DISSERTATION

THE IMPACT OF INSULIN LIKE GROWTH FACTOR 2 MRNA BINDING PROTEIN 1 (IGF2BP1) IN HUMAN AND CANINE OSTEOSARCOMA

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ABSTRACT

THE IMPACT OF INSULIN LIKE GROWTH FACTOR 2 MRNA BINDING PROTEIN 1 (IGF2BP1) IN HUMAN AND CANINE OSTEOSARCOMA

Osteosarcoma (OS) is a malignant bone tumor that afflicts over 10,000 dogs. Most dogs and approximately 30-40% of children with OS succumb to metastatic disease. We identified elevated insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1) as one of the biomarkers of poor prognosis in canine OS. IGF2BP1 is an oncofetal protein that regulates mRNA subcellular localization, nuclear export, stability, and translation. IGF2BP1 controls the expression of oncogene targets and correlates with poor outcome in a variety of human cancers.

Using microarray analysis, we identified elevated insulin-like growth factor II mRNA binding protein 1 (IGF2BP1) expression as a biomarker of poor prognosis in canine osteosarcoma. Also, our preliminary data show that IGF2BP1 knockdown (shRNA) in a human OS cell line increased sensitivity to doxorubicin by ≥ tenfold compared to control. Significant reductions in cellular migration, invasion, proliferation, and tumor growth in nude mice were also observed ($p < 0.05$). The current research explores mechanisms for increased IGF2BP1 expression in panels of human and canine osteosarcoma cell lines. Gene amplification, hypomethylation, increased transcription, and alterations in microRNA (miRNA) regulation directly or through 3'UTR shortening have all been hypothesized by many studies as mechanisms to increase IGF2BP1 expression in cancer. We evaluated the expression and alternative polyadenylation of IGF2BP1 using RT-qPCR and western blot analysis in human and dog osteosarcoma cell lines. We assessed transcriptional activation of IGF2BP1 using luciferase reporters containing promoter sequences

from the human and canine IGF2BP1 genes. To detect genomic amplification and methylation, we used qPCR to assess gene copy numbers and treatment with the DNA methylase inhibitor, 5-Azacytidine, to explore activation of gene expression through hypomethylation.

Using qPCR analysis, we observed genomic amplification in 35% of canine tumors and cell lines and correlated amplification with IGF2BP1 transcript expression ($p = 0.0006$, Pearson $r = 0.88$). We observed no genomic amplification in human cell lines. Significant loss of 3'UTR regulatory sequences was found in 20% of canine cell lines ($p < 0.05$). The promoter analysis showed that most regulatory elements were located within ~580bp from the translational start site in both species. Using pathway-focused luciferase reporter assays, we identified activation of the following factors: MYC, NF-Kappa B, AP-1, and TCF4: β-catenin. Thus, our data show that multiple mechanisms can contribute to elevated IGF2BP1 expression, and these results can be used to develop new treatment strategies that target elevated IGF2BP1 or regulatory mechanisms.

Using the McKinley canine OS cell line, we generated and validated stable overexpression of IGF2BP1 (IGF2BP1-pLVX-Puro, Clontech). The stable OS cell line pool and individual clones with a corresponding empty vector control were analyzed and tested for migration, invasion, proliferation, and resistance to standard chemotherapeutic agents. We analyzed migration and invasion using a scratch wound assay and measured cellular proliferation as a surface confluence for 90 hours on an IncuCyte Zoom. We also assessed the clones' sensitivity to doxorubicin over 48 hours using a bioreductive resazurin-based fluorometric assay. We assessed changes in transcript expression in response to IGF2BP1 from isolated total RNA analyzed on Affymetrix Canine 1.0ST microarrays (University of Colorado Cancer Center Genomic and Microarray Shared Resource).

The overexpressing IGF2BP1 clones had increased resistance to doxorubicin compared to the control, and the IC_{50} levels correlated with IGF2BP1 mRNA levels ($p < 0.05$, $r^2 = 0.89$). For cellular proliferation, we found that only the IGF2BP1-expressing pool, that represents random insertion of the plasmid without selecting isolated clones, exhibited a significantly higher rate of proliferation relative to the empty vector control ($p < 0.05$). However, one of the highest expressing IGF2BP1 isolated clones had significantly greater cellular mobility and invasion than this pool, and both the pool and isolated clone had significantly higher rates of migration and invasion that cells transfected with the empty plasmid ($p < 0.05$).

Microarray analysis of control and overexpressing cells was used to detect global changes in gene expression and to identify potential targets of IGF2BP1. Differentially expressed genes were cross referenced to the RNA Binding Protein Immunoprecipitation database, published by Conway et al. (2016) using human stem cells, to identify direct mRNA targets bound by IGF2BP1. We identified 162 genes that were differentially expressed between control and overexpressing cells (FC $\geq$2, FDR$<$ 0.05), and 13 of those genes have been previously reported to bind IGF2BP1 directly. Pathway analysis of these 13 genes identified enrichment for genes involved in the regulation of cell adhesion, migration, and the extracellular matrix. Altered expression and IGF2BP1 binding of a subset of these transcripts were confirmed using RNA immunoprecipitation and RT-qPCR. Our data suggest that IGF2BP1 plays a significant role in human and canine osteosarcoma. This study revealed the functional relevance of IGF2BP1 and identified it as a biomarker for aggressiveness in osteosarcoma. With this knowledge, new treatment strategies can be developed that target IGF2BP1 or it is signaling pathways for osteosarcoma, or any cancer that expresses high levels of IGF2BP1. This treatment may have a high impact on the cell's ability to metastasize.

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DEDICATION

I dedicate this dissertation to

my late brother Naif Mahdi Alyami (1982-2018).

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CHAPTER 1: LITERATURE REVIEW AND PROJECT RATIONALE

1.1 Osteosarcoma

1.1.1 Epidemiology of Osteosarcoma in humans

Osteosarcoma (OS) is typically a malignant bone tumor of mesenchymal origins. These osteoblast cells are neoplastic, actively proliferate, and produce osteoid and bone. OS can originate from any bone in the skeletal system. However, it primarily occurs at the metaphyseal plates of long bones, particularly the extremities [1]. In general, the most common locations for OS tumors are in the distal femur (40%), the proximal tibia (16%), and proximal humerus (15%). Another less common area is the proximal femur that accounts for only 5% of the cases [2].The most common symptoms of OS are swelling and localized pain of the affected limb, and in many cases, the tumor weakens the bone matrix structure, resulting in a bone fracture [3, 4].

OS is a rare disease, with approximately 900 new cases yearly in the United States, and represents less than 1% of cancer cases [5]. OS occurs mostly in children and adolescents between 10 and 20 years old. OS is the most common bone tumor in children younger than 20 years, comprising an estimated 56% of malignant bone cancers in the U.S. OS is more common in male patients than female patients, occurring at a 1.43:1 ratio [5, 6]. Nevertheless, OS is the most common bone cancer in adolescence and early adulthood, but the condition occurs less frequently than brain tumors and lymphomas in this age group [4].

The disease is usually diagnosed using histological methods; however, it can be difficult to characterize the subtype of OS. It is essential to correctly diagnose the type and classification of any bone cancer, as treatment method and prognosis often differ based on these characteristics.

OS can be classified into one of eight categories: conventional, telangiectatic, small cell, low-grade central, secondary, parosteal, periosteal, and high-grade surface, per the current World Health Organization classifications [7]. Prognoses and five-year overall survival rates vary across these different categories. For instance, the 5-year survival rate is 83% for periosteal OS, 46.1% for high-grade OS, 90% for low-grade central OS, and between 42% and 50% for small cell OS [7].

1.1.2 Metastasis and Treatment

OS is an extraordinarily malignant tumor that can generate deadly metastases all over the body [8]. Metastatic tumors in the lungs, central nervous system, or gastrointestinal tract are the leading cause of death in OS patients [9]. OS is one of the most aggressive cancers; it is estimated that up to 30-40% of OS patients have micrometastases in the lungs at the time of diagnosis [5, 10-12].

The standard treatment for OS in the 1970s was amputation of the affected limb. Surgery is an essential treatment to remove the primary tumor; however, with amputation only, the long-term survival rate for even a localized tumor is only 20%, and this percentage decreases with every recurrence. Surgical resection alone is certainly not enough for patients with metastasis at diagnosis, and their survival rate drops dramatically relative to those with a localized tumor (down to 4 to 10%) [2, 13-15]. In the last quarter of the 20th century, group studies and international collaborations showed that the most effective course of therapy for OS includes the combination of high-dose methotrexate, doxorubicin, cisplatin and ifosfamide/etoposide [16]. Now, current strategies for OS treatment consist of neoadjuvant chemotherapy, surgical removal of the primary tumor along with all evident metastatic disease, and then adjuvant chemotherapy. Occasionally, radiation therapy is used as palliative treatment. Introducing chemotherapy to treatment

significantly increases the survival rate from 20% with amputation alone to 70% for non-metastatic OS; this methodology is the result of many clinical trials through the 1970s and early 1980s that demonstrated the importance of chemotherapy types and combinations, as well as the timing of surgery [16]. In the past, amputation was necessary to manage and control OS, but in recent decades, improvements in detecting, imaging, and reconstruction techniques have made limb salvage surgeries more feasible in people [17-19]. Moreover, the development of advanced limb-sparing surgical technology has helped patients with pathological fractures or when amputation is not desirable [20, 21]. However, not removing the whole tumor can increase the likelihood of recurrence, but with an expert surgeon, studies have shown no differences in disease recurrence, and even an increased five-year survival rate. The 15 years follow up showed only a 33% risk of infection with limb salvage methods in people [22]. However, amputation can relieve the patient from a lifetime risk of infection or the body rejecting the endoprosthetic [23, 24].

Despite our increasing knowledge of anticancer therapies, the survival rate of OS has plateaued at levels reached in the 1980s, with 30 to 40% of diagnosed children still succumbing to the disease [25]. Patients can suffer from toxicity at any point of the multidrug neoadjuvant chemotherapy, which can slow the treatment plan. Researchers are now focusing on personalizing the treatment based on tumor biomarkers or gene expression to limit unnecessary toxicities [26-30]. Pharmacogenetics refers to the interaction between genomic technologies to identify biomarkers or genes that can indicate a drug efficiency or its toxicities [31-33]. However, in cases of OS, these studies are extremely difficult. Obtaining tumors from a rare pediatric disease is extremely difficult, and acquiring naïve tumors where the gene signature is not altered by cytoreductive treatments, is the main challenge facing OS researchers.

The causes of OS recurrence and chemoresistance remain unsolved; furthermore, studies of human OS pathogenesis and treatments are frequently hampered by the relatively infrequent occurrence of this cancer in the human population. However, a variety of animal models have contributed significantly to the understanding of OS pathogenesis.

1.1.3 Canine as a Model for Cancer

Cancers occur naturally in dogs, which gives them a fundamental advantage as an animal model for human disease. As pet dogs, they are exposed to the same environmental elements as their human companions. Additionally, since cancer occurs spontaneously in pet dogs, tumor development is analogous to human cancers, with the common features of escape from the host immune system, tumor heterogeneity, development of recurrent or resistant disease, and metastasis [25].

It is estimated that the rate of death from all types of cancer is 27.2 % in purebred dogs and 27.6% in mixed breeds. Often, specific dog breeds are more likely to develop specific types of cancer like sarcomas than others, such as the Airedale Terrier, Bernese mountain dog, Boxer, Golden, Bouvier des Flandres, Bullmastiff, Irish setter, and Scottish terrier [34]. There are similar predispositions toward cancers in human populations that increased the risk to large spectrum of children and adult onset cancers. Li Fraumeni syndrome is an autosomal dominant disease caused by germline mutations in the *TP53* gene that can be inherited [35]. Also, Ashkenazi Jewish individuals are at increased risk for breast, ovarian, melanoma, pancreatic cancers [36].

Dogs are treated with many of the same methods as for people. The large number of dogs with cancer can provide researchers with significant statistical power for clinical trials, especially for rare cancers, such as soft tissue sarcomas[37]. In addition, the larger body sizes of canines offer

the capacity to collect multiple tumor biopsies, blood, urine and other body fluids with greater ease than other animal models, and the similarity of the metabolic activity between humans and dogs allows for direct translation of treatment protocols between species [38-41]. Thus, in dogs, we can evaluate new anticancer treatments and even improve current chemotherapies to provide better strategies for both humans and dogs. For example, muramyl tripeptide (MTP) clinical trials in dogs with OS have translated well into humans, as the studies show an increase of the survival time in canines with OS and, correspondingly, in human OS patients [42-44].

Furthermore, there is a growing body of evidence that supports a significant parallel between the genomic profiles in canine and human cancers based on cross-species genomic analysis, which indicates that these diseases are also similar at the molecular level [45, 46]. Finally, the linkage-disequilibrium for any single breeds is up to 100-fold longer than within the human population or across all dog breeds, making individual dog breeds powerful and ideal subjects for genetic mapping of disease alleles [47].

1.1.4 Canine OS as a Model for Human OS

In contrast to the limited number of human OS tumor samples, canine OS is a common, spontaneous cancer with over 8,000 new cases annually [48]. Canine OS exhibits similar histological, biological, and pathological characteristics as human OS, including similar chromosomal aberrations and alterations in gene expression patterns [25]. It is the most common primary bone tumor in dogs, and the most frequently affected sites, similar to humans, are the metaphyseal regions of long bones, particularly the front limbs [49-51].

The histology and sub-classification of OS in dogs are also like that of human OS, according to the World Health Organization [34]. These subtypes include osteoblastic,

chondroblastic, fibroblastic, telangiectatic, giant cell type, and poorly differentiated. The type with the poorest prognosis and association with metastasis in both species is the telangiectatic subtype [34].

The age of onset for OS in humans is during the adolescent growth phase of the bones, suggesting a close relationship between rapid cell growth of metaphyseal bone and tumor development; children with the disease are usually taller than average [52]. Consistent with these observations, OS most commonly affects large and giant canine breeds, which share the rapid growth phase of the skeletal system seen in people with OS [53-56]. In contrast to human OS, canine OS appears to be heritable, with some breeds being predisposed to develop the disease [57].

Current studies of cancers are driven by lab animal models like transgenic mice and xenograft rodent models. However, OS mice/rodent models do not represent the complex microenvironment, genetic instability, and heterogeneity of the disease. For example, murine primary tumors are mainly located on the flat bones versus the metaphyseal plates of long bones. Furthermore, mice/rodent models fail to recapitulate the mechanisms of recurrence, metastasis and prognosis of OS exhibited by spontaneous cancers [25, 58, 59].

Pathological studies at the molecular level in dogs with OS have identified many factors and mutations that might drive OS pathogenesis that are like humans. These possible drivers include the overexpression of the tumor suppressor p53, which is correlated with a more aggressive phenotype in humans and dogs with OS [60, 61]. Also, dogs with greater micro-vessel density of primary tumors were at greater risk of developing pulmonary metastasis, which is also true in cases of human OS [62, 63].

