INVESTIGATING FIBROBLAST GROWTH FACTOR AND ITS ROLE IN CANINE OSTEOSARCOMA

BY

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THESIS

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ABSTRACT

Canine osteosarcoma (OS) is a malignant neoplasia of the osteoblast, most often identified in the appendicular skeleton, which is both locally aggressive and highly metastatic. The standard of care treatment of amputation and adjuvant chemotherapy yields a median survival time of 10-12 months, an improvement in which has not been noted despite active research. The biologic behavior of cancers seems to be correlated to the state of differentiation of the tumor cell population. Differentiation is taken into account when a histopathologic grade is assigned to a particular tumor; poorly differentiated tumors acquire a higher grade and are expected to behave more aggressively. The tumor microenvironment contains many cells and signaling molecules such as cytokines, chemokines and growth factors. Investigating these factors may identify a stimulus for a less differentiated and more aggressive neoplasia. This is even more compelling if the stimulus is a druggable target.

Fibroblast growth factors (FGFs) interact with fibroblast growth factor receptors (FGFRs) to initiate cell signaling that is important in embryonic development, wound healing, and angiogenesis. FGF2, also termed basic FGF (bFGF), plays an important role in osteoblast proliferation and maintaining osteoblasts in an undifferentiated state.

We hypothesize that 1) canine OS cells will express FGFRs and FGF2, 2) FGF signaling blockade will attenuate pro-tumorigenic properties in OS cells, 3) FGF signaling blockade will cause enhanced differentiation of the OS cell lines, and 4) circulating FGF2 will be increased in canine OS patients compared to healthy controls and increased in dogs with osteoblastic OS compared to those with osteolytic OS.

We investigated FGFR gene expression with reverse transcriptase polymerase chain reaction (RT-PCR) and FGF2 secretion via ELISA. The effects of FGF signaling attenuation on
OS cell pro-tumorigenic properties were evaluated by colorimetric proliferation assay and scratch migration assay. The effects of FGF signaling blockade with pan-FGFR inhibitor BGJ398 on OS cell differentiation were evaluated with Alizarin Red staining and quantification, alkaline phosphatase (ALP) bio-activity, and quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) for osteogenic genes alkaline phosphatase (ALP), osterix(OSTX), osteonectin (OSN), and runt-related transcription factor 2 (RUNX2). Circulating FGF2 levels were quantified in the plasma of dogs with naturally occurring OS (determined to be osteoblastic or osteolytic based on relative bone mineral density obtained via DEXA scan) and healthy controls via ELISA.

FGFR gene expression was noted in OS cell lines. FGF2 secretion was identified in all cell lines with secretion noted in a clear cell density-dependent manner in 2 of 3 cell lines. FGFR signaling attenuation inhibited OS cell migration while not affecting cell proliferation. FGFR signaling blockade increased differentiation in 1 of 2 cell lines evaluated, with a trend toward increased differentiation in the other cell line. The presence of naturally-occurring OS did not alter the level of circulating FGF2 in dogs. This study is the first to identify a link between FGF signaling attenuation and OS cell differentiation and migration in a cell line-dependent manner. These findings indicate that FGF blockade could be beneficial in some dogs with OS.
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Differentiation is an important indicator of tumor grade and prognosis, with poorly differentiated tumors behaving more aggressively than those that are well differentiated (1–3). It has been postulated that forcing cancer cells into a more differentiated state represents a possible form of cancer treatment, as evidenced by in vitro work with a multitude of cancer types (1,4–6). The use of differentiation agents has been employed against some human cancers as an adjuvant or primary treatment, including acute promyelocytic leukemia (5) and neuroblastoma (1). In humans, there is a clear link between osteosarcoma (OS) grade, which includes differentiation status, and prognosis (7–10). This link is a challenge to prove with canine OS, as this cancer is commonly highly aggressive and grading systems have not accurately and reliably predicted prognosis (11–16).

Fibroblast growth factors (FGFs) are a family of 18 polypeptides which interact with fibroblast growth factor receptors (FGFRs) to initiate cell signaling that is important in embryonic development, wound healing, and angiogenesis (17–25). FGF2, also termed basic FGF (bFGF), plays an important role in osteoblast proliferation and maintaining osteoblasts in an undifferentiated state (21,26,27). FGF2 has also been shown to maintain OS cells in a de-differentiated state, contributing to their aggressiveness (28). This ligand is present in the tumor microenvironment, shown to be associated with tumor associated macrophages. Osteoblasts may also secrete FGF2(27), which could lead to autocrine and paracrine signaling if this phenomenon is noted in the malignant counterpart. BGJ398 is a pan-FGFR inhibitor which has been evaluated in several human cancer cell lines, in xenograft models, and in clinical trials (29–41).
The purpose of this study is to investigate the role FGF signaling plays in canine OS differentiation and aggressiveness. The objectives of this study were to 1) identify FGFR expression and FGF2 secretion in canine OS cells, 2) evaluate the effects of the pan-FGFR inhibitor BGJ398 on OS cell pro-tumorigenic properties, 3) determine the effects of FGFR inhibition on OS cell differentiation, and 4) determine if circulating FGF2 levels are higher in dogs with OS compared to healthy dogs, or in dogs with osteoblastic OS compared to osteolytic OS.

We hypothesize that 1) canine OS cells will express FGFRs and FGF2, 2) FGF signaling blockade will attenuate pro-tumorigenic properties in OS cells, 3) FGF signaling blockade will cause enhanced differentiation of the OS cell lines, and 4) circulating FGF2 will be increased in canine OS patients compared to healthy controls and increased in dogs with osteoblastic OS compared to those with osteolytic OS.
CHAPTER 2
LITERATURE REVIEW

2.1 Differentiation

2.1.1 Overview

Differentiation is the process that determines the ultimate specialized phenotype and function of a particular cell within an organism(5). After the first several divisions of the fertilized egg, embryonal stem cells develop. These are considered totipotent stem cells, meaning that they have the ability to give rise to any tissue within the body. After many symmetric divisions, in which the embryonal stem cells give rise to more embryonal stem cells, the divisions become asymmetric – one of the daughter cells remains an embryonal stem cell while the other daughter cell becomes either a germinal stem cell or a somatic stem cell (progenitor cell). Germinal stem cells will give rise to reproductive cells such as sperm and egg, while somatic stem cells will ultimately give rise to the mature cells that make up the functional organism. The division of these more specialized stem cells remains asymmetric, with one daughter cell retaining the stem cell properties as the other daughter cell undergoes further differentiation through a series of cell divisions (transit amplifying cell). The tissue-determined stem cell will largely remain quiescent, as tissue renewal is primarily provided by the transit amplifying cells. The ultimate fate of the transit amplifying cell is to become the mature functional cell. As the cell divides and becomes more differentiated, the capacity for producing progeny of many different cell types (plasticity) is limited while specialized functions are gained. Terminally differentiated cells are the most highly specialized, no longer divide, and ultimately undergo apoptosis.

2.1.2 Differentiation in neoplasia
The determination of cancer cell differentiation is a process familiar to surgical pathologists, as this is an important criteria in identifying the histopathologic grade of the tumor (1). Well differentiated tumors structurally resemble the tissue of origin and typically carry a better prognosis, while poorly differentiated tumors do not resemble the tissue of origin, often with a concurrent reduction of cohesiveness and increased invasiveness into surrounding tissues, and carry a poor prognosis; tumors that are intermediately differentiated lay between. This spectrum of differentiation indicates that cancer cells may either de-differentiate or arise from a stem cell somewhere along the path of differentiation (1,3–5). The cancer stem cell theory is attractive, as it explains that genetic mutation is more likely to occur in a transit amplifying cell while it is rapidly dividing, prior to terminal differentiation, and maturation arrest at any point in the differentiation process will account for the degree of differentiation seen histopathologically. Additionally, the development of a tumor requires that the affected cells do not undergo apoptosis, a characteristic of stem cells that undergo asymmetric division(5). The act of tissue de-differentiation seems unlikely, however studies showing reversible differentiation in cell lines suggest this may indeed be possible(1,4,28). While the true origin of tumor differentiation status remains to be elucidated, the potential to target differentiation in cancer therapy remains.

2.1.3 Differentiation therapy

The concept of forcing poorly differentiated cancer cells to terminally differentiate, leading to an inhibition of cell division and eventual apoptosis, has been investigated in many tumor types(5,6,42,43). Differentiation therapy is currently clinically used in the treatment of acute promyelocytic leukemia and neuroblastoma(42,44).

Retinoids, derivatives of vitamin A, have been studied for their ability to induce differentiation(42,44–48). Acute promyelocytic leukemia (APL) is characterized by a
translocation between chromosome 15 and chromosome 17 which results in a PML-RARα fusion protein. RARα is typically heterodimerized with its receptor RXR and a co-repressor complex which inhibits transcription through histone deacetylation. RARα undergoes a conformational change and disassociates with the co-repressor in physiologic concentrations of all-trans retinoic acid (ATRA) and initiates normal differentiation and maturation of myeloid cells. In APL, the fusion protein does not allow for sufficient disassociation from the co-repressor and the myeloid cell differentiation is therefore arrested in the promyelocytic phase. Treatment with exogenous ATRA was found to induce terminal differentiation in these neoplastic cells and, ultimately, hematologic remission in 90% of patients (6,42,43).

