vismodegib.

The lungs from the mice were analyzed with two techniques in parallel. The most accurate method is digital analysis using multiple non-serial slides to give a three-dimensional representation of the pathology. This is very time consuming. Forensic researchers have developed species-specific primer pairs for DNA analysis [360]. The amount of canine DNA within the mouse lungs was able to be determined using this technology. It is slightly less accurate because the tissue blocks included the esophagus and trachea with the lungs. Despite this complication, the PCR data closely matched the pattern of the digital analysis. Plus the DNA analysis was 95% quicker. Future experiments planning to utilize DNA analysis should receive adequate tissue dissection to make this test even more accurate.

HER2-inhibition

The canine osteosarcoma biopsies evaluated were predominately positive for HER2. This is in agreement with the majority of human studies. In contrast, HER2 overexpression was not prognostic in dogs. Regardless, the identification of HER2 in so many cases supports using drugs like lapatinib in canine clinical trials.

Concluding Remarks

The future goal of this research is to merit beginning clinical trials in dogs with spontaneous osteosarcoma as a model for human osteosarcoma. Previous studies suggest that osteosarcoma micrometastases are present in up to 90% of canine osteosarcoma cases at the time of diagnosis. Even when these metastases are not visualized with radiographic techniques, experienced veterinarians understand that they are present. Metastases will grow months following amputation of the primary tumor. It makes sense to start TIC-inhibiting drugs at the time of diagnosis and prior to surgery, and to continue treatment, preferably in combination with chemotherapy or radiation, until the drugs are not effective. In patients where metastases are evident at diagnosis or in patients that are not a candidate for surgery (i.e. axial osteosarcoma), TIC-inhibiting drugs could intensify the effectiveness of adjuvant treatment. This is an exciting

new area of cancer research with prospective treatments that may bring relief from those suffering from osteosarcoma.

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