Further, to examine the increased incidence of OS in some dog breeds, one study compared the chromosomal aberrations in Golden Retrievers and Rottweilers that were diagnosed with

spontaneous OS. This study revealed a significant impact of the breed and the genetic background on the tumor cytogenetics and karyotypes [64]. Another example of OS heritability in dogs is found in the Scottish deerhound breed, which is predisposed to OS and shows a robust dominant heritability of the disease [65]. The connection between the breeds that are predisposed to OS may identify potential risk factor genes that can hopefully apply to human OS [66]. Thomas et al. (2009) showed that the genetic aberrations observed in human OS are also seen in canine OS with both breed-dependent and independent associations [67].

1.1.5 Clinical, Pathologic Factors and Prognosis for Canine with OS

Chemotherapeutic treatment for dogs typically involves doxorubicin and platinum-based drugs similar to regimens used for human OS therapy. These chemotherapeutic treatments increase the median survival time to 262-450 days [68]. In dogs with detectable metastases at diagnosis, the survival time is significantly decreased, but adding chemotherapy and radiation improves their survival time. However, most canine patients eventually develop metastasis, with 90% occurring in the lungs, similar to human OS [69-71]. Similar to humans where 30-40% of OS patients relapse within 3 years of diagnosis, in canine OS the one-year survival rate after diagnosis and treatment is less than 45%, with a marked decrease in the median survival to 79 days if metastasis is discovered at the day of diagnosis [72-74]. With dogs, the median survival time is less than 20 weeks (140 days) and the majority of dogs are euthanized within a year due to metastatic disease [70] if amputation is the only treatment. Unfortunately, limb salvage or amputation and adjuvant chemotherapy only increase the median survival time to 10-11 months in dogs, with a small percentage of patients surviving over one year [75-81].

The age distributions of OS in dog patients are also bimodal, with a small peak in young animals (18-24 months) and a second larger peak in older dogs (median age of diagnosis of seven years). However, adolescent canine patients have the most aggressive disease when compared to older dogs [50, 82, 83].

Many factors have been suggested to have a significant influence on the long-term survival of both species with OS. Studies have shown that dogs with lower body weight have a longer survival time, as OS occurs extensively in large and giant breeds [84-87]. Besides, tumor location has a high impact on survival time, as dogs with proximal humeral tumors have a shorter survival time Proximal humeral tumors may be due to increased body weight stress on the bones [75, 85, 88]. Increased levels of serum alkaline phosphatase in the blood are also associated with decreased survival time in dogs[85]. Also, the number, time, and sizes of the developed lung metastases or lymph node metastasis can indicate a poor response to the medication, thus decreasing the survival time [89-91].

1.1.6 Genetic Pathogenesis of OS

The causes of OS in humans and dogs are not known. We do know that the phenotypic risk factors for OS are associated with the physiologic growth spurts in tall children with high birth weights [92]. Furthermore, the loss of tumor suppressor genes, such as p53 and RB1, has been identified in many studies of OS, and is believed to be primary step in OS pathogenesis [93, 94]. Genomic instability is a common feature of both human and canine OS [41]. The accumulation of knowledge and understanding of molecular factors involved in other diseases can contribute to the identification of potential players.

1.2 General Features of Genetic Alterations

1.2.1 Chromosomal aberrations

One of the hallmarks of OS, both human and canine, is extensive genomic instability [41, 67, 95]. It has been hypothesized that a mutational crisis called chromothripsis is a possible trigger for OS. Chromothripsis occurs when a single catastrophic event causes hundreds of genomic rearrangements that are confined in a few chromosomes causing genomic instability [96]. Other studies hypothesize that the genomic instability is a result of the loss of the repetitive telomeric sequences at the ends of chromosomes. Telomerase enzymatic activity is responsible for maintaining the length of telomeres; in cancer cells, the telomeres can be maintained through a method termed alternative lengthening of telomeres (ALT). ALT is another mechanism to escape cellular senescent in cancer cells. These studies reported an association between OS outcome and telomere length, and found that ALT occurs more frequently in OS than other genomic instability-associated diseases [96-100].

1.2.2 DNA Helicase Genes

DNA helicase genes are a conserved gene family that code for an enzymatic activity leading to the separation of the DNA double-strand in multiple cellular processes, such as DNA replication and repair [101]. Multiple syndromes have mutations in RecQ protein-like (RECQL) helicases. DNA helicase family enzymes are essential for maintaining genome stability and dysregulation, and loss of function is an early step in the progression of osteosarcoma. Mutations in RECQL helicases family can case genomic catastrophe resulting in chromosomal rearrangements [102, 103]. Bloom syndrome is caused by mutation of RECQL2 gene, the mutation

of RECQL3 gene causes Werner syndrome, and Rothmund-Thomson syndrome is caused by the mutation of the RECQL4 gene [104-106]. It has been shown that patients with these syndromes are highly predisposed to OS [102, 103].

1.2.3 The TP53 Gene

Li-Fraumeni syndrome is a disease that predisposes the patient to multiple malignancies, and is considered one of the most common syndromes predisposing children to sarcomas; an estimated 30% of children with this syndrome will develop OS during their lives [107]. The syndrome is a result of a germline mutation of the *TP53* gene, which is the most commonly mutated gene in human cancers. *TP53* encodes for p53, a master transcription factor and pro-apoptotic tumor suppressor [108, 109]. Although mutations associated with cancer have been identified throughout the TP53 gene, the most frequently mutated region is the DNA-binding domain. [96]. Since p53 functions as a tetramer, mutated p53 can bind to and alter the activity of wild-type p53, thereby acting as a dominant negative inhibitor [110-114].

Furthermore, TP53 mutations in somatic cells are commonly observed in human (75%) and canine (83%) OS, with these mutations linked to OS pathogenesis or progression [115, 116]. Many studies have shown that TP53 mutations in canine OS primary tumors correlate with decreased survival time [100, 117-126]. Mutations and deregulation of other genes that are involved in p53 pathways, such as mouse double minute 2 homolog (MDM2), checkpoint kinase 2 (CHEK2), and cyclin-dependent kinase inhibitor 2 (CDKN2 or p14ARF), have been reported in human and canine OS cases [96, 127, 128].

1.2.4 The RB1 Gene

RB1 gene mutation leads to another hereditary syndrome that affects children. Germline modifications of the tumor suppressor gene RB1 cause hereditary retinoblastoma in children with OS as secondary tumors [129-132]. RB1 protein is a transcriptional repressor that prevents E2F1 from binding to many target genes that activate the cell cycle [133]. Somatic RB1 mutations in canine OS cell lines and human patients indicate that spontaneous mutation of the gene may promote OS genesis [134-136]. Supporting this hypothesis, other studies have shown that the loss of heterozygosity, losing both copies of the genes during chromosomal segregation, at the RB1 locus occurs in 60-70% of human OS, suggesting the presence of a spontaneous mutation that promotes OS [96, 137].

Furthermore, mutation or amplification of one or more cell cycle regulatory genes, such as cyclin-dependent kinase inhibitor 2A (CDKN2A) and cyclin-dependent kinase 4 (CDK4) can also manipulate RB1 pathways, and have been identified in OS [127, 138]. In dogs, three studies concluded that 45% to 75% of dogs with OS had imbalanced copy numbers of CDK4 and CDKN2A/B, and 81% had mutations in *CDKN2A/B* in germline cells. 17 of 38 dogs (45%) with either the loss of the $p16^{INKA}$ gene encoded by *CDKN2A* [11q15] or gain of *CDK4* [CFA 15q24.3]) showed evidence of a disrupted Rb pathway, mirroring RB1 mutations in humans [67, 115, 139].

1.2.5 The PTEN Gene

Phosphatase and tensin homolog (PTEN) is another tumor suppressor gene that was reported to have a loss of copy number in 41% (of the cohort) in canine tumors [67]. Moreover, 42% of OS cases (16/38 cases) and 4 out of 5 canine cancer cell lines have variations in PTEN. In

some dog breeds with an increased risk of OS, there is total copy number loss of the gene in their tumors [67, 95, 140]. In human OS, PTEN mutation or deletion is not as common as those in the other tumor suppressor genes mentioned above; according to one study, only 15% of human OS tumors have reported copy number losses of PTEN [141].

1.2.6 The MYC Gene

Alterations in the critical regulatory genes that activate the cell cycle, cell differentiation, and cell survival have been reported in many cancers. These altered genes are known as oncogenes. An example of a well-known oncogene is Myelocytomatosis (MYC), a viral oncogene homolog, which is frequently overexpressed in human and canine OS [124, 142-144]. Additionally, the expression level of this oncogene expression is correlated with inadequate response to chemotherapy in OS [145-147].

A study in 2018 found that MYC upregulation in metastatic human OS samples acts as a super enhancer for proliferation, migration, and invasion of OS cells [148]. The authors of an additional study were able to transform mouse bone marrow stromal cells (BMSCs) to yield overexpression of c-MYC and the loss of Ink4a/Arf , which caused the mice to develop OS [149].

MYC is a well-known oncogene that plays a role in promoting the transcriptional amplification of many genes that are an essential hallmark of cancer [150]. Interestingly, other factors that interact with MYC are found to be dysregulated in OS, such as Insulin-Like Growth Factor II mRNA Binding Protein I (IGF2BP1), which is also known as CRD-BP. IGF2BP1 is known for its role in binding to the coding region-determinant portion of MYC mRNA, thereby stabilizing the transcript and efficiently increasing its translation [151, 152].

1.3 Signaling Pathways Involved in OS

Molecular mechanisms of growth and progression of OS have many redundancies and alterations in growth signaling pathways in genes such as IGF, VEGF, HER2, ErbB-4, PTHR, and HGF, all of which play an important role in healthy cell development [153-157]. Alterations in these genes have a wide range of effects, illustrating the difficulty in developing a molecular target therapy for OS. However, several clinical trials using small molecule inhibitors targeting of signaling pathways are currently ongoing.

1.3.1 The Notch Pathway

The Notch pathway is a conserved pathway that maintains cell proliferation, survival, apoptosis, and differentiation. The pathway starts with Notch receptor (Notch1-4) activation by ligand (jagged 1, jagged 2, Delta-like 1 or delta-like 1, 3 or 4), which triggers cleavage of the internal part of the receptor (intracellular domain of Notch) to serve as a transcription factor in the nucleus [158]. This transcription factor stimulates the expression of genes that block differentiation, including, Notch2, Jagged1, HEY1, and HEY2[159]. Correspondingly, inhibiting the Notch pathway diminished OS cell proliferation both *in vitro* and *in vivo* [159]. Another study showed that an elevation of Hairy and Enhancer of Split 1 (HES1), a downstream target of the Notch pathway, might contribute to canine OS development and progression [160]. Although no specific mutations have been detected in Notch signal pathway molecules, inhibition of the pathway shows therapeutic promise [160].

1.3.2 The Hedgehog Pathway

The Hedgehog (Hh/Gli) pathway is also a conserved family that plays a key role in embryonic development, tissue differentiation, cell growth, and, recently, has been linked to the progression and metastasis of various cancers [161, 162]. The Hh/Gli signaling cascade is complicated, but generally starts with Hh ligands, such as Sonic hedgehog (Shh), Desert hedgehog (Dhh) or Indian hedgehog (Ihh), which bind to the 12 transmembrane Patched1 (PTCH1) and Patched2 (PTCH2) receptors. This binding represses the ability of PTCH to bind Smoothened, a seven transmembrane receptor, which then releases GLI1, GLI2 and GLI3 transcription factors from the SuFu –Gli complex to activate the pathway [163]. One study identified significant protein expression of ribosomal protein S3 (RPS3) in OS lung metastases, suggesting that RPS3 is one of the gene targets of GLI2. Interestingly, previous work by Joeson et al. (2007) also identified RPS3 in the large ribonucleoprotein (RNP) granules that are used as a shuttle to translocate mRNA via IGF2BP1 [164]. Consequently, RPS3 regulates the aggressiveness of OS by promoting cell invasion and metastasis to the lungs, providing an encouraging therapeutic target for OS patients [165]. Overexpression of Yes-associated protein 1 (Yap1), an oncogene and another target gene for the Hh/Gli pathway was also observed in osteoblastic OS [166].

1.3.3 The WNT Pathway

The Wnt pathway is an essential pathway in normal cell development and involved in many types of human cancer, particularly in epithelial cancer types [167]. In human osteosarcoma, activation of Wnt signaling contributes to OS development, particularly the formation of lung metastases [168, 169]. The Wnt signaling pathway starts with the binding of Wnt ligands to

Frizzled receptors and their co-receptors Lipoprotein Receptor-Related Protein 5/6 (LRP5/6). These ligand-receptor interactions lead to the phosphorylation of the APC complex and prevent the tagging of β-catenin for degradation by inhibiting GSK-3β. The accumulated β-catenin translocates to the nucleus and binds to TCF/LEF to activate many oncogenes, such as c-MYC, cyclin D1, metalloproteinases, and c-Met, which themselves regulate differentiation and bone development. One study reported that OS cell lines express Wnt ligands and receptors, whereas secreted Wnt antagonists, including secreted frizzled-related protein (sFRP) and Dickkopf (Dkk) family proteins, are commonly absent in OS cells [170]. Other studies have shown crosstalk between Wnt, Notch, and Hedgehog, which provides a novel therapeutic approach for OS as all these pathways are involved in healthy bone development. Three drugs are currently in clinical trials: RO4929097 for the notch pathway, vismodegib targeting the Hedgehog pathway, and cyclopamine, an antagonist of SMO [171].

Finally, many of these genes are commonly mutated in both human and canine OS. For example, previous research identified gene dysregulation in MYC, TP53, CDKN2A, PTEN, RB1, Wilms tumor 1 (WT1), and v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) oncogenes through both breed-dependent and independent cytogenetic abnormalities in dogs [67]. These genetic changes can assist in the identification of the molecular pathways contributing to OS. Although most of these genetic changes are indicators or prognostic factors, many are not directly targeted by treatment. Figure 1.1 illustrates some of the signaling pathways that are dysregulated and some potential agents that are in development to target these pathways.