Neuroblastoma, a childhood cancer of the sympathetic nervous system, has been found to spontaneously differentiate from this malignant neoplasm to a benign ganglioneuroma, a concept that stimulated investigation into differentiation therapy as a component of the treatment protocol for these children. Differentiation of neuroblastoma cells in vitro was noted with ATRA treatment and a phase III clinical trial investigating maintenance high-dose pulsed 13-cis-retinoic acid, with better pharmacokinetics than ATRA, showed reduced relapse from minimal residual disease following consolidation therapy (1,44). In addition to APL and neuroblastoma, ATRA has been evaluated with promising results in nasopharyngeal carcinoma cells in vitro (43).

Additional differentiation therapies elicit their effect by altering epigenetics. Sodium phenylbuturate (PB) and valproic acid (VA) seem to stimulate differentiation by inhibiting histone deacetylation. Sodium phenylbuturate has been evaluated in patients with advanced cancers, showing some positive effect in patients with prostate cancer, astrocytoma, and myelodysplastic syndrome. Valproic acid was shown to induce differentiation in vitro in colon, breast, and teratocarcinoma cell lines, as well as in leukemic blasts and hematopoietic progenitor
cells in patients with acute myeloid leukemia. Azacytidine, a methyltransferase inhibitor, has been shown to induce differentiation and have a positive response in patients with myelodysplastic syndrome. Arsenic has been shown to initiate differentiation of APL cells, especially when they have become resistant to ATRA; a large portion of arsenic mechanism of action is thought to be through apoptosis (6,49).

The use of differentiation therapy in cancer, either alone, in combination with other differentiation agents, or as an adjuvant to traditional therapies, is an area of active research with interesting and sometimes clinically relevant results.

2.1.4 Differentiation and canine osteosarcoma

Osteosarcoma is a mesenchymal tumor characterized by the production of osteoid and thought to be derived from the osteoblast. It is the most common primary bone tumor in dogs, accounting for approximately 85% of all skeletal malignancies (50–52). Canine OS has many similarities to pediatric OS, making it an attractive model for this devastating disease. These include the predilection for the appendicular skeleton, the presence of micrometastatic disease at the time of diagnosis, and similarities in treatment approach requiring surgical removal and chemotherapy (50,51). It is estimated that greater than 10,000 cases of canine OS are diagnosed in the United States each year, a higher incidence than the approximately 1,000 cases of pediatric OS (50,52). The frequency with which we encounter this disease in dogs allows a robust population for translational research. Despite decades of active research, the median survival time for dogs with OS has not be extended beyond the 10-12 months reported with amputation of the affected limb followed by adjuvant chemotherapy (52–54). While the response to treatment is improved in children undergoing similar treatment, the 5-year survival rate remains only 60-70% (50).
Another similarity between pediatric and canine OS is the prevalence of histologically high grade tumors\(^{(50,51)}\). There are several grading schemes for human OS which appear to have prognostic significance. All have a high emphasis on anaplasia within the tumor, with low grade tumors generally lacking this characteristic and high grade tumors carrying a loss of differentiation and worse prognosis \(^{(55,56)}\). OS is often histologically heterogenous, and so if a portion of a low grade tumor is found to be anaplastic then it is termed “dedifferentiated” and an aggressive biologic course is assumed\(^{(55,56)}\). While the literature on the significance of canine OS grading schemes is varied, the general trend is that a high histopathologic grade correlates with more aggressive biologic behavior. The grading scheme introduced by Straw et al in a 1996 review of 51 mandibular canine OS showed no prognostic significance \(^{(57)}\), a finding that was consistent when this grading scheme was used to evaluate OS of extracranial flat and irregular bones \(^{(14)}\). Kirpensteijn et al developed a new grading scheme evaluating many variables including cellular pleomorphism, amount of matrix, tumor cell density, percent necrosis, and number of mitoses with consideration also given to vascular invasion, estimated whirl formation, and estimated multinucleated giant cells; 166 primary and 34 metastatic canine OS samples were analyzed \(^{(11)}\). This study showed that the vast majority of these samples were high grade (grade III tumors made up 75% of the samples) and that grade III tumors were associated with a significantly shorter disease free interval and survival time. There was no significant difference in incidence of metastasis between grades in this evaluation. When comparing the primary tumor and paired metastatic lesion \(n=29\), no significant differences were noted among the variables evaluated, although the mean score for percent necrosis was significantly higher in the metastatic lesions when the data of all primary tumors were combined. All metastatic lesions and 26/29 primary tumors were grade III. The clinical relevance of the previously introduced Straw grading
scheme was further evaluated in 140 tumor samples by Loukpolous et al., showing a significant correlation between high grade and several prognostic factors such as metastatic status, appendicular location, patient age <4 years, and non-cranial location; survival time was not assessed in this review(13). Degree of nuclear pleomorphism, mitotic index, and percent necrosis was taken into account in this grading scheme; all samples were highly pleomorphic regardless of grade. The Straw grading scheme was used in a study evaluating the use of doxorubicin in combination with BAY 12-9566, an inhibitor of matrix metalloproteinases, and no prognostic significance was noted (16). In a recent meta-analysis of prognostic factors in canine appendicular OS, histopathologic grade was not included in the variables assessed, as the effect of only serum alkaline phosphatase (ALP), tumor location, and age at diagnosis on outcome were reported most frequently (12).

It is thought that OS may arise from a defect in differentiation from the mesenchymal stem cell (MSC) to the osteocyte (10,45–47,55,58). Mesenchymal stem cells are pluripotent bone marrow stromal cells which can differentiate into bone, fat, cartilage, muscle, and nervous tissue. The MSC differentiation cascade is tightly regulated and involves many signaling pathways and regulatory genes. Osteogenesis is influenced early-on by signals involved in chondrogenesis such as bone morphogenic proteins (BMPs) -2 and -7 and FGF. Key players in osteogenic differentiation include osteogenic BMPs (BMPs -2, -4, -6, -7, -9), Runx-2, and Wnts. Any disruption along the MSC osteogenic differentiation pathway could lead to a block in differentiation and subsequent tumorigenesis. The potential defects are not well understood and may include genetic or epigenetic changes in Wnt, Rb, p53, p27, among others. If a defect leads to a block in differentiation, the undifferentiated OS precursor would retain the ability to
proliferate, creating an imbalance between proliferation and differentiation that would lead to a malignant phenotype (Figure 2.1)(10).

Differentiation therapy has been investigated in OS, with several reports of reduced proliferation and increased differentiation markers in human OS cell lines with various non-specific differentiation-promoting agents(10,59). These include the nuclear receptor superfamily of proteins (PPARγ, retinoid, and 1,25-dihydroxyvitamin D3 activation; estrogen antagonists), parathyroid hormone/parathyroid related hormone (PTHrP), and BMPs with Runx-2 (BMPs alone did not induce differentiation). Similarly, investigations into differentiation therapy in canine OS has shown a reduction in tumor cell proliferation and increased differentiation markers with vitamin D, retinoids, BMP-2 and transforming growth factor β (TGFβ) treatment, although the effect varied between the cell lines(45–47,58). A study evaluating the effect of vitamin D3 and ATRA treatment in mouse models of primary and pulmonary metastatic OS, derived from the HMPOS canine OS cell line, showed a decrease in primary tumor and metastatic lesion weight, as well as a more osteoblastic differentiation histopathologically(48).

As investigations into differentiation therapy progress, more specific targets may be elucidated, as fibroblast growth factor has in human OS(28,55,60).

2.2 Fibroblast growth factors

2.2.1 Structure and function

Fibroblast growth factors (FGFs) are a family of 18 protein ligands which mediate many important cellular responses. These ligands interact with fibroblast growth factor receptors (FGFRs) to initiate cell signaling that is important in embryonic development, wound healing, and angiogenesis(17–24,27,61). Fibroblast growth factors range from ubiquitous to rare depending on the cell type, tissue, and stage of development. Fibroblast growth factors signal
through the FGFRs primarily in a paracrine fashion, although autocrine and endocrine signaling may also occur depending on the FGF. The FGFRs are receptor tyrosine kinases (RTKs) and have the typical structure of an extracellular domain, a transmembrane domain, and a cytoplasmic domain. The extracellular ligand-binding domain is comprised of three immunoglobulin (Ig) like domains (D1 – D3), the “acid box” (7-8 acidic residues in the linker between D1 and D2), and a positively charged region in D2 which acts as a binding site for heparin. The interaction between FGFs and FGFRs is facilitated by heparin or heparan sulfate proteoglycans (HSPGs), which bind to both the receptor and the ligand, stabilizing the connection and acting as a reservoir for FGFs within the extracellular matrix. The D3 region of the extracellular domain undergoes alternative splicing in FGFRs 1-3, but not FGFR4. The N terminal half of D3 is static while the C-terminal half may be alternatively spliced to a IIIa or IIIb isoform. The IIIb isoform is expressed exclusively in epithelial tissue and the IIIc isoform is expressed exclusively in mesenchymal tissue. It is this specific expression of the D3 isoforms that allows interaction between the epithelial and mesenchymal layers in response to different FGFs during development. The FGFRs are promiscuous in that they may bind and become activated by several different FGFs, however the receptor D3 splice variant and HSPG interaction contribute to ligand-binding specificity. Once the FGF/HSPG/FGFR complex is initiated, receptor dimerization occurs, leading to autotransphosphorylation and initiation of the RTK signaling cascade within the cytoplasm. This ultimately results in activation of the MAP/ERK pathway leading to cell proliferation, the Akt pathway leading to cell survival, and the PLCγ pathway leading to cytoskeletal alterations (Figure 2.2).