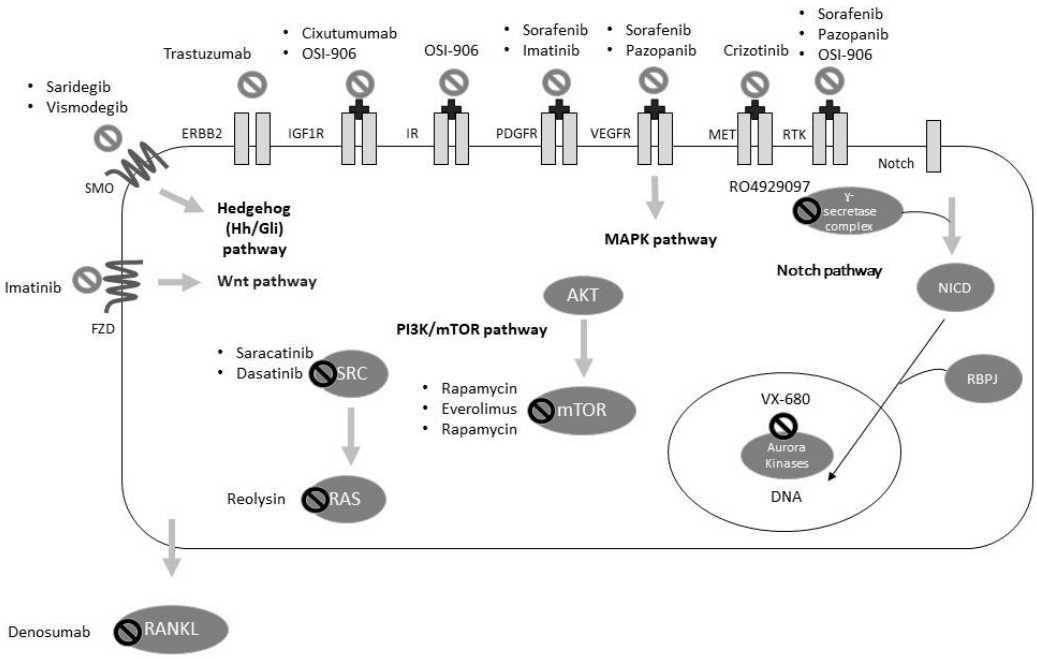

Figure 1.1 Molecular pathways and potential therapeutic drugs for OS.
Drug targets include specific receptor tyrosine kinases (RTKs). ERBB2, insulin-like growth factor 1 receptor (IGF1R), insulin receptor (IR), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR) that activate MAPK pathway, and hepatocyte growth factor receptor (MET). Other possible targets include, mTOR, smoothened (SMO) that activates the Hedgehog (Hh/Gli) pathway; Notch, Notch intracellular domain (NICD) and recombination signal binding protein for immunoglobulin κJ region (RBPJ) as Notch pathway targets. Frizzled (FZD) which activates the Wnt pathway. Aberrant activation of these signaling molecules and pathways in OS may promote tumor cell proliferation, survival, migration, angiogenesis and/or metastasis. Modified from [171].

1.4. IGF2BP1 and Osteosarcoma

Previously, our lab analyzed gene expression profiles using Affymetrix Canine 2.0 microarrays on OS tumors from two groups of canines. One group had a disease-free interval (DFI) of <100 days following amputation and chemotherapy, while the second group derived from canines with DFI >300 days. IGF2BP1 transcript levels were very low in microarray analysis of healthy dog bone, which correlates with the transcript levels observed in human osteoblasts. However, there was significantly elevated expression in tumors from dogs with a DFI >300 days (132-fold), and an even higher level of expression in tumors from dogs with a DFI of <100 days (915-fold), as compared to the control. Thus, the tumors taken from the patients with the shortest disease-free interval had higher expression levels of IGF2BP1 than long DFI patients (7-fold, p=0.047) [172].

Insulin-Like Growth Factor II mRNA Binding Protein I (IGF2BP1), also known IMP1, CRD-BP, VICKZ, ZBP, Vg1RBP/Vera, or KOC is a member of a highly conserved RNA binding protein (RBPs) family. This family consists of oncofetal proteins, which means they are normally only expressed during embryonic development. The three members of this RBP family (IGF2BP1, IGF2BP2, and IGF2BP3) are tightly regulated and shown to promote cancer phenotypes in adult cells [173]. *IGF2BP1* knockout mice were deficient in gut development, with increased mortality and dwarfism. Conversely, transgenic mice expressing IGF2BP1 in mammary tissue developed breast cancer [173, 174]. Increased IGF2BP1 expression correlates with poor outcome in a variety of human cancers, including melanoma, breast, ovary, liver, and colorectal cancer [175-179]. IGF2BP1 binds to mRNA targets and regulates their stability, translation, subcellular localization, and nuclear export [180]. IGF2BP1 protein is known to interact with the mRNAs of various genes

that have a high impact on cancer progression including: ACTB, β-catenin, MYC, CD44, GLI 1, IGF2, MAPK4, MDR-1, PPP1R9B, PTEN, HCV and KRAS (Table 1. 1).

The genomic DNA encoding Insulin-like growth factor II mRNA binding protein 1 gene is located on chromosome 17q21 near epidermal growth factor receptor 2 (HER2/neu) in humans and on chromosome 9 in dogs. The gene has a total of 15 exons in humans and dogs. Additionally, in both species, IGF2BP1 promoter contains CpG islands that may regulate gene expression via methylation. The translated protein consists of 577 amino acids with six conserved domains: four K-homology (KH1KH2 and KH3KH4) domains and two RNA recognition motifs (RRM1 and RRM2). Studies have shown that the KH domains are the primary domains that recognize and target the mRNA; however, which KH domain interacts may depend on the targets [181-183]. Also, IGF2BP1 contains two nuclear export signals between KH2 and KH4 facilitate the protein entering the nucleus [184]. A recent study shined a light on the ability of IGF2BP1 to read m(6)-methyladenosine (m(6)A), a modification on mRNAs that determines the mRNA fate, using KH3KH4 domains [185]. These same domains are required to recognize ACTB mRNA, but all four KH domains are essential to recognize c-MYC , CD44 and KRAS mRNA [181, 182, 186, 187]. The KH1KH2 domains to create a high affinity platform for the binding and modification of mRNA [188].

Doyle et al. (2000) identified amplification of IGF2BP1 gene copy number in human breast cancer. In this study, amplification and increased expression of IGF2BP1 was associated with the increased stability of c-MYC mRNA [176]. Despite its proximity to the HER2 gene, Doyle et al. (2000) results indicate that approximately one-third of human breast cancers have independently amplified IGF2BP1 from human epidermal growth factor receptor 2 (HER2/neu). Gene amplification of IGF2BP1 has also been observed in neuroblastoma patients [189]. Additionally,

IGF2BP1 can enhance MYC N expression, doxorubicin resistance in *vitro*, migration, invasion, and cell self-renewal in neuroblastoma [189]. In addition to gene amplification, chromosomal rearrangement with IGH (immunoglobulin heavy chain), resulting in an IGH-IGF2BP1 fusion protein, might also play a direct role in the pathogenesis of B-ALL (B-cell Acute lymphoblastic leukemia) by up-regulating IGF2BP1 expression [190].

Another mechanism for IGF2BP1 regulation is through microRNA (miRNA). miRNAs are small noncoding oligonucleotides (20 to 25 nucleotides), which play a critical role in regulating gene expression. By targeting the mRNA of many genes through complementarity between a miRNA and its target, miRNAs can induce mRNA decay or represses mRNA translation [191]. The primary microRNA transcripts (pri-miRNAs) are processed by Drosha–DGCR8 (DiGeorge syndrome critical region gene 8) and generating the precursor miRNAs (pre-miRNAs). Then the processed pre-miRNA is exported to the cytoplasm via Exportin 5 and binds to the ribonuclease Dicer cleaves double-stranded RNA (pre-miRNAs) to the mature miRNA[192]. Then mature miRNAs are associated with Argonaute 2 (AGO2) protein, which uses the miRNA to targets mRNA and using catalytically activity to cleave the mRNA, that are the main players in miRNA-induced silencing complexes (miRISCs) [191]. This complex can regulate many processes that are involved in; cellular development, differentiation, proliferation, and cell death [193].

A study shows that the loss of DICER resulted in the elevation of subset of miRNAs targets that are reinforced with IGF2BP family members are resistance to the reactivated miRNAs and increased tumorigenicity of the cells [193]. The increased tumorigenicity of these cells can be reduced with knockdown of IGF2BP1 family genes, suggesting that these oncofetal proteins, upon

activation by loss of miRNA signaling, can then sustain oncogenic signaling through their RNA binding capabilities[193].

A recent study by Huttelmaier's group found that for mRNAs that are RISC targets can escape the posttranscriptional regulation by binding to IGF2BPs [194]. Both IGF2BP1 and AGO2 bind within the 3'UTR of target mRNAs; however, no direct interaction between the proteins was found. Interestingly, there was no evidence that IGF2BP1 directly covers the miRNA binding sites (MBS)—in contrast, IGF2BP1 preferred to bind away from MBS by 40 nucleotides. Huttelmaier's group hypothesized that the conformational changes that IGF2BP1 generates close to the MBS may protect the mRNAs from the RISC complexes. Thus, IGF2BP1 promotes oncogenic potential by protecting oncogene mRNAs from miRNA regulation [194].

In many studies, the expression of the let-7 miRNA family correlates negatively with IGF2BP1 [195-197]. Boyerinas et al. (2008) generated point mutations at the binding sites for let-7 in IGF2BP1 mRNA and measured higher luciferase activity of a 3'UTR reporter construct showing a direct downregulation by let-7 [195]. Another study by Mayr et al. (2009) observed a general shortening of mRNA 3'UTRs, including IGF2BPs family, by alternative polyadenylation (APA) in cancer cells, which activated oncogenes by allowing them to escape post-transcriptional regulation by miRNA [198].

IGF2BP1 can act as a central mediator in a feedback loop that leads to the cancer stem cell phenotype. LIN28B (Lin-28 homolog B) promotes the stem cell phenotype by impairing let-7 family miRNA biogenesis. Loss of let-7 reduces IGF2BP1 mRNA post-transcriptional regulation. IGF2BP1 feedback enhances the expression of LIN28B and HMGA2 (High Mobility Group A class of proteins which bind to AT-rich DNA), creating, as Busch, B et al. (2016) described, an oncogenic triangle feedback loop [199]. In addition to regulating this stem cell feedback loop,

IGF2BP1 protein helps determine an mRNA's fate by regulating the nuclear export, stability, translation, and subcellular localization of its targets. These IGF2BP1 interactions and the potential impact on carcinogenesis are described in Table 1.1.

A recent publication shows other miRNAs or long noncoding RNAs (lncRNAs) regulate IGF2BP1 mRNA that impacts cancer formation and initiation [200-202]. miR-196b is known to induce cell apoptosis under hypoxic stress. Hypoxia can increase chemoresistance and regulates miR-196b expression to target the 3'UTR of IGF2BP1 mRNA[200]. Similar studies have shown miR-873, miR-423-5p, miR-491-5p, miR-372 miR-150, miR-708, miR-506, miR-4500, and miR-98-5p all serve to suppress IGF2BP1 expression at the mRNA level to inhibits metastasis [201, 203-209]. The upregulation of lncRNA, such as LINC01093 and HULC, in hepatocellular carcinoma (HCC) impairs the ability of IGF2BP1 to stabilize oncogenic drivers [210, 211].

IGF2BP1 may have its greatest impact through regulation of the tumor microenvironment and the ability to develop metastases. The overexpression or knockdown IGF2BP had a significant impact on the microenvironment and the development of lung metastases through the extracellular release and activity of exosomes [212]. Similarly, IGF2BP1 overexpressing melanoma cell lines release exosomes that have a significant role in modifying the formation of distant metastases [212, 213].

Given the importance of IGF2BP1 in cancer progression, a group of researchers identified a small molecular inhibitor that interferes with IGF2BP1 and c-MYC mRNA binding. BTYNB is a novel small molecular inhibitor that inhibits the interaction of IGF2BP1 with multiple mRNA targets, such as eEF2 and β-TrCP1 that induces NF-Kappa B activity [214]. BTYNB exhibited low systemic toxicity and selectively targeted cells with IGF2BP1 overexpression indicating future therapeutic potential[214].

Despite the multiple biological activities ascribed to IGF2BP1, it is currently unclear exactly what role IGF2BP1 plays in the development and metastatic progression of OS, and what factors may contribute to its elevated expression. Our overall objective here is to determine the mechanisms and elements that lead to increased expression of IGF2BP1, as well as the functions of the IGF2BP1 protein that contribute to OS metastasis and chemoresistance.

Table 1. 1 IGF2BP1 protein can interact with the mRNA of various genes that have a high impact on cancer progression.

Target	Binding mRNA	Regulation of the target mRNA	Hallmarks of cancer
ACTB (actin beta)	3'UTR	Inhibition of mRNA translation and mRNA transport	Cell migrating [215, 216]
β-catenin	3'UTR	Inhibition of mRNA decay	Invasion and Metastasis [217]
MYC	CDS	Inhibition of mRNA decay	Invasion, metastasis, proliferation, insensitive to growth signal and apoptosis [218, 219]
CD44	3'UTR	Inhibition of mRNA translation and mRNA transport	Cell migrating [220]
GLI 1	unclear	Inhibition of mRNA decay	Invasion, metastasis, proliferation, angiogenesis, insensitive to growth signal and apoptosis [221]
IGF2 (Insulin-Like Growth Factor 2)	5'UTR	Inhibition of mRNA translation	Proliferation [222]
MAPK4 (Mitogen-Activated Protein Kinase 4)	3'UTR	Inhibition of mRNA translation	Cell migrating [223]
MDR1 (Multi-Drug-Resistance Factor 1)	CDS	Inhibition of mRNA decay	Resistance to apoptosis [224]
PPP1R9B (tumor suppressor)	3'UTR	mRNA transport	Proliferation [181]
PTEN	CDS	Inhibition of mRNA decay	Cell migrating [223]
HCV (Hepatitis C virus)	5' and 3'UTR	Enhance mRNA translation	Proliferation [225]
KRAS	CDS and 3'UTR	Inhibition of mRNA decay	Invasion, metastasis, proliferation, insensitive to growth signal and apoptosis [226]

1.5 Project Rationale

Despite the medical progress by OS researchers, we still do not know the etiology or the cause of OS. Also, to develop a novel therapeutic strategy for OS, we need an animal model that exhibits similar histological, biological, and pathological chromosomal aberrations and alterations in gene expression patterns to human OS [25]. Using Affymetrix Canine 2.0 microarrays, our lab previously identified Insulin-like Growth Factor 2 mRNA binding protein 1 (IGF2BP1) as a biomarker that was elevated in canine tumors relative to normal bone and was further elevated in tumors taken from dogs with a short disease-free interval. Subsequent studies showed that IGF2BP1 expression was also elevated in human osteosarcoma cell lines with the highest levels in MG63.2 cells, a highly metastatic clone of the MG63 parental line. Gene knockdown of IGF2BP1 in MG63.2 cells increased sensitivity to doxorubicin and reduced cellular proliferation, migration, and invasion that reduced tumor growth in nude mice. These results suggest that IGF2BP1 could be involved in the pathogenesis and metastatic progression of canine and human osteosarcoma [172].

Analysis of the mechanisms that drive elevated expression of IGF2BP1 can provide both new clues regarding the pathogenesis of osteosarcoma and potential targets for treatment. Therefore, we first explored mechanisms that can lead to elevated IGF2BP1 expression in both humans and canines in Chapter 2 (Different Factors Contribute to Increased Expression of IGF2BP1 in Human and Canine Osteosarcoma). We hypothesize that four possible mechanisms act to increase IGF2BP1 protein levels: 1) the shortening the 3'UTR to break away from post-transcriptional regulation by miRNA, 2) genomic amplification of IGF2BP1, 3) the activation of transcription through multiple potential transcription factor binding sites (c-MYC and β-catenin,

NF-κB, AP1, and E2F), and 4) methylation of CpG islands within the gene promoter may block transcription in adult cells, and thus this loss of promoter methylation may alter expression. In this chapter, we explore the mRNA and protein expression of IGF2BP1 through qRT-PCR, genomic amplification via quantitative PCR (Q-PCR), and western blot. We also use a Luciferase Reporter promoter and 3'UTR analysis systems to quantify miRNA regulation of the 3'UTR and gene promoter activity in human and canine OS cell lines. We generated a series of 5' deletion constructs and utilized a TRANSFAC database to predict critical transcriptional regulatory elements on IGF2BP1 promoters for both the human and canine genes. Additionally, we assessed the role of DNA methylation in the regulation of IGF2BP1 in these cell lines by assessing IGF2BP1 expression following treatment with the demethylating agent (*5'-Azacytidine*) that inhibits DNA methyltransferase.

In Chapter 3, (Illuminate the Functional Role and Transcript Targets of IGF2BP1 in Osteosarcoma Progression), we assessed the functional role of IGF2BP1 in osteosarcoma progression by overexpressing IGF2BP1 in canine cells. This work was conducted after previous data from our lab using gene knockdown strategies indicated a significant role of IGF2BP1 during human osteosarcoma progression. We over-expressed IGF2BP1 and analyzed cellular proliferation, migration/invasion, and chemosensitivity.

In the second part of chapter 3, we identified the transcript targets of IGF2BP1 in OS progression using Affymetrix Canine 1.0ST microarrays and by comparing the gene expression of cells overexpressing IGF2BP1 to those stably transfected with the empty plasmid (negative control). Results from this study were comprehensively analyzed to identify genes differentially regulated by the overexpression of IGF2BP1, as those genes that may be directly bound and regulated by IGF2BP1. These studies confirm the functional role of IGF2BP1 in the development

and progression of OS, identify the mRNA targets through which it acts, and characterize the mechanisms that contribute to its increased expression in OS. The results of these studies can inform future research to identify new therapeutic strategies for OS. Agents targeting the signaling pathways that increase IGF2BP1 expression can be developed to improve the survival rates of OS patients, as well as other metastatic cancers that express IGF2BP1, as explained in chapter 4 under General Conclusions.

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CHAPTER 2: DIFFERENT FACTORS CONTRIBUTE TO INCREASED EXPRESSION OF IGF2BP1 IN HUMAN AND CANINE OSTEOSARCOMA

SYNOPSIS

Background: Osteosarcoma is an aggressive malignant bone tumor that afflicts greater than 10,000 dogs, but only 400 adolescents yearly. Most dogs and approximately 30% of children eventually succumb to metastatic disease. Using microarray analysis, we identified elevated insulin-like growth factor II mRNA binding protein 1 (IGF2BP1) expression as a biomarker of poor prognosis in canine osteosarcoma. Gene amplification, hypomethylation, increased transcription, and alterations in microRNA (miRNA) regulation directly or through 3'UTR shortening have all been hypothesized by many studies as mechanisms to increase IGF2BP1 expression in cancer. The current research explores these mechanisms in panels of human and canine osteosarcoma cell lines.

Methods: We evaluated the expression and alternative polyadenylation of IGF2BP1 using RT-qPCR and western blot analysis in human and dog osteosarcoma cell lines. We assessed transcriptional activation of IGF2BP1 using luciferase reporters containing promoter sequences from the human and canine IGF2BP1 genes. To detect genomic amplification and methylation, we used qPCR and 5-Azacytidine drug for three days then evaluate the gene expression.

Results: We investigated mechanisms that contribute to increased IGF2BP1 expression in panels of human and canine osteosarcoma cell lines and tumors. Using qPCR analysis, we observed genomic amplification in 35% of canine tumors and cell lines and correlated amplification with IGF2BP1 transcript expression ($p = 0.0006$, *Pearson* $r = 0.88$). We observed

no genomic amplification in human cell lines. Significant loss of 3'UTR regulatory sequences found in 20% of canine cell lines ($p < 0.05$). The promoter analysis showed that most regulatory elements located within ~580bp from the translational start site in both species. Using reporter transcriptional assays, we identified activation of the following factors: MYC, NF-Kappa B, AP-1, and TCF4: β-catenin.

Conclusions: Overall, our data suggest that increased IGF2BP1 expression contributes to the development and progression of human and canine osteosarcoma. Multiple mechanisms can contribute to elevated IGF2BP1 expression, and these results can be used to develop new treatment strategies that target the elevated IGF2BP1 or any of the transcription factors.

2.1 Introduction

Osteosarcoma (OS) is the most common malignant bone tumor in children (those younger than 20 years), and approximately 400 children are diagnosed with osteosarcoma in the U.S. each year [1]. OS is one of the most aggressive cancers because it is estimated up to 80% of the patients will have micrometastases (usually to the lungs) at the time of diagnosis. However, multi-agent dose-intensive therapies have resulted in long-term survival rates as high as 75%. Unfortunately, for those patients with metastasis at diagnosis, their survival rate is only 30% [1]. There remains a need for new models and strategies to identify and treat this and other highly metastatic cancers.

Strikingly, dogs show a similar progression of OS, sharing the same prognostic factors and metastatic sites. Also, both people and dogs are predicted to have micro-metastasis at the time of diagnosis [2]. Sadly, OS is exceptionally resistant to radiation therapy, and the metastases that develop are also generally resistant to chemotherapy [3]. Dogs are great animal models because they share the same environment with people and develop OS spontaneously at a higher (10X) rate [4]. Furthermore, dogs' tumors share many key features with the human OS; most interestingly the development of chemotherapy-resistant metastases [5].

Furthermore, there are growing bodies of evidence to demonstrate a significant parallel at the molecular level between the genomic profiles in dog and human cancers based on cross-species genomic analysis [6, 7]. These results show dog breeds as robust and ideal subjects for genetic mapping of disease alleles. Thus, there is suggestive evidence that canine and human OSs are very analogous at the molecular level.

We first identified IGF2BP1 as a potential biomarker using microarray analysis of canine tumor samples with different disease-free intervals. We analyzed the gene expression profiles using Affymetrix Canine 2.0 microarrays on two groups of canine OS tumors; one group with a

disease-free interval (DFI) of < 100 days following amputation and chemotherapy, against those from canines that had a DFI >300 days. Furthermore, comparing the transcript levels between control (normal dog bone) and the two groups of tumor samples show significant elevation by 132-fold in the tumors from dogs with a DFI >300 days, and 915-fold for those with a DFI of <100 days. Correspondingly, the tumors taken from the patients with the shortest disease-free interval had higher transcript levels (7-fold, p=0.047) of IGF2BP1 than long DFI patients [8]. We found a similar elevation of IGF2BP1 in the highly metastatic human MG63.2 subline compared to normal human osteoblasts and the parental MG63 cell line. MG63.2 cell expression of *IGF2BP1* transcripts was 17.8-fold higher than cultured human osteoblasts.

Insulin-Like Growth Factor II mRNA Binding Protein I (*IGF2BP1*), also named *IMP1,VICKZ, ZBP,* and *CRD-BP* is expressed only during embryonic development (an oncofetal protein), which indicates that *IGF2BP1* is tightly regulated [9, 10]. IGF2BP1 functions by binding to the mRNA to regulate subcellular localization and nuclear export, and monitors mRNA stability and translation. Binding to mRNAs of the cell, IGF2BP1 protein can generate a complex network that interacts with various genes that have a high impact on cancer progression. Increased IGF2BP1 expression correlates with poor outcome in a variety of human cancers including melanoma, breast, ovary, liver, and colorectal cancers [11-15].

This gene is located on chromosome 17q21 near epidermal growth factor receptor 2 (HER2/neu) in humans and is located on chromosome 9 in dogs. Also, the *IGF2BP1* promoter region contains CpG islands that may regulate gene expression via methylation to block transcription in adult tissues [10].

Mice with IGF2BP1 knockout ($IGF2BP^{-/-}$) were deficient in gut development, had increased mortality and had dwarfism symptoms, yet, transgenic mice expressing IGF2BP1 in

mammary tissues developed tumors [9, 10]. The deficiency in knockout mice shows the critical role of the *IGF2BP1* during development while reactivation in adults causes uncontrolled cell growth.

The potential role in cancer development and progression correlates with our preliminary data using human OS cell lines. Using knockdown methods to reduce IGF2BP1 levels in MG63.2 (highly metastatic human osteosarcoma cell lines), we found that IGF2BP1 expression is essential to maintain cellular proliferation, migration, and invasion. Interestingly, the knockdown of IGF2BP1 in human OS cells increases doxorubicin sensitivity and reduces tumor growth in athymic nude mice (Kalet et al, unpublished data). These *in vitro* and *in vivo* data suggest that IGF2BP1 drives growth, metastasis, and chemoresistance in OS.

Herein, we identify the mechanisms that contribute to the regulation of IGF2BP1 expression in human and canine OS. Our objective here is to determine the mechanisms and elements that increase the transcriptional expression of *IGF2BP1*. Based on the gene features and functionality we investigated four mechanisms to modulate IGF2BP1 in OS: amplification, promoter regulatory elements, polyadenylation signals, and demethylation.

2.2. Materials and methods

2.2.1 Cell culture

All the cell lines were validated for species and genetically identified using short-tandem-repeat analysis [16]. The human OS cell lines MG63, SJSA-1, and U-2OS were purchased from ATCC (Manassas, Virginia). MG63.2 human osteosarcoma cells are a metastatic subline of the MG63 cell line developed through Fiddler selection of MG63 cells metastasizing to the lung [17]. These cells were a gift from Dr. Hue Luu (University of Chicago, Illinois). Canine cell lines Abrams, Gracie, D17, OSA8, OS2.4, McKinley, HMPOS, Yamane, Moresco, and Vogel. Abrams cells were derived from metastatic OSA nodules whereas McKinley, Vogel, and Yamane derived from primary tumors (Supplemental Table 1). Canine cell lines were generated by the referenced originators and provided by Dr. Douglas Thamm. All cells were cultured in DMEM High Glucose (Thermo Scientific, Cat#SH30022.01) supplemented with 2X MEM vitamins, 100 mM sodium pyruvate, 1X MEM non-essential amino acids, and 10% fetal bovine serum (FBS, Atlas Biological, Fort Collins, Colorado). For the methylation experiment, we used a demethylating agent (*5′-Azacytidine*) that inhibits DNA methyltransferase at a low dose from (Sigma, Cat#A2385). We dissolved the drug for each experiment in phosphate buffered saline (1X PBS, Corning cellgro, Cat#45000-446) and stored at -80ºC as a 1000x or 500x stock solution. Cells were cultured to ~70% confluence, and separate plates were treated with *5- Azacytidine* at (5 and 10µM) for 72 hours or with only PBS for the control. We changed the media and gently washed with PBS each time we added the drug, after 24 hours. After 72 hours of treatment, the cells were collected for RNA extraction and cDNA was prepared for RT-qPCR analysis.

2.2.2 RNA Isolation, cDNA, DNA, Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR) and Quantitative Polymerase Chain Reaction (q-PCR) Analysis

Quantitative RT-qPCR was performed on total RNA from human and canine OSA cell lines. cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) with 1µg input RNA. Total RNA was extracted using RNeasy kit (Qiagen) with on-column DNase I (Qiagen) based on the manufacturer's protocol (San Diego, California). Primers were designed to be intron-spanning using Primer-BLAST, to amplify all possible isoforms noted in National Center for Biotechnology Information (NCBI). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) expression was performed using the iQ SYBR Green Supermix and Stratagene Mx3000P instrument. The mixture contained cDNA from 25ng of RNA and 100nM of forward primer and 300nM of the reverse in a 25µL volume. To assess genomic amplification, DNA was extracted using the DNeasy kit (Qiagen), then the quantitative polymerase chain reaction (qPCR) expression was performed as described above, but the total reaction contained 50ng of genomic DNA.

In all cases, the amplification efficiencies were equal or greater than 90%, and both amplicon size (70 to 200 bp) and sequence were confirmed. Furthermore, the ΔΔCt method used for data analysis of gene expression levels as described by Livak et al. (2001) [18]. To normalize the data, we used hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) as a housekeeping gene. *HPRT1* was shown to be expressed consistently across all cell lines cultured in-house. We used *HPRT1* to normalize the data in both RT-qPCR and qPCR.

Thermal cycling was performed on the Mx3000p instrument with the following parameters: 95°C for 10m followed by 40 cycles of 95°C for 30s and 60°C for one minute. Data collection was performed at the end of the 60°C step. Dissociation curve ramps were presented at

the end of the cycle to verify that only a single product was generated. Data analysis was performed with the Mx3000p software. Primers (Table 2.1) were designed based upon NCBI RefSeq mRNA sequences (Integrated DNA Technologies, Coralville, IA, USA) and checked for specificity using NCBI blast and UCSC In-Silica PCR [19, 20]. The q-PCR Primers (canine) for IGF2BP1 were designed to amplify exon 14, and for the human primers, we used primers designed against exon 14 that have been published in Bell et al. (2015) [21] (Table 2.1 and 2).

Table 2. 1 Primer sequences and amplicon sizes used in RT-qPCR for selected genes.

Primer	Species	Sequence (5' to 3')	Amplicon Size
HPRT1 Forward	Human & Canine	TGCTCGAGATGTGATGAAGG	192 base pairs (bp)
HPRT1 Reverse	Human & Canine	TCCCCTGTTGACTGGTCATT	
IGF2BP1 Forward	Human	CATCAGCAGCTGGCCGGGTC	240bp
IGF2BP1 Reverse	Human	TCCGTGCCTGGGCCTGGTTA	
IGF2BP1 distal poly A Forward	Human	TGTATCCTTCCTCCCATCCTT	106bp
IGF2BP1 distal poly A Reverse	Human	ACTGGAGTGTTCGTTCATCCA	
IGF2BP1 Forward	Canine	AAGGACAACGGGCTGAAATCG	109bp
IGF2BP1 Reverse	Canine	CAAGCAAGTGGGCAAACCTGA	
IGF2BP1 distal poly A Forward	Canine	TGAGAGAGGCCGCTTCTGAAT	109bp
IGF2BP1 distal poly A Reverse	Canine	TCAGAAGGGAAGGGACGCATC	

Table 2. 2 Primer sequences and amplicon sizes used in q-PCR for selected genes.