2.2.2 The role of FGF signaling in skeletal development and bone remodeling
FGF signaling is important in both endochondral and intramembranous bone formation, as evidenced by the identification of FGFR mutations in genetic diseases that affect bone development (17,19,20,62). From the earliest development of the limb bud, FGF signaling has been identified, with the binding specificity from alternative splicing playing an important role. FGFR2-IIIb is expressed in the ectoderm and FGFR1-IIIc is expressed in the limb mesenchyme. FGF10 is expressed in the mesenchymal tissue and signals with FGFR2-IIIb to form the apical ectodermal ridge. FGF8 is then expressed in this ridge and signals back to FGFR1-IIIc in the limb mesoderm, exemplifying the reciprocal signaling required for outgrowth and patterning of the limb. FGFR1 and FGFR2 are expressed in mesenchymal condensation, the first morphologic event leading to bone formation. FGFR3 is first identified as chondrogenesis begins. Mutations in the FGFRs account for several human skeletal dysplasias, including dwarfing chondrodysplasia syndromes such as achondrodysplasia caused by FGFR3 mutation, and craniosynostosis syndromes characterized by premature fusion of cranial sutures. Mice that overexpress FGF2 develop enlarged bones, while loss-of-function inhibits bone growth.

FGF2 is a ubiquitous ligand and binds to all four FGFRs with highest affinity for FGFR1 and FGFR2. It is involved in fracture repair, and activated fibroblasts in the fracture microenvironment express this ligand during the healing process (27,63). The effect of FGF2 on fracture healing appears to be dependent on the amount and duration of FGF2 exposure; limited exposure of FGF2 in conjunction with BMP enhanced calcification while large amounts of FGF2 reduced the amount of bone formed and continuous FGF2 inhibited osteoblast differentiation. FGF2 induces osteoblast proliferation and accelerates vascularization, both aiding in fracture repair.

2.2.3 FGF signaling and neoplasia
2.2.3.1 Overview

With the extensive functions of FGF signaling that allow for its significant contribution to embryonic development and wound healing, as well as maintenance of stem cell populations, it is no surprise that FGF signaling could be manipulated to enhance tumorigenesis. Specifically, FGF signaling has been shown to contribute to cancer cell proliferation, migration, survival and chemoresistance, epithelial-to-mesenchymal transition (EMT) phenotype, and angiogenesis(17,37,64–69). These manipulations may be acquired through the following: 1) ligand-independent signaling by genomic alteration of FGFR (including activating mutations, gene translocations, or gene amplification), 2) paracrine loop formation between FGF produced by the tumor stroma and the FGFR due to FGFR splicing (altered ligand specificity) or amplification of FGFR gene (FGFR expressed out of context), 3) autocrine loop development by increased expression of FGF ligand by the cancer cell or FGFR expression out of context so it has binding specificity to the autocrine FGF when it would not have otherwise, and 4) the effect of FGF on tumor stroma such as angiogenesis(Figure 2.3). Multiple specific incidences of FGFR mutations leading to tumorigenesis have been identified in human cancers, such as FGFR3 mutations in bladder cancer and upregulation of FGFR3 in multiple myeloma (Figure 2.4). The effect of FGF signaling differs depending on the specific ligand-receptor binding, the tissue type, and the stage of development. In this vein, it is also possible to see FGF signaling as a tumor preventative, as is noted with FGFR2-IIIb in mouse models(64,70–72). FGF signaling has been evaluated in canine neoplasia to a lesser degree. Gene expression of FGFRs 1-4 and FGFR1 protein expression was identified in canine soft tissue sarcomas (73) and a significantly higher concentration of FGF2 was found in the urine of dogs with bladder transitional cell carcinoma compared to normal dogs and dogs with urinary tract infections (74).
FGF signaling inhibition may be acquired through the use of small molecule tyrosine kinase inhibitors (TKIs), FGFR antibodies, and FGF ligand traps (64,67,68,75). FGF ligands to stimulate FGFRs may be of interest in cancers where FGF2R-IIIb appears to be protective. TKIs, which act by inhibiting the catalytic activity of the tyrosine kinase domain by inhibiting ATP binding, are the most clinically relevant. There are several such inhibitors available for use, although the promiscuity of these TKIs often result in off-target side effects which may make antibodies or ligand traps more attractive. BGJ398 is a selective pan-FGFR TKI with predominant activity against FGFR1, FGFR2, and FGFR3, which has been evaluated in several human cancer cell lines, in xenograft models, and in clinical trials (29–41,76).

2.2.3.2 FGF signaling in OS

FGF signaling has been evaluated extensively in human OS in recent years. FGFR expression has been identified in OS tumor samples (77) and along with FGF2 in cell lines (78,79). A study using flow cytometry showed only low expression of FGFR1 and FGFR3 in OS cell lines (80). Amplification of FGFR1 was noted in 1/7 OS cell lines and 1/17 spontaneous OS tumor samples (76), which led to a follow-up study identifying FGFR1 amplification in 9.6% of primary OS tumors (81). This study showed that 18% of patients that were poor-responders to neoadjuvant chemotherapy had FGFR1 amplifications while none of the good-responders had this genetic alteration. Additionally, all of the 4 paired metastatic samples in the FGFR1 amplification positive group also harbored this amplification. FGF1 and FGF2 stimulation has been shown to increase proliferation of OS cell lines (28,79,82). FGF2 was found to be highly expressed in OS tumor stroma and led to maintenance of OS cells in an undifferentiated state, increased proliferation and migration, and conferred resistance to doxorubicin chemotherapy (28). It has been shown that the Sox2 transcription factor inhibits Wnt signaling to maintain
undifferentiated osteoblasts, which appears to be regulated by FGF signaling (83). This same group went on to show that Sox2 is required for OS cell self-renewal and that Wnt activity is increased in Sox2 knock-down OS cells, a phenomenon they contribute to FGF signaling given the dose-dependent decrease of Sox2 noted with exposure to a FGFR inhibitor (84). OS cell migration was shown to be increased with cooperation between parathyroid hormone and FGF2, as this led to a decrease in biglycan extracellular matrix content (78). In regards to the role of FGF2 in OS angiogenesis, it was shown thatapurinic/apyrimidic endonuclease 1 (APE1), an upstream effector of angiogenesis-related molecules, upregulated FGF2-FGFR3 angiogenesis as shown by an increase in microvessel density (MVD) (85). Additionally, APE1, FGF2/FGFR3, and MVD levels correlated to a poor prognosis. Histone demethylases JMJD2B and JMJD2C were shown to be increased in OS cells compared to normal osteoblasts and OS tumor sample compared to adjacent normal (86). FGF2 was shown to be upregulated by these histone demethylases, in turn leading to an increase in OS cell proliferation, migration, and invasion. Serum FGF1 was found to be 2.5 times greater in patients with OS compared to healthy controls; FGF2 concentration was not significantly different, although it was found to be greater in patients with OS than those with osteochondroma (87,88). The FGF concentrations did not confer a clinical significance in these studies, however a Chinese study evaluating VEGF and FGF2 serum levels in OS patients with and without metastasis compared to healthy controls found an increase in VEGF and FGF2 in OS patients compared to controls (89). The serum concentration of both factors decreased post-operatively, however they remained elevated compared to controls. The pre-operative concentrations were positively related to the size of the primary tumor. There was a significant difference in VEGF and FGF2 concentrations in patients with recurrence/metastasis compared to those without, and the pre-operative and post-operative values
were found to be an independent factor for recurrence or metastasis after surgery. There is compelling evidence that FGF signaling plays a role in human OS tumorigenesis, however this signaling axis has not been investigated in canine OS to date.
CHAPTER 3
MATERIALS AND METHODS

3.1 Cells and reagents

Three canine (Abrams, K003, HMPOS) OS cell lines were used in this study. The HMPOS cell line was provided by Dr. James Farese, University of Florida. The Abrams cell line was provided by Dr. Douglas Thamm, Colorado State University. The K003 cell line was provided by Dr. Chand Khanna, National Cancer Institute. All cell lines were cultured at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with glutamine (2 mmol/L), penicillin (100 IU/ml), streptomycin (100 IU/ml), and 10% fetal bovine serum (FBS) in a humidified atmosphere supplemented with 5% CO₂. Cell cultures were maintained in subconfluent monolayers and passaged two to three times weekly as necessary.