Primer	Species	Sequence (5' to 3')	Amplicon Size
HPRT1 Forward	Human & Canine	GACAAGTTTGTTGTAGGATATGCCC	163bp
HPRT1 Reverse	Human & Canine	GTCTGTTCAAATTATGAGGTGCTGG	
IGF2BP1 exon 14 Forward	Human	TCTCTTGCCTGTTCTTGCTG	101bp
IGF2BP1 exon 14 Reverse	Human	CTGGGCCTGGTTACTCTGTC	
IGF2BP1 exon 14 Forward	Canine	TTGCAGAATTTGACAGCGGC	123bp
IGF2BP1 exon 14 Reverse	Canine	CAAGAGTGCCATACGTACCTG	

2.2.3 Western blot analysis

Cells were harvested for western blots from ~70% confluent plates after washing with PBS buffer twice on ice. Then the cells were lysed for 5 minutes on ice with Radioimmunoprecipitation assay buffer (RIPA buffer) that contains: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Disodium EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate with Complete, EDTA-free Protease Inhibitor Cocktail Tablets (Roche). The lysates were collected in 1.5ml tubes and repeatedly passed through a 26-gauge needle or were sonicated three times for 10sec at power 6. Finally, the lysed cells were centrifuged for 15 minutes at 13,000 x g, and the supernatant was transferred to a new tube and stored at -80°C before analysis. Total protein was quantified with the BCA Assay Kit (Pierce, Cat# 23227) and 10 to 20 µg of complete protein was denatured in 2 or 6X Laemli Buffer and run on a (4 -20%) Mini-PROTEAN TGX Stain-Free Gel (BIO-RAD, Cat#456-8095) at 50V for 5 minutes then 250V for 25-30 minutes, prior to transfer to a PVDF membrane using the semi-dry transfer unit Trans-Blot Turbo (BioRad).

The membrane was dried, blocked for 1 hour in 10% nonfat dry milk in PBS at room temperature and washed three times using Tris-buffered saline containing 0.05% Tween-20 (PBS-T) for 5 minutes. The blots were incubated with the anti-IGF2BP1 mouse monoclonal antibody (1:5000, MPL code no. RN001M) in 1% milk/PBS overnight at 4 °C. The membrane was washed three times for five minutes each in PBS-T and incubated with goat-anti-mouse-horseradish peroxidase (HRP) conjugate (1:10000, BioRad Cat# 170-6516) in 5% milk in PBS for 1 hour.

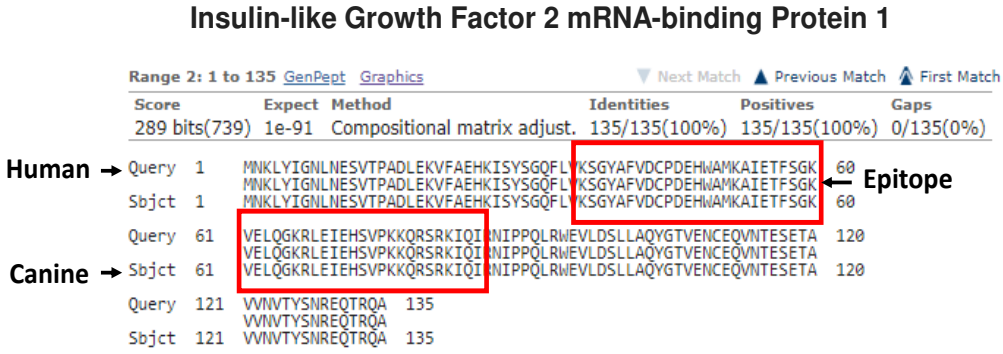

Figure 2. 1Alignment of the IGF2BP1 antibody epitope and canine IGF2BP1.
The region probed by the antibody has 100% identity between the canine and human proteins. The 576 AA canine protein is 99% identical to the human protein. The matching blocking peptide is identical in sequence to the epitope.

2.2.4 Polymerase Chain Reaction (PCR), Digestion, ligation and transformation preparation

PCR was performed using human and canine OSA cell lines genomic DNA as a template. DNA was extracted using DNeasy kit (Qiagen), and primers were designed to amplify specific regions with a restriction site added at the 5' end to digest and ligate the fragment with the plasmids. Also, we created two-point mutations at the E box (CACGTG » CAGCTG) binding site, multiplex PCR, in both promoters 583bp for human and -582bp dog (Table 2.3 and 4). Primers were designed based upon NCBI RefSeq DNA sequences (Integrated DNA Technologies, Coralville, IA, USA) and then checked for specificity using NCBI blast and UCSC In-Silica PCR [22, 23]. Based on the target size we used different DNA polymerases. Following the manufacturer's protocol, we used Phusion DNA polymerase (New England, Bio Lab Cat# M0530) to amplify the 3'UTR. For the promoter amplification, we used GoTaq® Flexi DNA Polymerase (Promega, Cat#M8291).

Then we loaded the samples on an agarose gel (Benchmark scientific, Cat#A1705) and the gel percentage ranged from 0.5-2% based on the fragment sizes. After confirming the fragment size, we gel extracted the fragment using QIAquick Gel Extraction Kit (Qiagen), and we sent the pieces for sequencing at Genewiz.

After confirming the insert sequences, we digested 1µg or more of the insert, vector and positive control with approximately 10 U of restriction enzyme overnight at 37°C in 30 µl reactions. Digested fragments were isolated by gel extraction for ligation.

For ligations we combined a 3:1 molar ratio of insert to vector DNA (100 ng) in a 20µl reaction with 1 U/ µl of T4 DNA ligase (NEB). The mixture was incubated at 16°C overnight. Ligation reactions were transformed in 50µl DH5α competent *E. coli* cells mixed with 2-5 µl of

the ligation reaction and incubated on ice for 10 minutes. After that, we heat- shocked the mixture for 20 seconds at 42°C and incubated again on ice for 2 minutes. We added Super Optimal broth with Catabolite repression (SOC media, 100µl 2M Glucose, 100µl 2M MgCl, and LB media up to 10ml), and incubated with shaking for 1 hour (250 rpm) at 37°C. Transformations were plated on Agar LB media plates (Agar, BD Cat# 214050, LB Broth, Miller, Fisher) with 100 µg/ml Ampicillin for selection (Ampicillin Trihydrate 50mg/ml, Fisher, Cat# BP902-25) and incubated for 18 hours at 37°C.

Using the empty digested vector as a negative control, we selected 5 to 10 colonies from the plate for growth into 3ml of LB media, 6µl of Ampicillin (Trihydrate 50mg/ml) and incubated them with shaking (250 rpm) at 37°C for 18 hours. Plasmid DNA was isolated from the bacterial cultures using alkaline lysis purification and diagnostic digests were conducted as mentioned above. For positive plasmids (correct insert sizes, and sequencing) we prepared DNA from bacteria grown in 50-500ml LB media (QIAprep Spin Midiprep or Megaprep Kit) for transfection into mammalian cell cultures.

Table 2. 3 Primer sequences and amplicon sizes for series of 5' deletion constructs of IGF2BP1 promoters form human promoters.

Primer	Species	Sequence (5' to 3')	Amplicon Size
IGF2BP1 promoter Reverse –68 to ATG (ATG translation start codon 1+)	Human	CTAGGCCAAGAGGCGGAGA	
IGF2BP1 promoter primer Forward -262 to ATG	Human	AGGGAAGAAGCTGCGCCGT	194bp
IGF2BP1 promoter primer Forward -340 to ATG	Human	GATTTTATTTAGAGGCGGCGC	272bp
IGF2BP1 promoter primer Forward -583 to ATG	Human	GCCAGGTTCTTGCAAAGGG Mutated E box primer Forward CTGTCACCA(**GC**)TGGCTTCTCC Mutated E box primer Reverse GGAGAAGCCA(**GC**)TGGTGACAG	515bp Wild E box CA(**CG**)TG
IGF2BP1 promoter primer Forward -664 to ATG	Human	ATGTGAGATCTGGGCTGGAC	569bp
IGF2BP1 (NheI, restriction enzyme) -745 to ATG	Human	GCTAGCA	720bp
IGF2BP1 promoter primer Forward -820 to ATG	Human	TGCGACCCTCTCCTGAAG	752bp
IGF2BP1 promoter primer Forward -2797 to ATG	Human	AAAGTTCCCGGGCAACAGAG	2551bp

Table 2. 4 Primer sequences and amplicon sizes for series of 5' deletion constructs of IGF2BP1 promoters form canine promoters.

Primer	Species	Sequence (5' to 3')	Amplicon Size
IGF2BP1 promoter Reverse –68 to ATG (ATG translation start codon 1+)	Canine	CCCTGAAGTTGTCCGGAGCC	
IGF2BP1 promoter primer Forward -281 to ATG	Canine	GGGTTTCGGACAGAAGGGAA	149bp
IGF2BP1 promoter primer Forward -347 to ATG	Canine	GATTTTATTTAGAGGCGGCGC	214bp
IGF2BP1 promoter primer Forward -489 to ATG	Canine	CTGGAGATCGGTGGGCTG	357bp
IGF2BP1 promoter primer Forward -582 to ATG	Canine	GGGCAATTAGAGCTTCGGGA Mutated E box primer Forward CTTTCACCA(GC)TGGCTTCGCC Mutated E box primer Reverse GCGAAGCCA(GC)TGGTGAAAG	450bp Wild E box CA(CG)TG
IGF2BP1 promoter primer Forward -693 to ATG	Canine	AAGAGGTGGGAGATTCTGGG	561bp
IGF2BP1 (NheI, restriction enzyme) -788 to ATG	Canine	GCTAGCA	620bp

2.2.5 Plasmids and Reagents

We inserted a series of 5' deletion constructs into the promoterless luciferase reporter vector pGL4.17 [*Luc2/Neo*] (Promega, Cat#E6271). TRANSFAC database was used to predict the transcription factor binding sites on both the human and canine *IGF2BP1* promoters (Table 2.2) [24]. Cells were seeded at ~20,000 cells/well on 96-well plates in 100 µl of media, allowed to attach overnight, and transfected with 0.18µg of the target plasmid (Luciferase) and 0.14µg of a Renilla control plasmid (pRL-SV40). We use the Renilla control to normalize for transfection efficiency and Attractene (0.75µl, QIAGEN) as a delivery method for the plasmid mixtures in the cells. The plasmids were mixed with the Attractene, incubated at room temperature for 15 minutes, and mixed with sterilized TE buffer to a final volume of 50µl. The mixture was vortexed and applied to the cells and incubated for 24 hours in the CO_2 incubator.

After the 24 hours, we aspirated the media and added 50µl fresh media to each well. We assayed for firefly and renilla luciferase using the Dual-Glo Luciferase Assay System (Promega, Cat#E2820) following the manufacturer's directions. Cell lysates were transferred to white Opaque Tissue culture plate (Falcon, Ref#353296) and read for the Luciferase and Renilla signals, with 15 minutes incubation for each step on a BioTek Synergy HT plate reader. We normalized the results for each reaction with the Renilla signal that is generated from the same wells and expressed the data as the activity relative to the empty plasmid control.

Following the same protocol, we also used pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Cat#E1330) as a reporter that evaluates miRNA activity in each cell line with *IGF2BP1* 3'UTR fragments inserted and amplified using the genomic DNA from canine cells. In this assay we transfected 0.3µg of each respective pmirGLO construct into canine cell lines using Attractene and followed the same steps as previously described (Table 2.5).

Finally, we used Clontech pathway profiling system (Cat# 631910), with specific cis-acting DNA binding sequence (enhancer elements) that are located upstream the luciferase reporter gene. This method allows us to monitor the binding of transcription factors to enhancer elements and screen for the induction of luciferase reporter. We tested the transfection factors and pathways for: MYC/MAX using E-box promoter, AP-1 using c-jun/c-fos binding sequences, and CRE using ATF2/CREB, E2F using E2F/DP1, NFκB based on the manufacturer's instructions with the appropriate controls. Also, for the β-catenin transcription factor we used M50 and M51 super8 TOPflash reporters that also activate a luciferase reporter.

Table 2. 5 Primer sequences and amplicon sizes for 3'UTR constructs of IGF2BP1 canine promoters.

Primer	Species and restriction enzyme	Sequence (5' to 3')	Amplicon Size
IGF2BP1 Forward 3'UTR 614bp (short, first poly A-signal)	Canine (NheI)	GTGG*CTAGC[CCAGCCCCTTCCTGTCAC]	614bp
IGF2BP1 Reverse 3'UTR 614bp (short, first poly A-signal)	Canine (AccI)	GACGT*CGAC[AACTATAGATGCTGGTGTGTCTTCTC]	
IGF2BP1 Forward 3'UTR 3495bp (long)	Canine (NheI)	GTGG*CTAGC[GGGAACTGACGCATTGCTTT]	3495bp
IGF2BP1 Reverse 3'UTR 3495bp (long)	Canine (XhoI)	GACC*TCGAG[CCCTTCCCAGCACACTTGAT]	
IGF2BP1 Forward 3'UTR 2981bp (long, last poly A-signal)	Canine (NheI)	GTGG*CTAGC[AGAAGAGGTGGTGCAGGATC]	2981bp
IGF2BP1 Reverse 3'UTR 2981bp (long, last poly A-signal)	Canine (XhoI)	GACC*TCGAG[ATTTTCAACCTGGCCGCAAT]	

*Indicate where the restriction enzyme cut.
[] Indicate the primer sequence.
GTG and GAC are three base pairs added to allow the restriction enzymes to cut the site.