The pan-FGFR inhibitor BGJ398 was purchased from Selleck Chemicals, Houston, TX (catalog# S2183).

3.2 Canine FGFR RT-PCR

Total RNA was collected from Abrams, HMPOS and K003 OSA cells with a commercially available kit (RNasey mini kit, Qiagen, Valencia) and 1 μg of total RNA was reverse-transcribed to cDNA (SuperScript First-Strand cDNA Synthesis Kit, Invitrogen, Carlsbad, CA). 5 μL of reverse transcribed product was used as a template in a 50 μL polymerase chain reaction containing 10 mM of each oligonucleotide, 2.5 U of Taq DNA polymerase, and forward and reverse primers for canine FGFR1, FGFR2, FGFR3, and FGFR4 (Integrated DNA Technologies, Coralville, IA), as listed in Table 3.1. Reactions were performed in a PTC-200 Peltier thermal cycler with the following cycling conditions for FGFR1 and FGFR2: 5 minutes at 94°C denaturing step, followed by 40 cycles (60 seconds at 94°C, 90 seconds at 58°C, and 60
seconds at 72°C), and concluded by 72°C for 10 minutes. FGFR3 and FGFR4 followed similar cycling conditions with the exception of annealing temperatures of 61°C and 62°C, respectively.

### 3.3 *In vitro* FGF2 ELISA

The Abrams, K003, and HMPOS cell lines were plated in 1% FBS DMEM media at 37°C and 5% CO2 with seeding densities of 50,000, 25,000, 12,500, and 6,500 cells per well in a 96-well plate for 48 hours. Cell culture supernatants were collected and the concentration of secreted FGF2 was quantified with a commercial kit (Human FGF basic Immunoassay #HSFB00D, R&D Systems, Minneapolis, MN) using a monoclonal antibody specific for human bFGF, which was previously validated with canine urine(74). The optical density of each well was read 490 nm with wavelength correction set to 630 nm, yielding pg/mL of bFGF based on the standard curve. This was performed in duplicate.

### 3.4 Cell Protein Collection

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

3.5 Cell proliferation assay

Abrams, HMPOS, and K003 cell lines were transferred to a 96-well plate at concentrations of 100,000, 50,000, 25,000, 12,500, 6,250, 3,125 and media control per well and cultured overnight in DMEM supplemented with 1% FBS (1% media) to adherence. Cell proliferation was measured with the use of a colorimetric proliferation assay (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI). Assays were performed in triplicate and in three separate experiments. The steepest point of the curve was estimated to be around 15,000 cells/well for all cell lines at 24 and 48 hours; this determined the seeding number for subsequent cell proliferation assays.

Abrams, HMPOS, and K003 cell lines were transferred to a 96-well plate at a concentration of 15,000 cells per well and cultured overnight in DMEM supplemented with 1% FBS (1% media) to adherence. Three experimental conditions for a duration of 72 hours were evaluated including DMEM supplemented with 1% FBS (1% media) only, 1% media with BGJ398 pan-FGFR inhibitor at 200nM, and 1% media with vehicle (DMSO). Cell proliferation was measured with the use of a colorimetric proliferation assay. Assay was performed in triplicate and in three separate experiments. The Abrams cell line was also seeded at a concentration of 5,000 cells per well with the same conditions in triplicate and three separate experiments. Based on these results, the Abrams cell line was excluded from subsequent cell proliferation assays.
HMPOS and K003 cell lines were transferred to a 96-well plate at a concentration of 15,000 cells per well and cultured overnight in 1% media to adherence. Eight experimental conditions for a duration of 24, 48, and 72 hours were evaluated including 1% media only, 1% media with DMSO, and 1% media with BGJ398 pan-FGFR inhibitor at 6.25 nM, 12.5 nM, 25 nM, 50 nM, 100 nM, and 200 nM. Cell proliferation was measured with the use of a colorimetric proliferation assay. Assay was performed in quadruplicate and in three separate experiments. This was repeated at 24 and 48 hours with 12 replicates in one experiment using fluorescence to confirm results.

To determine if there was a difference in effect between two aliquots of BGJ398 pan-FGFR inhibitor, the K003 cell line was evaluated as noted above with each aliquot (inhibitor #1 and inhibitor #2) in triplicate. Cell proliferation was measured with the use of a colorimetric proliferation assay. This experiment was performed once.

3.6 Migration scratch assay

Qualitative analysis of cell migration was performed using the “scratch assay” method (90). The Abrams, HMPOS, and K003 cell lines were grown to 80% confluence in 6-well plates in DMEM supplemented with 10% FBS (complete media). Three experimental conditions for a duration of 24 and 48 hours were evaluated including DMEM supplemented with 1% FBS (1% media) only, 1% media with BGJ398 pan-FGFR inhibitor at 200 nM, and 1% media with vehicle (DMSO). A standardized acellular gap was created through cell monolayers using a 200 µL pipette tip in the middle of each well. Images of the acellular gap were captured at time 0 (maximal gap) and 24 or 48 hours later for each experimental condition (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1). The average width of 5 representative acellular gaps per experiment conditions and cell line were used for quantitative comparisons at 24 and 48
hours and 3 independent experimental conditions were performed. Each of the treatment conditions was performed in duplicate and the assay results are representative of three separate experiments (HMPOS and K003) and 2 separate experiments (Abrams). Data was analyzed with Image J software (National Institutes of Health, Bethesda, MD).

3.7 Differentiation Assays

Abrams, HMPOS and K003 cell lines were plated into 6-well plates at a concentration of 100,000 cells per well and allowed to adhere overnight in DMEM supplemented with 10% FBS (complete media). The next day, the cells were washed with PBS and the culture medium was changed to DMEM supplemented with 1% FBS, 5% pen/strep, 100 nM dexamethasone, 10nM β-glycerolphosphate, 550 µL ascorbic acid, termed osteogenic media (OG media). Upon completion of a pilot study to assess osteogenesis via Alizarin Red staining in OS cell lines at 21 days in OG media, the Abrams cell line was no longer assessed with differentiation assays.

HMPOS and K003 cell lines were seeded and maintained as noted above. Three experimental conditions were evaluated including OG media only, OG media with BGJ398 pan-FGFR inhibitor at 200nM, and OG media with vehicle (DMSO). The medium was changed every third to fourth day until qRT-PCR and Alizarin Red staining/quantification at day 7, yielding discordant results which were concerning for cytotoxicity. To determine the concentration at which BGJ398 no longer influenced cell confluence, a subjective assessment of cytotoxicity was performed by daily microscopic evaluation of cells seeded as above and maintained with a titration of BGJ398 at 0 nM, 6.25 nM, 12.5 nM, 25 nM, 50 nM, and 100 nM for 10 days. Based on these results, experimental conditions for the subsequent differentiation assays were altered to include OG media only, OG media with vehicle (DMSO), and OG media...
with BGJ398 pan-FGFR inhibitor at 6.25 nM, 12.5 nM, and 25 nM. Cells were collected at days 0, 1, 4, 7, and 10 based on the assay to be performed, as noted below.

3.7.1 qRT-PCR

3.7.1.1 RNA isolation

Total RNA was isolated from the cell monolayers using the phenol-based dissociation agent, TRIzol® (Invitrogen Corporation, Carlsbad, CA). 700 µL of TRIzol® was added into each well. The cell lysates were scraped from the cell surface, transferred to 1.5 ml eppendorf tubes on dry ice and stored at -80°C. 700 µL ethanol was added and the samples were mixed. 700 µL of the mixture was loaded into a Zymo-Spin IIC Column in a collection tube and centrifuged at 12,000 rpm for 30 seconds. The column was moved into a second collection tube and the remaining 700 µL of the mixture was added and the tube was again centrifuged for 30 seconds. The column was moved to a new collection tube and 400 µL of RNA wash buffer was added followed by centrifugation for 30 seconds. A 15:1 mixture of DNA Digestion Buffer and DNase was vortexed and briefly centrifuged. 80 µL of this mixture was added to each sample column. The samples were incubated at room temperature for 15 minutes and then centrifuged. 400 µL Directzol RNA Prewash was added to the column which was then centrifuged, the flow-through was discarded, and these steps were repeated. 700 µL RNA Wash Buffer was added, the column was centrifuged for two minutes, the flow-through was discarded, and the column was again centrifuged. RNA was eluted with 50 µL DNase/RNase Free Water which was centrifuged after sitting for 1 minute. The flow-through was collected in a microcentrifuge tube.