-2835
AAAGTTCCCGGGCAACAGAGCCACACGTTCTGGCCAAGATAATTTTTTCCCCCCTGGACTCTAATTTTCATCA
ATTCTCTTTGCTGTGCTGTTTGTATTAGCTTGTTTTTCATTTAAAAAAATGTTTATTGAGTGCCTACTATGTGGC
ATGCCTGTTTCCTACTCATTGGTTTGTTTAAAAATAATGCTTGTGACCAACAGCTAAGTATTAAGAGTCAGATA
AGGGGGGAGGGTCGGAGGTGTGTGTGTGGGGGTGAGACACAGATGTCTCCCACCTTCTTGGACAGAAAATAT
TCTCTTTCTACTCAACAACAAATAGCTCCTAGTACTTTAATTCTTGTTGTTCCATGGGAAAAAAAAATCACAAA
AACAAAAATCCGACAAACACGTGGCTGGGTAGAACAAAACGCTCATTGGGGAGGGTGGGCTCTAAGGTGGT
GGAGGATATAGAGAAACCGAGTTGGAAGCCCTCCCCCGCCCCTAAGTCCCAGCCCCATTTCTTTTCAGCGCGC
CGGGAAAACGGGGAGGGGGACAAAGGTGCTGCGTGCTGTCTTTCAACTCCCGACTTTTTGAATGGCATACAA
TCGTCCGGCCGCAGAGCGGTGAGCCAAAGTCGGAGTCAGCTCAGACTCTGCAAACGAAAAATAATAACAATA
AAAAAAATTCAAGGCATTCATATCGGTGTAGTCAACACGTGTTGCGGAGTCGGCCCGGGCCGCAGGCGACTG
AGACAATGGGGTCGGGGACTGGGGAAGGGGAGTGACCCCCTTCCTCACCTGCCCTCCCAGCACGCGTGCCCC
CGCTAGAATTATCCTCCCTCCCACAGAGTCCGGAGTAACACCCCAGTTTTCGCCCTGTTCAAGGGGATTCCAG
AATTTCTGGGGGTCGACCCCTCCTTTTTCCCCGTTCCCTGGGCGAGGGGGTTCCAGGGTCTCCGGGCCGCGCT
CTCGGCCACCCCTGGCGCCATCCCAGCCTCCGCCGCTAGAAACTCGTTGGCCTCGCTCGCCGCTCCGCCGTGG
AGCCGGGGCTTGCCCGTGGACCGCGCGTCCCGCTCTCCGCCGCGTATCCGGGACTCCGAAACGCGCGGCGAG
CAGCCCCCTCCCCCACCGCCCAGACGGGGTGCGACCGCCCACGTGTCGCCCCTTGCCCAGTCGGGTCCTTCCC
TCGGGCTCCGGGAGCCGGAGGATCCGGAATGAGTTGGTCAAAGCCGAGGGAAGGGACCCAGGCGGATCCGC
TTCCCCCTCCCCAGGAACGGCGGGAGGAGGAGGGGAGTGGCCGCCGCCCCCTCCCCTAAGCTCCCGCCCCTC
GCTTCCCCGTGCGCGGAGCGGAAATTACCCCCTCCCCCCGCGGGTCTGACCTGGGGACGTGGGCGGGGCTTCC
CGCTTAGCCCCCCGCCCTCCGTCCCTCTCCCGCCTTCCCGCCAGGATCGGAATCCTGCACCCCAAACAAAGTT
CCGAGACCCGAACCTAGGGCCTAGAGAGCTGCCAGCAGTGTCCCGGGTGGTGCAGGCTCTGGAAACTCCACC
TGTCTGTCCCCGACTCTGGGAGGGAAAAGGGGACCATTCAAATCTCCACCCGGTCACTCCCTCCCTAAGACCT
CCCCCTTCAGTTCGCTCCGGTTAGCCCTAGCTATCACTCCTCTTCCTTGTCTCCCACCGTTCCTGTCTCCTGGAG
TCATCTCCGGCCAGTTCCGCGTGTTCCCCTCAGGACCCCGAAACGTTATCATTTAACAGCCGGACCCCGGGTG
CGAACCACCCTTTCTCCCGTGGGGATCTCAAAACATTATTTCCTGCGGCTTCCTCGTTCTCCACTGGGATACCC
CGTTCCCAGCTTTCCAGTCCCGCGCTCGCAGGGTCCCCCGGTCCTGCATCCCCGGAGGCGGGGGAGTTTCGTG
GGGCTGGCGTGGGGAAGGCGGAGACCCCCCCCCATTTAAACATCTAGGGAAAAGGTTCCTTTCTTTTTAAATCA
CCAGCTCGTGCGACCCTCTCCTGAAGACCCCAGAGTCTTCGGCTTCTCTTTGTCTGTCTCGGCCGTCCCCCGCC
TGTCAGTC**GCTAGCA**GTTAACCGGGGAGGGGTGAGCAACCCCGCCCCCCAAAACGAATGAAAAATAACC
GTACATTTTACCCTTTAAGACATGTGAGATCTGGGCTGGACCATCCTCCCCCAGTTTGGGCTGGGGTAGGGGG
ATGGGTGGGGGCGGGGCGCTTCCCTAGGGGCCAGGTTCTTGCAAAGGGGGAAGGGGCCTGTAGAGCTTCAGG
GACGCTGCCGCACCGCCCCAGTTTACCCCGGGGGTGAGTTGAGTTCCCCACCCTCAGGCGGGAGATTATCGG
GTTTGGCCGGAGGGCCGAGGGGCCCTGTGGCGTCGGGGGTCTTTCCCTGCAGGGTGGGTGGGGGCTGTCACC
ACGTGGCTTCTCCTTTTTTTTTTTTTTTTTTTTCCGCTCCATTTTTTTTCAAGTCGATTTTATTTAGAGGCGGCG
CCAGGGCGGCCGCGGAGAAACGTGACACACCAGCCCTCTCGGAGGGGTTTCGGACCGAAGGGAAGAAGCTG
CGCCGTGTCGTCCGTCTCCCTGCGCGCCGCGGGCACTTCTCCTGGGCTCTCCCCGAACTCTCCCGCGACCTCTG
CGCGCCCTCAGGCCGCCTTCCCCGCCCTGGGCTCGGGACAACTTCTGGGGTGGGGTGCAAAGAAAGTTTGCG
GCTCCTGCCGCCGGCCTCTCCGCCTCTTGGCCTAGGAGGCTCGCCGCCCGCGCCCGCTCGTTCGGCCTTGCCC
GGGACCGCGTCCTGCCCCGAGACCGCCACCATG(+1)

-1000
GGTCCCGCGCTCGCAGGGTCCTTCGGTGCCGGATTCCCGGAGGCAGGGGAGTCTCCCGGGGCCGGGGCGGGC
GAGGCCGAGACCCCTCACTAAAAAATCTAGGGGAAGAGCGCCTTTCTTTAAAAATCACCGGCTTGCGCGACC
CCCGCCTGGAGACCCCAGAGTCCTCGGCTTCTCTTTGTCTATCTCGGCCGTTCCCCGCCTGTCAGTC**GCTAG**
CAGTTAACCGGGGTAGGGTGGGGAAACTCTCGCCCCCCACCCCCGCCCCGACCCCCACACGAATAAAAAATA
ACCGCACATTTCACCCATAAAGAGGTGGGAGATTCTGGGCCCGACCGCCCTCCCCCAGCCGGGCCCGGGGTA
GGGGGATGGGTGGGGGCGGGGCGCCTCCCTAGGGGCCAGATTCTTGCCAAGGGGGAGGGGGCAATTAGAGC
TTCGGGAACGCAGCCCCCACCGCCCAGTTCCCCGCCCGGGGTGGGGGGGGGTTGAGTGCCCCCTCCCTTCCCA
CCCGGGGCTGGAGATCGGTGGGCTGGGCCCGGCCGGAGGGCCGGTGGGCCCAGAGGCGTCGGGGGTCTTCCC
GAGCCGGGTGGGTGGGGGCTTTCACCACGTGGCTTCGCCCTTTTTTTTTTTTTTTTCCGCTCCATTTTTTTCA
AGTCGATTTTATTTAGAGGCGGCGCCAGGGCGGCCGCGGAGAAACGTGACACACCAGCCCGCTCGGAGGGGT
TTCGGACAGAAGGGAAGGAGCAGCGCCGCGTCGTCCGCCTCCCAGCGCGCCGCGGGCACTTCTCCCGGGCCT
CCCCGAACTCTCCCGCGACCTCTGCGCGCCCTCAGGCCGCCCTCCGCGCCGCGGGCTCCGGACAACTTCAGGG
GTGGGGTGCAAAGAAAGTTTGCGGCTGCCGCCGCCGGCCTCCCGCCTCTCGGCCTAGGAGGCTCGCCGCCCG
CGCCCGCGCCCGCCCGCTCGGCCTTGCCAAGGGACCGCGTCCCGCCCCGACACCGCCACCATG(+1)

Figure 2. 2 Sequence of human and canine IGF2BP1 promoters.
The first sequence showing the human IGF2BP1 promoter starting at -2835 and ended with ATG translation start codon is denoted and designated as +1. The second sequence showing the canine IGF2BP1 promoter starting at -1000 and ends with ATG translation start codon is denoted and designated as +1. The underlined sequences indicate the binding sites of the primers and the bold sequence indicates NheI restriction site.

2.3 Results

2.3.1 Genomic Amplification of IGF2BP1 Occurs in Canine OS but not in Human OS

The amplification of chromosomal region 17q21.32, where *IGF2BP1* is located in the human genome, was reported in several cancer types and hypothesized to contribute to oncogenesis [12, 25, 26]. We chose to quantify the DNA copy number of *IGF2BP1* in OS cell lines from both species. For this investigation, we selected five human OS cell lines: MG63, MG63.2, SJSA1, Saos2, and U2OS and ten canine OS cell lines: Abrams, Gracie, D17, OSA8, OS2.4, McKinley, HMPOS, Yamane, Moresco, and Vogel. Also, we tested genomic DNA from 20 tumors and control normal DNA extracted from dogs with OS.

qPCR was used to evaluate the relative copy numbers of *IGF2BP1* at exon 14 in both species relative to healthy human and canine genomic DNA [26]. We used BT474 cells (human breast cancer) as a positive control since they have previously been shown to have amplification of this region [27]. Consistent with prior findings; we identified a 10-fold increase in *IGF2BP1* genomic DNA in the BT474 cell line relative to normal human genomic DNA. Among the human cell lines, no significant increases in genomic copy numbers were observed (Figure 2.3 A).

Next, we tested the mRNA levels using RT-qPCR to predict any correlation with genomic amplification. We have seen an increased level of expression of *IGF2BP1* in the highly metastatic human OS cell line (MG63.2) relative to normal human osteoblasts and the parental MG63 cell line [28].

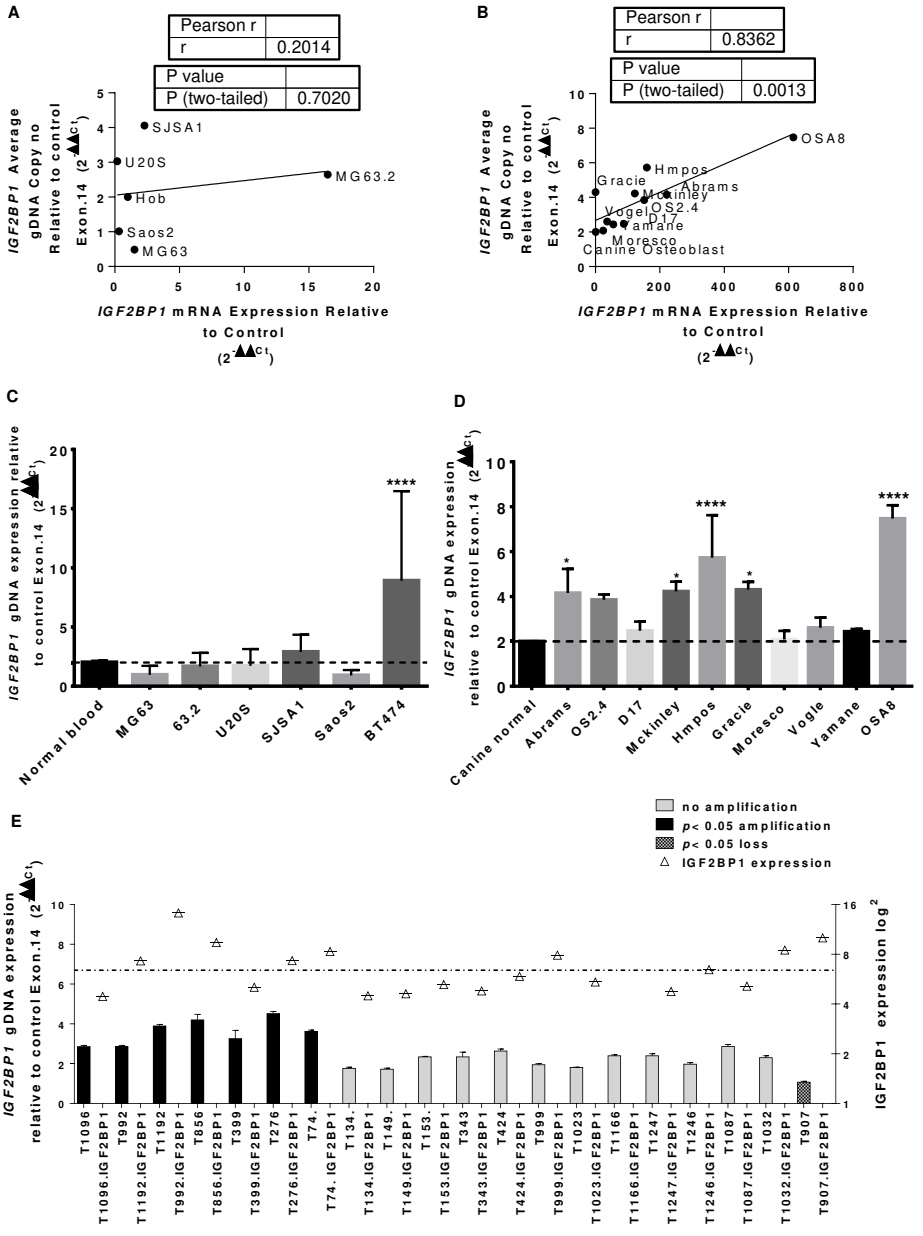

Figure 2. 3 Detecting the correlation between the expression and amplification of IGF2BP1.
Quantitative PCR of genomic DNA with primers directed against IGF2BP1 exon 14 was used to assess gene copy numbers relative to normal genomic DNA. IGF2BP1 expression was quantified by RT-qPCR. A and C) There was no significant amplification or correlation between gene expression (RT-qPCR) and genomic copy number of IGF2BP1 in human OS cell lines. BT474 is a breast cancer cell line used as a positive control. B and D) canine cell line genomic copy number is significantly amplified and correlated with gene expression (RT-qPCR). E) Bars indicate copy number (mean and standard deviation 3 technical replicates) relative to matched normal DNA. 35% of the samples show amplification of genomic copy number for IGF2BP1. Triangles represent Log 2 expression values from Affymetrix Canine 2.0 microarrays. Line represents average gene expression across all 20 samples. C and D represent data from three different experiments and DNA extractions. One-way ANOVA and Dunnett's range test (* = $p < 0.05$).

However, the 17.8-fold increase in IGF2BP1 transcript abundance does not correlate with the slight non-significant increase in DNA copy number (Figure 2.3 B). These results are evidence that genomic amplification is not responsible for increased transcript abundance in these human OS cell lines.

We observed that 5 of the canine OS cell lines (McKinley, HMPOS, Gracie, Abrams, and OSA8) had 2 to 4-fold increases in the copy number of IGF2BP1 genomic DNA relative to control ($p < 0.05$, Pearson $r = 0.89$).

We detected IGF2BP1 genomic amplification in 7 (35%) canine tumors and above average IGF2BP1 expression in 8 (40%) of the 20 canine tumors. Of the 8 tumors with high IGF2BP1 expression, five had IGF2BP1 amplification suggesting that gene amplification may account for elevated expression in 62.5% of these tumors. While 60% of the tumors show no evidence of genomic amplification, two have elevated IGF2BP1 gene expression. One sample with significant genomic deletion also had higher gene expression. Thus, alternate methods of regulation are required for 37.5% of the tumors with elevated IGF2BP1 expression. These results indicate that the canine OS cell lines and tumors used multiple methods to overexpress IGF2BP1 at two different levels, transcription and post-transcription regulation. Moreover, the human cell lines appear to primarily elevate expression at the transcriptional and post-transcription level.

2.3.2 Promoter activity of IGF2BP1 requires different transcription factors in human versus canine OS cell lines

One of the possible mechanisms regulating *IGF2BP1* expression is altered signaling to activate gene transcription. To explore the promoter activity, we identified elements at the

proximal promoter that might drive transcription of IGF2BP1 using promoter-luciferase reporter constructs in panels of human and canine cell lines.

The activity of the luciferase reporter constructs indicated that the human and canine cell lines (SJSA1, U2OS, Abrams, and Gracie) have factors capable of activating their respective *IGF2BP1* promoters. The canine -925 base pair (bp) promoter had higher promoter activity than any of the human promoter constructs in both human and canine cells. The human -820 and -2793bp promoters had similar luciferase activities, indicating that the -820bp proximal promoter contained the essential elements required to drive transcription (Figure 2.4) and that the addition of nearly 2000 bp did not significantly increase activity. To try to define the critical elements located within this proximal promoter, we generated a series of 5' deletion constructs for both the human and canine promoters (Figure 2.5 and Figure 2.6).

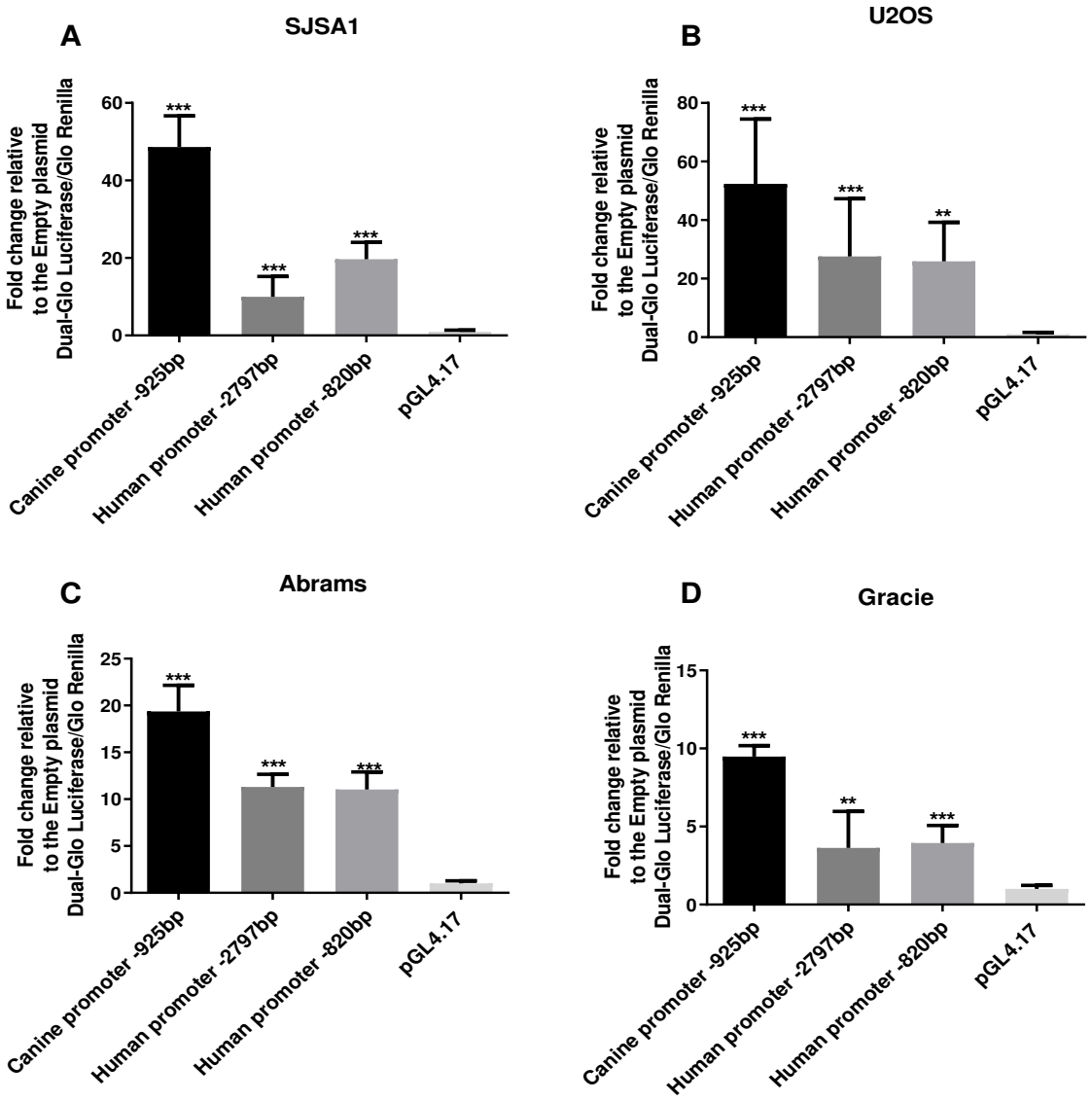

Figure 2. 4 Analysis of human and canine IGF2BP1 promoter constructs in both human and canine OS lines relative to empty plasmid (pGL4.17).

Luciferase reporter constructs were cotransfected in human and canine OS lines with pRL-SV40 using Attractene as described in materials and methods. At 24 hours post-transfection cells were harvested and assayed for luciferase and Renilla luciferase activity. Luciferase activity was normalized to Renilla luciferase activity and expressed as fold increase over the empty pGL4.17 plasmid. All the human (A and B) and canine (C and D) cell lines show significant luciferase activity. The highest was the canine promoter at -925bp and the human -820 and -2793bp promoters had similar luciferase activities, indicating that the -820bp proximal promoter contained the essential elements required to drive transcription. This graph represents data from three different experiments with two plasmid preparations for each per analysis. Bars represent mean and standard deviation. Bars represent mean and standard deviation. One-way ANOVA and Dunnett's range test (* = $p < 0.05$).

We transfected OSA8, D17, and Gracie canine cell lines with 6 sequential deletion constructs of the human *IGF2BP1* promoter (-820, -745, -664, -583, -340, and -262 bp relative to the translational start site) for 24 hours, and the results indicated different luciferase expression between the promoters. The maximum promoter-luciferase activity was generally maintained from the -820bp through the -583bp deletion constructs (Figure 2.5B). Deletion of the region between -820 and -745bp resulted in a significant loss of luciferase activity in the D17 line, but no substantial change was observed in the other two canine cell lines. However, the most significant and consistent loss in signal in the three canine cell lines occurred with deletion of the area between -582bp and -340 bp.

When these same human promoter constructs were transfected into the human SJSA1 and U2OS OS cell lines, we again observed a significant decrease in luciferase activity. In U2OS cells, an initial decrease is observed with the deletion to -745bp, after which there is a considerable drop with deletion of the region between -583bp and -340bp (Figure 2.5C). With SJSA1, no significant loss in activity is observed until deletion to -664bp. Consistent with the results from the canine cell lines, the most consistent decrease in activity occurred with the removal of the section between -583 and -340 bp (Figure 2.5C). A significant reduction in activity was also observed with the deletion from -340 to -262 in the D17 canine cell line (Figure 2.5B).

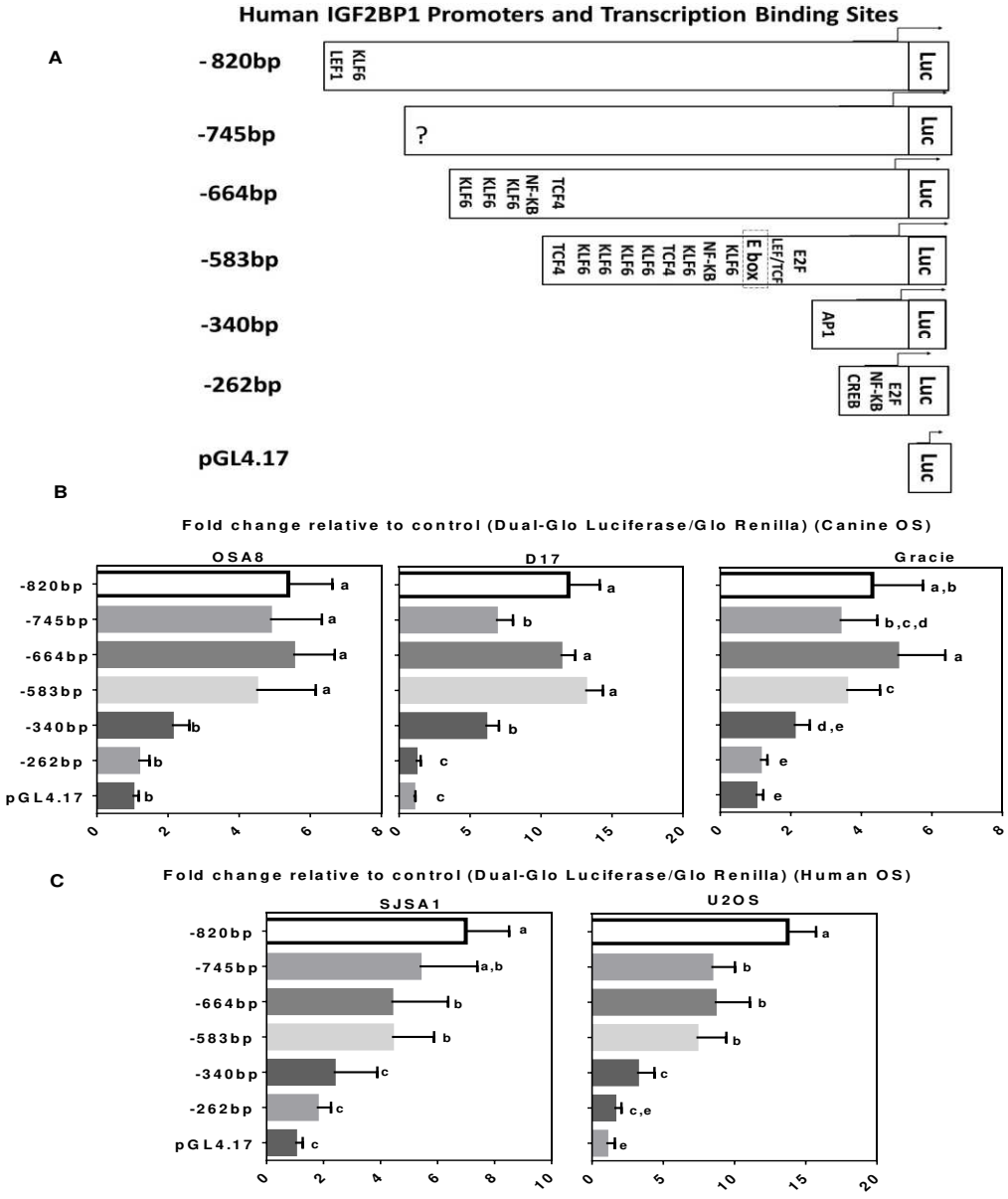

Figure 2. 5 Analysis of human IGF2BP1 promoter constructs in canine and human OS lines relative to empty plasmid control (pGL4.17).

Luciferase reporter constructs were cotransfected in human and canine OS lines with pRL-SV40 using Attractene as described in materials and methods. At 24 hours post-transfection cells were harvested and assayed for luciferase and Renilla luciferase activity. Luciferase activity was normalized to Renilla luciferase activity and expressed as fold increase over the empty pGL4.17 plasmid. A) Pictorial representations of the human IGF2BP1 promoter with transcription factor binding sites. B) Luciferase activity of the human promoter constructs in canine cell lines. C) Luciferase activity of the human promoter constructs in human cell lines. Bars are mean + standard deviation of 2 plasmid preparations per construct transfected in 3 separate experiments. Bars bearing different letters are significantly different analyzed by one-way ANOVA and Tukey's multiple comparison's test ($p < 0.05$).

When the canine and human cell lines were transfected with a series of deletion constructs of the canine proximal promoter, significant decreases in activity were observed with deletion of the regions between -582 and -347bp in 4 of the five cell lines (Figure 2.6B and C). We want to note that the OSA8 cell had the highest expression of IGF2B1 among the canine cell lines and exhibited the most significant decrease with deletion of the region from -582 to -489 in the canine promoter (Figure 2.6B). In the U2OS cell line decreases were only observed with deletions from -788bp to -693bp and -347 to -281bp (Figure 2.6C). Using the canine promoter constructs, the SJSA1 human cell line shows a significant drop of luciferase activity with deletion to -788bp, to -693bp, between -582 and -489 bp, and again with deletion to -347bp -925bp (Figure 2.6C). U2OS had two significant reductions in the luciferase signal with deletion from -788 and -693bp and from -347bp to -281bp. The canine promoters had higher fold activation in both the human and canine cell lines.

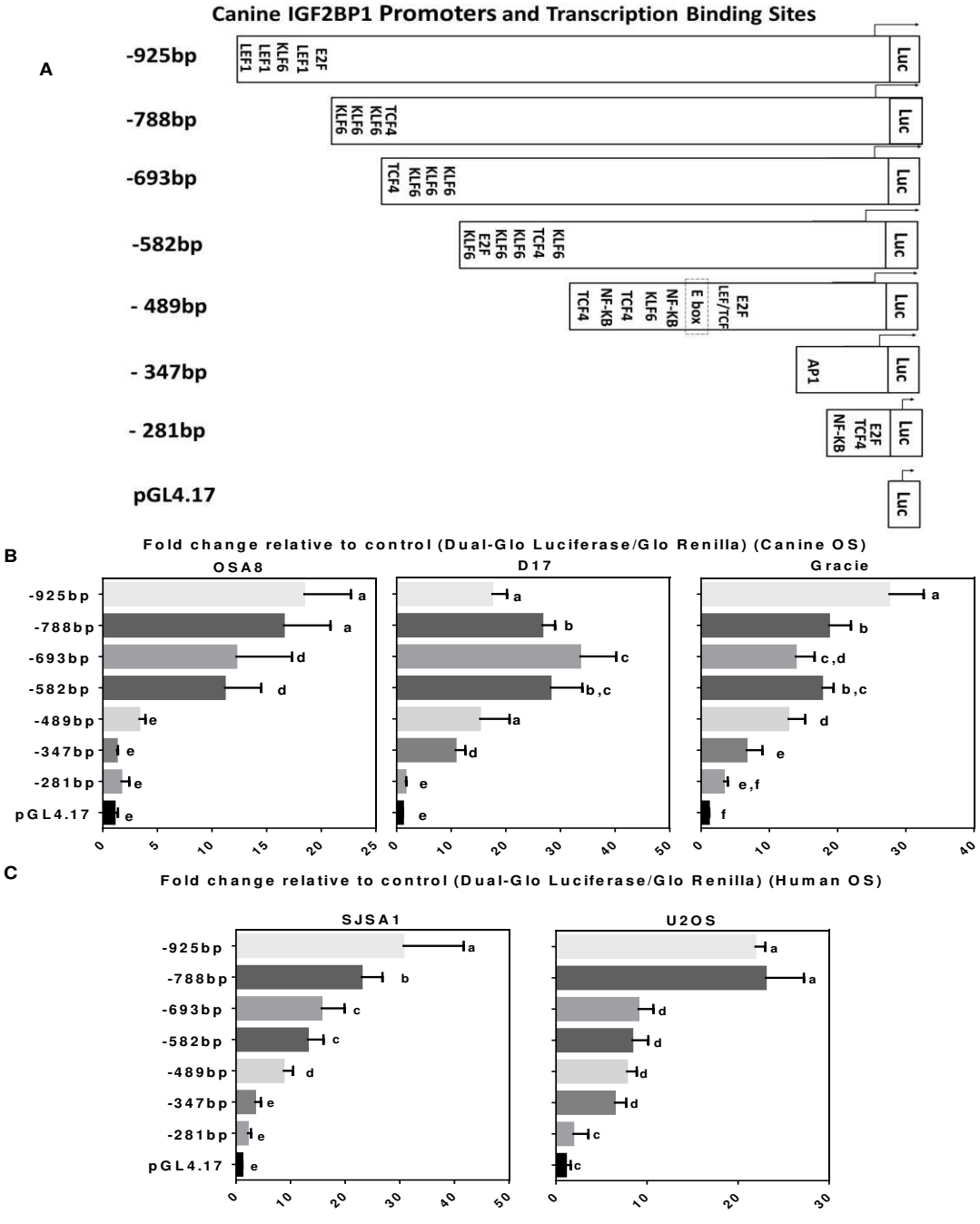

Figure 2. 6 Analysis of canine IGF2BP1 promoter constructs in canine and human OS lines relative to empty plasmid control (pGL4.17).