The concentration of RNA (1:40 aliquot of each sample) was determined by measuring the absorbance at 260 nM (A260) and 280nM (A280) in a spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, MA). Total RNA was calculated as follows:
RNA in μg/μL = \frac{\text{OD at 260 nM} - \text{OD at 280 nM} \times 40}{1000} \times \text{dilution factor} \times \text{coefficient}

3.7.1.2 Reverse transcription

A commercially available reverse transcription kit (Superscript III First-Strand Synthesis System for RT-PCR, Invitrogen) was used to generate cDNA. Briefly, the volume of each sample containing 1 μg of total RNA was calculated and brought up to 8 μL total with DEPC water. A master mix (2 μL per sample) containing 1 μL random hexamers primers and 1 μL 10 mM dNTPs was added into each tube. Following incubation at 65 °C for 5 minutes (Bio-Rad DNA Engine Peltier Thermal Cycler), the samples were placed on ice for 1 minute to facilitate binding of the oligonucleotides. A master cDNA Synthesis mix (10 μL per sample) containing 2 μL 10x First Strand Buffer, 4 μL 25 mM MgCl2, 2 μL 0.1 M DTT, 1 μL RNase OUT and 1 μL (50 units) SuperScript II Reverse Transcriptase (RT) was added into each tube. The samples were mixed and incubated on the Thermal Cycler at 25°C for 10 minutes, 50°C for 50 minutes, and 85°C for 5 minutes. The samples were chilled on ice for 1 minute and then 1 μL of RNAse H was added to remove the RNA from the sample, leaving only cDNA. The samples were incubated at 37 °C for 20 minutes. The cDNA was dilute 1:5 with DEPC water to achieve 100 μL cDNA.

3.7.1.3 PCR amplification

The relative expression of osteogenic genes including ALP, OSTX, OSN, RUNX2 and the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was assessed by quantitative real-time reverse transcription PCR (qRT-PCR) induplicate cell layers (osteogenically-induced cultures) subjected to the previously noted conditions collected on days 0, 1, 4, 7, and 10.
The primers used for qRT-PCR analysis are listed in Table 3.2 (Integrated DNA Technologies, Coralville, IA). Lyophilized primers were reconstituted in DEPC water to generate 100 μM stock solution which was then diluted 1:10 to yield 10 μM working stocks. The total volume used for the PCR reaction was 25 μL; therefore, the final concentration of each primer used was 0.4 μM (or 400 nM). Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed using 4 μL of diluted cDNA template combined a mixture composed of 12.5 μL Power SYBR Green PCR Master Mix (Cat# 4367659 Applied Biosystems, Foster City, CA), 1.25 μL each of the forward and reverse primers, and 6 μL DNase/RNase-free water in a 96-well microplate. The reactions were performed according to the default program for SYBR green protocol in ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The level of expression for each target gene was calculated as 2 Δ Ct and the comparative ΔCt method was used to determine relative gene expression levels, using the day 0 OG MediaControl value as the nominal reference level.

3.7.2 Alkaline Phosphatase Bio-activity

Alkaline phosphatase activity was assessed in duplicate samples of osteogenically-induced cultures subjected to the previously noted conditions. At days 0, 1, 4, 7 and 10 of culture, the cells were harvested in 1 ml of PBS and stored as pellets at -80°C until further processing. The ALP activity was determined using a commercial colorimetric kit (SensoLyteNPP Alkaline Phosphatase Assay Kit, AnaSpec, Inc, Fremont, CA). Each sample was lysed with Triton X-100 and assay buffer, incubated at 4°C for 10 minutes under agitation, and centrifuged at 3500 rpm for 10 minutes at 4°C. The supernatants were assayed for ALP activity. 50 μL of a p-NitrophenylphosphatepNPPALP substrate working solution was added to each well of a transparent 96-well microplate. Then, 50 μL of each sample (in duplicate) and
standard of ALP solution was added and the plate was incubated at 37°C for 30 minutes. According to the manufacturer’s instructions, the absorbance was recorded at 405 nm, yielding ng of ALP based on the standard curve.

Protein concentrations were determined in aliquots of the cell extracts via BCA analysis. This cell layer protein was used as an index of cell number which could have varied after exposure to the different conditions. The relative activity of each sample was determined by normalizing the results of each quadruplicate sample (two experiments in duplicate) to the mean protein content of a duplicate BCA. Then, a mean and standard deviation were calculated for each quadruplicate sample.

3.7.3 Alizarin Red staining and quantification

A 2% Alizarin Red solution was used in duplicate cell layers (osteogenically-induced cultures subjected to the previously noted conditions) to identify calcium deposition on days 7 and 10 of exposure to OG media. Alizarin Red forms complexes with calcium ions. Following fixation with 10% formalin for 30 minutes, cell layers were washed 2-3 times with distilled water. One ml of 2% fresh Alizarin Red (Sigma-Aldrich) solution (pH 4.1) was added to each well. Following incubation at room temperature for 20 minutes, the stain was removed and washed 4-5 times with water until the rinsed solution was clear. Mineral deposits within the cell layers were stained bright red. Representative pictures of stained monolayers were obtained (Leica Microsystems, Leica Application Suite -LAS- version 2.6.R1). Plates were stored in -20°C.

Quantification was performed as previously described(91), initially with mineral oil and ultimately with a commercial kit (Alizarin Red S Staining Quantification Assay, ScienCellResearch Laboratories, Carlsbad, CA) and Parafilm M® in place of mineral oil.
Briefly, 800 µL 10% acetic acid was added to each well and incubated at room temperature for 30 minutes with shaking. Cells were collected with a cell scraper, transferred to a 1.5 mL microcentrifuge, and vortexed for 30 seconds. Samples were sealed with Parafilm M® (alternatively topped with 500 µL mineral oil) and heated at 85 ºC for 10 minutes. Tubes were incubated on ice for 5 minutes prior to centrifugation at 14,000 rpm for 15 minutes. 500 µL of supernatant was transferred to a new tube and 200 µL 10 % ammonium hydroxide was added to neutralize the acid prior to transfer to a 96-well plate. Absorbance was read at 405 nm with a plate reader.

Protein concentrations were determined from one separate 6-well plate per cell line and collection day, prepared at the same time and in the same conditions, via BCA analysis. This cell layer protein was used as an index of cell number which could have varied after exposure to the different conditions. Alizarin Red S concentration was corrected for the cell protein layer by normalizing the results of each quadruplicate sample (two experiments in duplicate) to the mean protein content of a duplicate BCA. Then, a mean and standard deviation were calculated for each quadruplicate sample.

3.8 PlasmabFGF ELISA

Heparinized plasma was collected from dogs with OS and healthy control dogs that were evaluated at the University of Illinois Cancer Care Clinic. All OS dogs had a diagnosis of appendicular OS confirmed by either histopathology or cytology with concurrent positive ALP staining(92). Heparinized plasma was collected at the time of initial presentation and samples were stored at -80°C until analysis. The OS dogs were separated into osteoblastic (n=23) and osteolytic (n=25) groups based on results of pre-treatment DEXA scans and the generation of primary bone tumor mineral density (rBMD). Primary tumors that were very osteoblastic and
produced a large amount of bone had rBMD> 1.4, while primary tumors that were very osteolytic and did not produce much bone had rBMD<0.7. PlasmabFGF was evaluated in treatment naïve OS dogs and ten healthy control dogs with a commercial ELISA test kit (Human FGF basic Immunoassay #HSFB00D, R&D Systems, Minneapolis, MN) using a monoclonal antibody specific for human bFGF.

3.9 Statistical analysis

The distribution of the continuous variable data was evaluated using the Kolmogorov Smirnov test. Data that were not normally distributed were log transformed to meet the assumption of normality. One-way ANOVA was used to evaluate for differences between groups, with Dunnett’s comparison test to detect a difference between control and experimental conditions. When less than 12 data points were present, indicating that results of normality tests would not be reliable, a normal distribution was assumed. Kruskal-Wallis with Dunn’s comparison was used when a non-normal distribution was noted which was not corrected with log transformation of data. An unpaired t-test was used to compare the MTS assay results between each concentration of the two inhibitors. P < 0.05 was considered statistically significant for all analyses. Statistical analysis was carried out using a commercially available software program (GraphPadInStat, Version 3.10).
CHAPTER 4

RESULTS

4.1 FGFRs are expressed in osteosarcoma cell lines

Transcription of genes for FGFR1, FGFR2, FGFR3, and FGFR4 was identified in canine OS cell lines Abrams, HMPOS, and K003 (Figure 4.1).

4.2 FGF2 is secreted by Abrams and K003 in a cell density-dependent manner

Active secretion of the FGF2 ligand was demonstrated by titration studies in three canine OS cell lines (Figure 4.2). At all cell densities evaluated, total FGF2 concentrations achieved physiologically relevant and active concentrations in the pg/mL range. The K003 cell line demonstrated the greatest capacity to secrete FGF2, approximately doubling to tripling the concentrations liberated by Abrams. HMPOS secreted a minimal amount of FGF2 which was not notably cell density-dependent.

4.3 FGFR inhibition has no consistent effect on canine OS cell proliferation

To determine if abrogation of FGF signaling effects OS cell proliferation or survival, MTS assays were performed at varying concentrations of BGJ398. A pilot assay using BGJ398 at 200 nM yielded a trend toward decreased proliferation with the inhibitor in HMPOS and K003, however inconsistent results were noted in the Abrams cell line which was therefore excluded from further MTS assays (data not shown). At 24, 48, and 72 hours with a titration of BGJ398 concentrations, no consistent difference in cell proliferation was noted in HMPOS or K003 cell lines at any concentration of inhibitor (Figure 4.3 a-b). HMPOS showed a significant increase in proliferation with BGJ398 at 50 nM, 100 nM, and 200 nM at 24 hours and with 12.5 nM and 100 nM at 48 hours, while no significant differences were noted at 72 hours. K003 showed variable results with a significant increase in proliferation with 12.5 nM and significant
decrease with 25 nM and 100 nM at 24 hours, significant increase with DMSO and decrease with
25 nM and 50 nM at 48 hours, and no significant differences at 72 hours.

It was noted that the inhibitor may be expired based on the data sheet, and so a new
inhibitor was purchased and the assay was repeated in the K003 cell line after 48 hours of
incubation with both inhibitors. No statistically significant difference was noted (Figure 4.4) and
a consultation with the company indicated that further quality assessment on the inhibitor
revealed that the compound stability exceeded that which was previously published, therefore the
first inhibitor was not truly expired. Based on these results we conclude that FGFR blockade has
no definitive pro- or anti-proliferative effect on canine OS cells.

4.4 Fibroblast growth factor signaling affects migration of osteosarcoma cells in vitro

Migration through the microenvironment to gain access to vasculature is an important
step in cancer cell metastasis. We therefore evaluated the effects of inhibition of the FGF
signaling pathway using BGJ398 on all three canine OS cell lines. An acellular gap was created
in confluent cultures and the gap was measured at 24 and 48 hours after treatment. Data are
represented as a percentage of the original gap (time 0), with 0% representing a completely
closed gap. In the HMPOS cell line, there was a significant difference in gap closure with
BGJ398 at the 24 hour time point (p<0.01) (Figure 4.5a). In the K003 cell line, there was a
significant difference seen with BGJ398 at both 24 and 48 hours (p<0.01 and p<0.05,
respectively) (Figure 4.5b). For Abrams (Figure 4.5c), the difference in gap closure with BGJ398
approached significance at 48 hours (Dunnett’s q value=2.061, >2.33 is significant), while a
Turkey-Kramer multiple comparison test showed a significant difference between DMSO and
BGJ398 wells at 48 hours (p<0.01).

4.5 FGFR inhibition increases mineralization in the HMPOS cell line
4.5.1 FGFR blockade causes a trend toward increased expression of osteogenic genes in HMPOS

HMPOS and K003 cell lines were exposed to OG media only, OG media with BGJ398 pan-FGFR inhibitor at 200nM, and OG media with vehicle (DMSO). qRT-PCR for osteogenic genes (ALP, OSN, OSTX and RUNX2) was performed at day 7, yielding inconsistent results in both cell lines (Figure 4.6). These findings, along with those noted in the corresponding Alizarin Red stain (see section 4.5.3.) were thought to be related to cytotoxicity. Specifically, a mild decrease in ALP expression was noted in the HMPOS cell line with BGJ398 treatment while the K003 cell line showed an increase in ALP and OSN expression. A BCA assay performed on protein collected from plates prepared in exactly the same fashion at the same time showed a decrease of protein in the inhibitor well compared to the OG media control and DMSO wells (data not shown). To determine the concentration at which BGJ398 no longer influenced cell confluence, a subjective assessment of cytotoxicity was performed by daily microscopic evaluation of cells maintained with a titration of BGJ398 for 10 days. Percent confluence was recorded daily and graphed, showing that cells reached a plateau of confluence and subjective viability at inhibitor concentrations of 6.25 nM and 12.5 nM (Figure 4.7).

qRT-PCR was repeated with cells exposed to titrated concentrations of BGJ398 (6.25 nM, 12.5 nM, and 25 nM) and collected on days 0, 1, 4, 7, and 10. FGFR blockade leads to a trend toward an increase in expression of osteogenic genes in the HMPOS cell line by day 10, while the osteogenic gene expression decreases by day 10 in the OG media and DMSO controls (Figures 4.8 a-d). The lack of statistical significance in the increase of osteogenic gene expression on day 10, despite a notable increase of the mean value, is likely due to the high standard deviation and the loss of the second 6.25 nM sample. The K003 cell line exhibits
variable and inconsistent changes in osteogenic gene expression in response to FGFR blockade, typically seen as a decrease in gene expression compared to control OG media (Figure 4.8 e-h), with the exception of a significant increase in Osterix expression in the 25 nM BGJ398 cells on day 10. The remaining osteogenic genes show an increase in mean value at 25 nM on day 10, although this change was not statistically significant.

4.5.2 FGFR blockade increases alkaline phosphatase bio-activity in HMPOS cells

Alkaline phosphatase activity in the HMPOS cell linewas noted to increase in all wells on day 4. The increase in the inhibitor wells appeared delayed compared to the control wells. On day 7 there was a trend toward increased ALP activity in the 25 nM well (p=0.0722). ALP activity was significantly increased with all concentrations of BGJ398 compared to OG media control on day 10 (p<0.01) (Figure 4.9a). K003 cells showed a significant decrease in ALP bioactivity compared to OG media control with BGJ398 at 6.25 nM (p<0.01) and 12.5 nM and 25 nM (p<0.05) on day 4, and with DMSO and all inhibitor concentrations on days 7 and 10 (p<0.01) (Figure 4.9b).

4.5.3 Alizarin Red staining intensity and concentration increases following FGFR inhibition

Alizarin Red forms complexes with calcium ions, therefore Alizarin Red staining was used to identify calcium deposition on days 7 and 10 of exposure to OG media and titrated concentrations of BGJ398 pan-FGFR inhibitor. A pilot study was performed to determine if osteogenesis would be detected in the cell lines, which were exposed to OG media alone with the intent to perform Alizarin Red staining at 21 days. The Abrams cell line was too aggressive and would not reliably survive beyond 5 days in the 6-well plates, and so this cell line was excluded from differentiation assays. HMPOS and K003 struggled to stay viable to 21 days, however
Alizarin Red staining at 18 days (K003) and 21 days (HMPOS) confirmed positive staining and mineral deposition (Figure 4.10).

HMPOS and K003 cell lines were exposed to OG media only, OG media with BGJ398 pan-FGFR inhibitor at 200 nM, and OG media with vehicle (DMSO). Alizarin Red staining performed at day 7 showed an unexpected subjective decrease in stain intensity in the inhibitor wells. Corresponding quantification results were discordant to the stain intensity and the use of mineral oil contributed to the technical difficulty of the assay and loss of sample (Figure 4.11). The quantification results were discarded and the stain intensity results were concerning for cytotoxicity. The aforementioned BCA assay performed on protein collected from plates prepared in exactly the same fashion at the same time and subjective assessment of cytotoxicity performed by daily microscopic evaluation of cells maintained with a titration of BGJ398 for 10 days (see section 4.5.1) influenced the final concentrations used for the Alizarin Red assay.

The final Alizarin Red staining and quantification assays were then performed using a commercial kit with the following conditions: OG media, OG media with DMSO, and OG media with BGJ398 pan-FGFR inhibitor at 6.25 nM, 12.5 nM, and 25 nM. A subjective increase in Alizarin Red stain intensity was noted in the inhibitor wells on day 10 for the HMPOS cell line. Quantification confirmed a statistically significant increase in mineralization compared to OG media control with 25 nM BGJ398 on day 7 and with all concentrations of BGJ398 on day 10 (Figure 4.12a-b). The difference was considered very significant with p<0.01 for all significant values compared to OG media control. In the K003 cell line, Alizarin Red stain intensity was not notably different in inhibitor wells compared to OG media control or DMSO. Quantification revealed an increase in Alizarin Red in the 25 nM well on day 7 which approached significance.
(p=0.0733) and a statistically significant increase in this well compared to control on day 10 
(p<0.01) (Figure 4.12c-d).

4.6 Osteosarcoma does not influence the concentration of circulating FGF2

The concentration of FGF2 was measured in heparinized plasma in treatment-naïve dogs 
with osteoblastic and osteolytic OS as well as healthy control dogs. There was no statistically 
significant difference between any of the groups (Figure 4.13)
CHAPTER 5
DISCUSSION AND CONCLUSIONS

Fibroblast growth factor signaling plays an important role in embryonic development, including the maintenance of stem cells and skeletal development, as well as in wound healing and angiogenesis(17–24,27,60–64). Aberrant FGF signaling is involved in tumorigenesis of many cancer types, including OS, by contributing to cancer cell proliferation, migration, and invasion, maintenance of an undifferentiated state, and tumor angiogenesis(64,65,67,68,70–72,76–79,81–86,88,89). Inhibition of FGF signaling has been shown to abrogate these properties in vitro with promising results in clinical trials(29–41). FGFR expression has been shown in canine soft tissue sarcomas(73) and the concentration of FGF2 was found to be higher in dogs with bladder cancer compared to normal dogs(74). Until this current investigation, FGF signaling has not been evaluated in canine OS.

In keeping with what has been identified in human OS cells, canine cell lines express genes for all four FGFRs(77,78). While all three of the canine OS cell lines secrete FGF2, the response is varied. K003 and Abrams both secrete FGF2 in a cell-density dependent manner with K003 secreting the highest concentration. HMPOS did secrete a lower concentration of FGF2, although this was not cell-density dependent. The concentration of circulating FGF2 is 0.6 pg/mL in normal conditions and up to 6 pg/mL in pathologic conditions(22). The level of FGF2 secretion from HMPOS is negligible at about 0.4 pg/mL, less than that seen in circulation physiologically. The varied secretion of FGF2 between cell lines is similar to what was seen with human OS cells(79). While the tumor microenvironment is the most likely source of FGF2, the identification of FGF2 secretion by OS cells indicates that FGF signaling may occur in an autocrine and paracrine manner.
The effects of FGFR inhibition on OS cell proliferation and migration were evaluated. Interestingly, there was no clear time-dependent or dose-dependent effect of FGF inhibition on proliferation in any of the cell lines, despite the use of inhibitor concentrations that greatly surpassed the IC50 for all four of the FGF receptors (FGFR1=0.9 nM, FGFR2=1.4 nM, FGFR3=1 nM, FGFR4=60 nM). This indicates that OS cell proliferation may rely on other signaling pathways. FGFR inhibition showed a decrease in migration in all three cell lines which was significant in HMPOS and K003. This is consistent with the finding that FGF signaling increases migration of human OS cells(28,78). This may confer a role for FGF signaling in OS metastasis. Both fluorescence and colorimetric assays were used to assess proliferation; ideally, each replicate should have been performed with the same plate reader to allow for the most cohesive data.

FGF signaling has been shown to maintain osteoblasts and human OS cells in an undifferentiated state(27,28,55,83,84). In this study, FGFR inhibition was used to see if a more differentiated phenotype may be achieved, as identified by analyzing osteogenic gene expression, ALP activity, and mineralization. The finding of unexpected and varied gene expression, a decrease in mineral production, and a decrease of protein noted in the initial differentiation assays at 7 days with the solitary inhibitor concentration of 200 nM was suggestive of cytotoxicity. This is in contrast to the results of the proliferation assay, which do not show a consistent cytotoxic effect of BGJ398 at 200 nM. It is possible that laboratory error may have contributed to the loss of cells in the inhibitor wells. Additional replicates of the differentiation assays with higher inhibitor concentrations may clarify this finding.

In the final differentiation assays using the titrated doses of BGJ398 of 6.25 nM, 12.5 nM, and 25 nM, the HMPOS cell line showed an increased expression of osteogenic genes,
increased ALP activity, and increased mineralization by day 10, supportive of maturation of the OS cells with FGFR inhibition. The difference in osteogenic gene expression was not statistically significant due to a large standard deviation and the loss of one of the wells at the time of cell collection. Ideally, this experiment should be repeated to allow for more robust statistical data.

The variability in response to FGFR inhibition between cell lines is again noted by the inconsistent response of the differentiation assays in the K003 cell line. A trend toward increased osteogenic gene expression was noted on day 10 at a concentration of 25 nM, with a significant increase in Osterix expression at this time. Osteogenic gene expression appeared decreased compared to control in at other concentrations and time points. ALP activity trended toward a decrease with FGFR inhibition. Mineralization as indicated by Alizarin Red staining and quantification was increased on day 10 in 25 nM BGJ398. It is possible that FGFR inhibition could influence K003 cell maturation and mineralization if given at higher doses and for longer duration.

Reports of serum levels of FGF2 in humans with OS are varied with most failing to show a significant increase compared to healthy controls(87–89). Our lab has previously shown that serum ALP directly correlates to absolute size of the tumor burden (93)and primary tumor relative bone mineral density (94). This led us to investigate whether decreased tumor bone mineral density may correlate an increase of FGF2 in circulation, as these tumors may be less differentiated and therefore produce less bone. We separated dogs into osteoblastic or osteolytic OS based on DEXA imaging and included healthy dogs as a control. We found no significant difference in plasma FGF2 concentration between dogs with osteoblastic OS compared to those with osteolytic OS. The plasma FGF2 concentration was also not significantly different in dogs with OS compared to healthy controls. Evaluating the tumor stroma would be a more accurate
measure of FGF2, as the extracellular matrix in normal tissues and tumor associated
macrophages in OS have been shown to harbor and secrete this ligand previously(27,28,37).

Several limitations in our study must be addressed. Most notably, the FGFR protein
expression was not confirmed, and evaluating cell pellets and spontaneous tissue samples with
immunohistochemistry for these receptors, as well as FGF2, would help to solidify the presence
of the FGF signaling axis in canine OS. Additionally, confirmation of the FGF signaling cascade
by identifying phosphorylation of downstream targets such as Erk 1/2 would strengthen the
findings from this study. FGF2 stimulation was not included in these assays. Both the standard
and the osteogenic media are supplemented with fetal bovine serum (FBS), which contains FGF,
and so stimulation may have been acquired by exposure to the media. In order to limit the effects
of this exogenous FGF in our studies, however, media containing only 1% FBS was used in our
assays rather than the standard 10% FBS. Performing these assays with titrated concentrations of
FGF2 may help to confirm the results noted. BGJ398 has not been evaluated in dogs, and so it is
not known if the concentrations used in this study are biologically achievable and therefore
clinically relevant. The effect of FGFR signaling inhibition on K003 differentiation was varied.
Extending the differentiation assays to include a larger range of BGJ398 doses and extending the
exposure to conditions to 14 days may clarify these results. While the osteogenic gene
expression experienced a clear increase in mean values on day 10 with exposure to the inhibitor
in the HMPOS cell line, this was not statistically significant. Increasing the number of replicates
would allow for more robust statistical data. This study did not evaluate other key tumorigenic
effects of FGF signaling such as cell survival/chemoresistance and angiogenesis. Evaluating the
effect of FGF2 stimulation and FGFR inhibition on OS cells exposed to chemotherapeutics
would enhance our knowledge of this signaling pathway in canine OS. Additionally, the effect of
FGF2 on OS angiogenesis may be evaluated via immunohistochemical staining of spontaneous tumor stroma for FGF2 expression in association with MVD. This study was primarily in vitro, limiting the clinical relevance. Extending this to include orthotopic murine models of canine OS to allow for investigation of the effect of BGJ398 treatment would further validate the use of FGFR inhibition for treatment of this devastating cancer in dogs.

In conclusion, our study showed that canine OS cells express all four FGFR receptors and secrete FGF2, with 2 out of 3 cell lines doing so in a cell density-dependent manner. FGFR blockade impedes OS cell migration, however it has no clear pro- or anti-proliferative effect. FGF receptor antagonism led to a more mature phenotype in the HMPOS cell line, as indicated by an increase in osteogenic gene expression, ALP activity, and mineral production. Circulating FGF2 concentration is not influenced by the presence of canine OS, regardless of whether it is osteoblastic or osteolytic, although investigation into the tumor stroma is warranted. These findings indicate that FGF blockade could be beneficial in some dogs with OS.
## FIGURES AND TABLES

Table 3.1: Primers utilized in RT-PCR reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense Primer</th>
<th>Antisense Primer</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>FGFR1</td>
<td>5’TCCAGTGCTCAAGCACATC</td>
<td>5’ATTTGGCTGTGGAAAGTGCCT</td>
<td>58°C</td>
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<td></td>
<td>(376 bp)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGFR2</td>
<td>5’GGCTCCATTAATCACAGGTAC</td>
<td>5’CCACCATAAGGGAATTAGG</td>
<td>58°C</td>
</tr>
<tr>
<td></td>
<td>(601 bp)</td>
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<td>FGFR3</td>
<td>5’AGGCCATCGGTATTGACAAGC</td>
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</tr>
<tr>
<td></td>
<td>(383bp)</td>
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<td>FGFR4</td>
<td>5’TCGCTTGGAGGCATTCC</td>
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Table 3.2: Primers utilized in quantitative RT-PCR reactions

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<td>GAPDH</td>
<td>5’ ATCACTGCCACCCAGAAGAC</td>
<td>5’ GTGGAAGCAGGGATGATGTT</td>
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Figure 2.1  (a) The osteogenic differentiation cascade, from mesenchymal stem cells (MSCs) to osteocyte. Effectors in the signaling pathway are noted, including Bone morphogenic proteins (BMPs) and downstream mediators (inhibitor of DNA binding (Id) proteins and connective tissue growth factor (CTGF)), which are early markers in the cascade. Runx-2 and Wnt are important in determining osteoblast commitment and differentiation. Markers of bone formation include alkaline phosphatase (ALP) and Osterix (early/middle markers) and osteocalcin and osteopontin (late markers). (b) Defects in the osteogeneic differentiation cascade leads to a malignant phenotype. The potential defects are not well understood and may include genetic or epigenetic changes in Wnt, Rb, p53, p27, among others. If a defect leads to a block in differentiation, the undifferentiated OS precursor would retain the ability to proliferate, creating an imbalance between proliferation and differentiation that would lead to a tumorigenic phenotype. (Wagner ER, Luther G, Zhu G, Luo Q, Shi Q, Kim SH, et al. Defective Osteogenic Differentiation in the Development of Osteosarcoma. Sarcoma [Internet]; 2011. Figure 1.)
Figure 2.2 Fibroblast growth factor (FGF) receptor tyrosine kinase signaling cascade resulting in (a) cell proliferation, (b) cell survival, and (c) cell motility. (Goetz R, Mohammadi M. Exploring mechanisms of FGF signaling through the lens of structural biology. Nat Rev Mol Cell Biol. 2013 Mar;14(3):166–80. Figure 2.)
Figure 2.3  Mechanisms of FGF signal alterations implicated in tumorigenesis. (a) Ligand-independent signaling by genomic alteration of FGFR. (b) FGFR splicing (altered ligand specificity) or amplification of FGFR gene (FGFR expressed out of context) could lead to a paracrine loop with FGF expressed by the tumor stroma. (c) Autocrine loop may develop by the increased expression of FGF ligand by the cancer cell or FGFR expression out of context so it has binding specificity to the autocrine FGF when it would not have otherwise. (d) Effect of FGF on tumor stroma such as angiogenesis. (Turner N, Grose R. Fibroblast growth factor signaling: from development to cancer. Nat Rev Cancer. 2010 Feb;10(2):116–29. Figure 3.)
Figure 2.4  Select FGFR aberrations leading to tumorigenesis in humans. (Haugsten EM, Wiedlocha A, Olsnes S, Wesche J. Roles of Fibroblast Growth Factor Receptors in Carcinogenesis. Mol Cancer Res. 2010 Nov 1;8(11):1439–52. Figure 2.)
Figure 4.1  Reverse-transcriptase polymerase chain reaction (RT-PCR) for FGFR1, FGFR2m, FGFR3, and FGFR4 gene transcription. Amplicons generated by RT-PCR for FGFR1–FGFR4 genes (top panels) and the house-keeping gene, GADPH (lower panel). Respective osteosarcoma cell lines evaluated as indicated: Lane 1 – Abrams, Lane 2 – HMPOS, Lane 3 – K003.

Figure 4.2  FGF-2 is secreted by K003 and Abrams in a cell density-dependent manner. HMPOS secretes a minimal amount of FGF-2.
At 24, 48, and 72 hours with a titration of BGJ398 concentrations, inconsistent differences in cell proliferation were noted in HMPOS and K003 cell lines. (a) HMPOS showed a significant increase in proliferation with BGJ398 at 50 nM, 100 nM, and 200 nM at 24 hours and with 12.5 nM and 100 nM at 48 hours, while no significant differences were noted at 72 hours. (b) K003 showed a significant increase in proliferation with 12.5 nM and significant decrease with 25 nM and 100 nM at 24 hours, significant increase with DMSO and decrease with 25 nM and 50 nM at 48 hours, and no significant differences at 72 hours. *p<0.05, **p<0.01.
Figure 4.4  There is no difference noted in K003 cell proliferation at any concentration when comparing inhibitor #1 and inhibitor #2.
Figure 4.5  Treatment with BGJ398 inhibits cell migration in a scratch assay. A monolayer of cells were scratched and then treated with 200 nM BGJ398. Coverage of the defect by cells was documented by photomicrographs after incubation for 24 and 48 hours. (a) Treatment with BGJ398 inhibited scratch closure in the HMPOS cell line after 48 hours (**)p < 0.01). (b) Treatment with BGJ398 significantly inhibited the K003 cell line at 24 hours (**)p < 0.01) and 48 hours (**p < 0.01). (c) In the Abrams cell line, a trend toward inhibited scratch closure was noted which was not significant.

![Image showing photomicrographs and graphs of BGJ398 effects on cell migration in HMPOS and K003 cell lines at 0h, 24h, and 48h]
Figure 4.5 (cont.)

b) Effect of BGJ398 on K003 cell migration after 24h incubation

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Effect of BGJ398 on K003 cell migration after 48h incubation

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c) Effect of BGJ398 on Abrams cell migration after 24h incubation

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Effect of BGJ398 on Abrams cell migration after 48h incubation

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Figure 4.6  qRT-PCR for osteogenic genes (ALP, OSN, OSTX, and RUNX-2) was performed after exposure to OG media, OG media + 200 nM BGJ398, and OG media + DMSO for 7 days. (a) A mild decrease in ALP expression was noted in the HMPOS cell line with BGJ398 treatment. (b) The K003 cell line showed an increase in ALP and OSN expression with BGJ398 treatment.
Figure 4.7 The effect of a titrated concentration of BGJ398 on microscopic evaluation of cell confluency as a subjective measure of cytotoxicity showed a plateau of cell confluence at 6.25 and 12.5 nM for both HMPOS and, more notably, K003 cell lines.
Figure 4.8  qRT-PCR for osteogenic genes (ALP, OSN, OSTX, and RUNX-2) was performed after exposure to titrated concentrations of BGJ398 (6.25 nM, 12.5 nM, and 25 nM) for 0, 1, 4, 7, and 10 days. (a-d) HMPOS cells treated with BGJ398 exhibit a trend toward an increase in expression of osteogenic genes by day 10, while the expression decreases by day 10 in the OG media and DMSO controls. (e-h) K003 cells exhibit variable changes in osteogenic gene expression following BGJ398 treatment, typically seen as a decrease in gene expression compared to control OG media, with the exception of a trend toward increased expression in the 25 nM BGJ398 cells on day 10 which is significant in OSTX. The remaining osteogenic genes show an increase in mean value at 25 nM on day 10 which was not statistically significant. * p<0.05, ** p<0.01.
Figure 4.8 (cont.)

b)

![Graph showing the effect of BGJ398 on OSN Gene Expression in HMPOS.]

Effect of BGJ398 on OSN Gene Expression in HMPOS

![Graph showing the effect of BGJ398 on RUNX2 Gene Expression in HMPOS.]

Effect of BGJ398 on RUNX2 Gene Expression in HMPOS

c)
Figure 4.8 (cont.)
d)

**Effect of BGJ398 on OSTX Gene Expression in HMPOS**

![Graph showing effect of BGJ398 on OSTX gene expression in HMPOS.]

e)

**Effect of BGJ398 on ALP Gene Expression in K003**

![Graph showing effect of BGJ398 on ALP gene expression in K003.]

53
Figure 4.8 (cont.)

f) Effect of BGI398 on OSN Gene Expression in K003

g) Effect of BGI398 on RUNX2 Gene Expression in K003
Figure 4.8 (cont.)
h)

Effect of BGJ398 on OSTX Gene Expression in K003

[Bar chart showing gene expression levels with error bars for different conditions.]

mRNA Relative Fold Increase

* p < 0.05, ** p < 0.01
Figure 4.9  The effect of BGJ398 on alkaline phosphatase activity was evaluated at days 0, 1, 4, 7, and 10. (a) HMPOS ALP bioactivity increased significantly at all concentrations of the inhibitor on day 10. (b) K003 ALP bioactivity decreased with the inhibitor at all concentrations on day 4 and with DMSO and all concentrations of the inhibitor on days 7 and 10. ALP concentration normalized to protein content and expressed both as a ratio and as fold difference to better visualize changes.

a)
Pilot study with positive Alizarin Red staining confirms that K003 and HMPOS show signs of mineralization with osteogenic media over time.
Figure 4.11  Effect of 200 nM BGJ398 on differentiation of canine OS cells. (a-b) HMPOS and K003 showed a subjectively decreased stain intensity with a discordant increase in quantification.

(a)
Figure 4.12 The effect of BGJ398 on mineralization on OS cell lines was assessed subjectively with Alizarin Red stain intensity and confirmed with quantification. (a-b) HMPOS experienced a statistically significant increase in mineralization with (a) 25 nM BGJ398 on day 7 and with (b) all concentrations of BGJ398 on day 10. (c-d) K003 experienced a (c) trend toward increased mineralization with 25 nM BGJ398 on day 7 and a (d) significant increase in mineralization with 25 nM BGJ398 on day 10. Microscopic images at 10x magnification. ARS concentration normalized to protein content and expressed both as a ratio and as fold difference to better visualize changes.

Microscopic images at 10x magnification.
Figure 4.12 (cont.)

b) Effect of BGI398 on HMPOS ARS concentration Day 10

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HMPOS Day 10 ARS/Protein Fold Difference

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c) Effect of BGI398 on K003 ARS concentration Day 7

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K003 Day 7 ARS/Protein Fold Difference

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Figure 4.12 (cont.)

Figure 4.13  Circulating FGF-2 concentration is not influenced by presence of osteoblastic or osteolytic osteosarcoma.
REFERENCES


25. HistolHistopathol, Vol 30, Feng et al. [Internet]. [cited 2017 Sep 8]. Available from: http://www.hh.um.es.proxy2.library.illinois.edu/Abstracts/Vol_30/30_1/30_1_13.htm