A) Pictorial representations of the canine IGF2BP1 promoter with transcription factor binding sites. B) Luciferase activity of the canine promoter constructs in canine cell lines. C) Luciferase activity of the canine promoter constructs in human cell lines. Bars are mean + standard deviation of 2 plasmid preparations per construct transfected in 3 separate experiments. Bars bearing different letters are significantly different analyzed by one-way ANOVA and Tukey's multiple comparison's test ($p < 0.05$).

We used the TRANSFAC and UCSC database to predict the binding sites for transcription factors along with histone modifications and CpG islands across these proximal promoters for human and dog IGF2BP1 [24, 29]. After further investigation of the region, we identified binding sites for oncogenic and tumor suppressor transcription factors that had significant correlations in gene expression with IGF2BP1 using Affymetrix Canine 2.0 microarray results for the canine OS cell lines.

We identified a significant positive correlation between the expression of MYC (myelocytomatosis viral oncogene homolog) and NF-Kappa B (nuclear transcriptional factor-kappa B) with IGF2BP1. Correspondingly, KLF6 (Krueppel-like factor 6), a tumor suppressor specific to osteogenesis, showed a negative correlation with IGF2BP1 transcript expression in the canine OS cell lines (Figure 2.5 and 6). Two studies have shown a positive feedback loop between MYC and β-catenin with IGF2BP1 levels [30, 31]. In addition, there appear to be binding sites for KLF6 and NF-Kappa B in the IGF2BP1 proximal promoter (Figure 2.5 and 2.6).

We also assessed the activity of TFs that we identified in the proximal promoter of IGF2BP1 using pathway profiling luciferase reporter constructs containing tandem repeats of binding sites for MYC, NF-Kappa B, LEF/TCF, CREB (cAMP response element-binding protein), E2F, and AP-1 (Figure 2.7). β-catenin binds to the promoter through interactions with the LEF/TCF family.

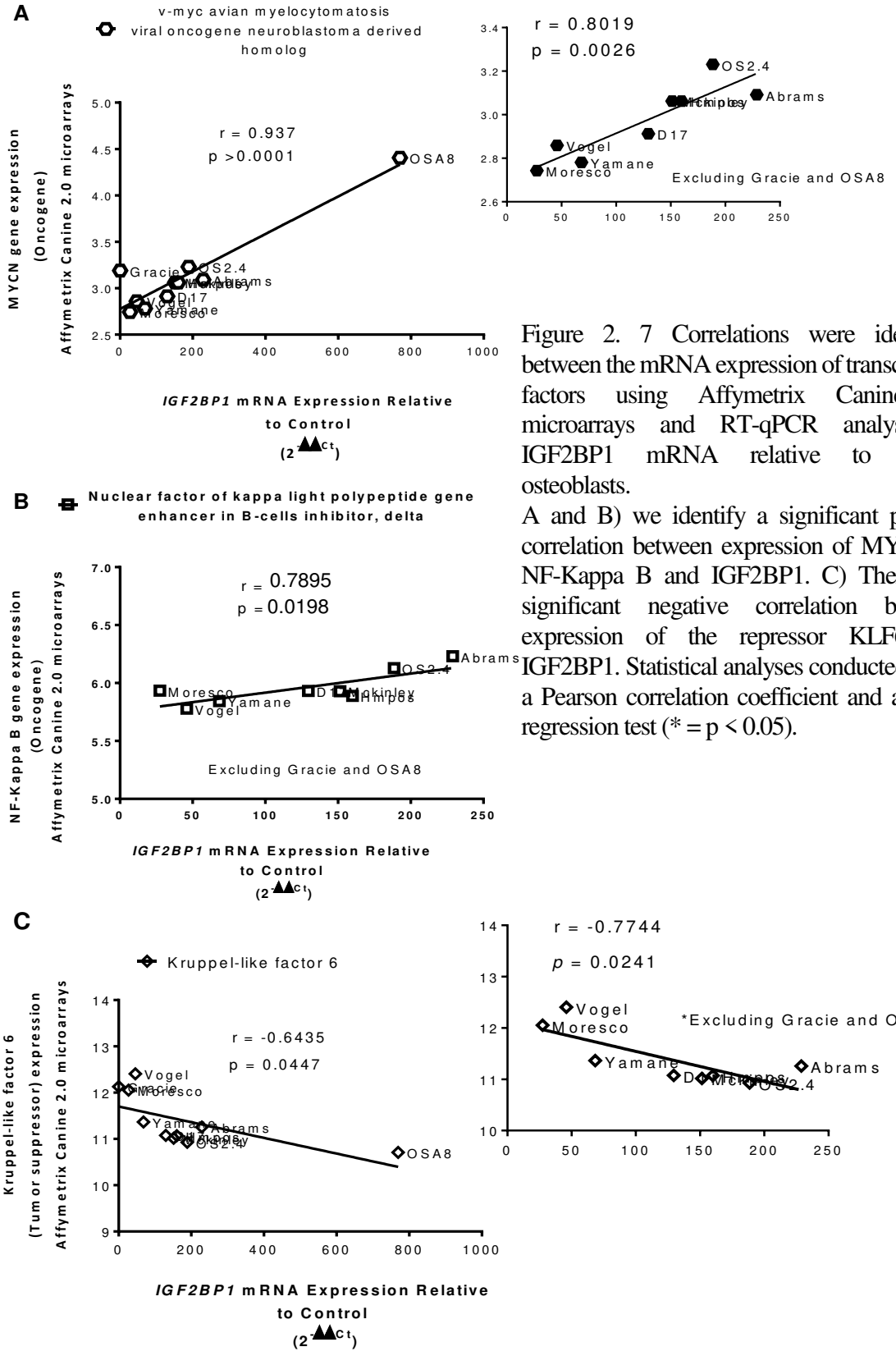

Figure 2. 7 Correlations were identified between the mRNA expression of transcription factors using Affymetrix Canine 2.0 microarrays and RT-qPCR analysis of IGF2BP1 mRNA relative to canine osteoblasts.

A and B) we identify a significant positive correlation between expression of MYCN or NF-Kappa B and IGF2BP1. C) There is a significant negative correlation between expression of the repressor KLF6 and IGF2BP1. Statistical analyses conducted using a Pearson correlation coefficient and a linear regression test (* = $p < 0.05$).

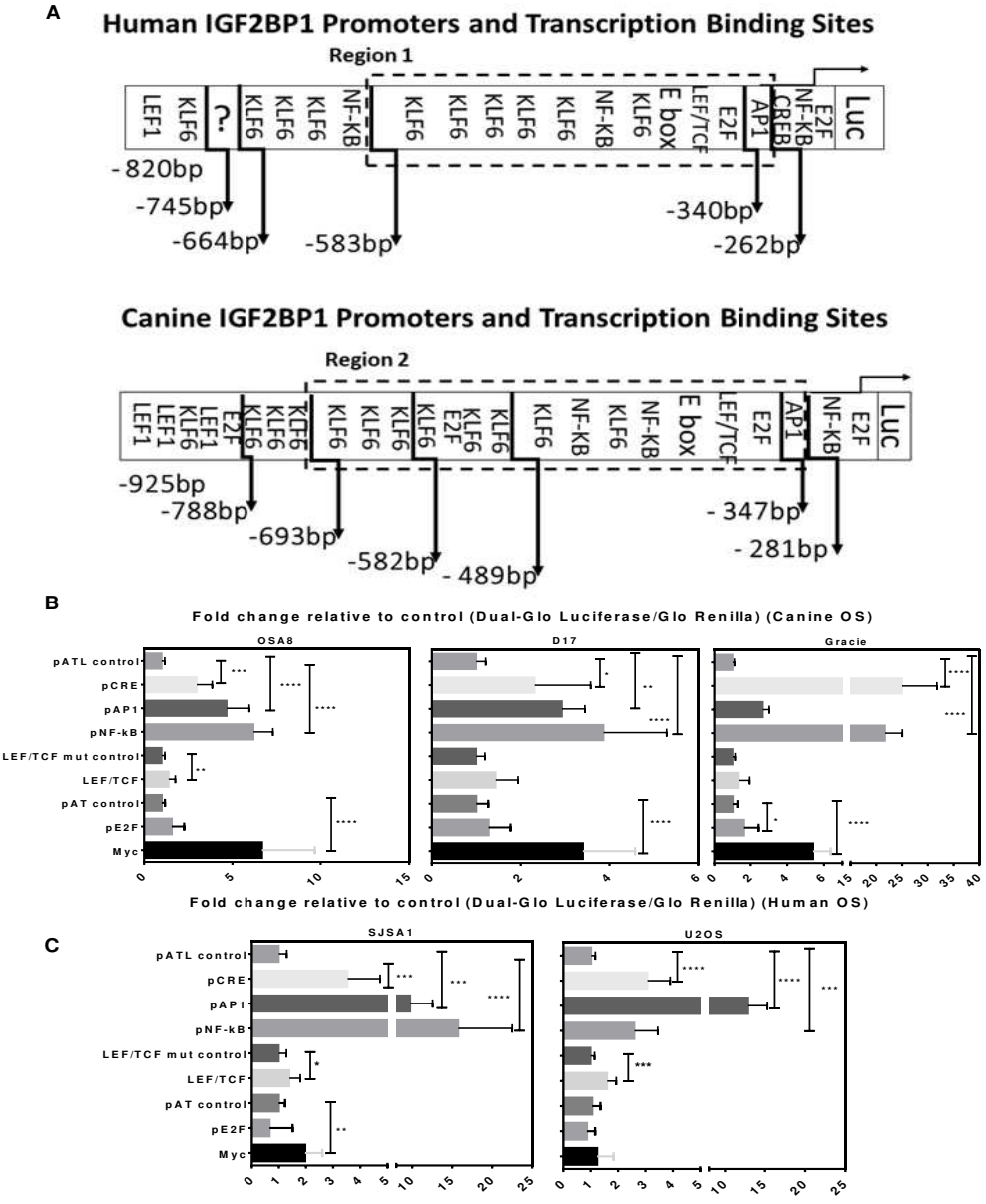

Figure 2. 8 Transcription Factor activity for relevant signaling pathways in the canine and human cell lines. Clontech pathway signaling luciferase constructs were cotransfected in human and canine OS lines with pRL-SV40 using Attractene as described in materials and methods. At 24 hours post-transfection cells were harvested and assayed for luciferase and Renilla luciferase activity. Luciferase activity was normalized to Renilla luciferase activity and expressed as fold increase over empty or mutated plasmids. A) IGF2BP1 pictorial representations for the human and canine promoter sequences with their binding sites. Regions 1 and 2 indicate the binding sites for TFs that resulted in significant loss of luciferase activity in the deletion constructs. B) Pathway profiling in the canine cell lines. C) Pathway profiling in human OS cell lines. Bars represent mean and standard deviation of transfection data from triplicate samples in three different experiments with two plasmids for each experiment. * = $p < 0.05$ using One-way ANOVA and Dunnett's range test.

By comparing the results of the deletion constructs and the transcription factor specific reporters, we can start to identify the primary drivers of IGF2BP1 transcription in these canine and human OS cell lines.

Looking back to the promoter deletion constructs, we identified a shared region between all the OS cell lines where the luciferase activity decreased gradually or dramatically before reaching the empty plasmid signal. These regions were region 1 in the human promoter constructs (-583bp to -340bp) and region 2 in the canine promoters (-693bp to -347bp) (Figure 2.8 A-R1 and R2).

Regions 1 and 2 share bindings sites for the following TFs; TCF4, NF-Kappa B, AP-1, CREB, E2F, E box, and LEF/TCF. With the exclusion of the U2OS cell line, each of the human and canine cell lines tested exhibited significant activation of the NF-KB reporter. The U2OS cell line exhibited significant activation of the AP1 reporter, as did the other cell lines except for the Gracie cell line (Figure 2.6 B and C). The Top-flash TCF4: β-catenin reporter was only active in the OSA8 cell line among the canine cell lines but was active in each of the human cell lines. Finally, the MYC reporter was strongly activated in all the canine cell lines, but only showed an approximate 2-fold increase in activity in the SJSA1 human cell line and was unchanged in the other human cell line. The loss of activity across the region from -583 bp to -340 bp in the human promoter and from -489 to -347 in the canine promoter suggest that the E-box and the NF-KB binding site are critical elements in this region for all the cell lines except the U2OS cell line. The MYC:MAX and TCF4: β-catenin transcription factor partners can bind to the E box, which could explain the significant impact of deletion of this region on the promoter activity. MYC and β-catenin are also bound and stabilized by IGF2BP1 to generate a feedback loop, as other studies

have shown. For the U2OS cell line, the primary drivers appear to be TCF4 binding sites across the region from -788 to -693 and the AP1 site located between -347bp to -281 bp.

To further investigate the role of the E box, which is a common TF binding site in both the human and canine promoters, we generated a mutated E box binding site (CACGTG » CAGCTG) in the human -583bp and canine -582bp promoters and compared their activity to the wild type promoter and a construct deleting the entire region. The E box is a DNA motif with the consensus sequence CANNTG (where N can be any nucleotide). An extensive list of TFs can bind to this DNA motif in its roles as a core promoter, proximal core promoter, and an enhancer [32]. The mutation we generated in the E box should destroy the MYC:MAX consensus binding site CACGTG » CAGCTG, but not the TCF4: β-catenin site (CANNTG » CAGCTG) (Figure 2.9 and 10).

Strikingly, SJSA1 and U2OS human OS transfected with human and dog wild type and mutant E box (-583/582bp) constructs showed significant losses in luciferase activity with mutation of the E-box, such that luciferase activity for this construct was not statistically different ($p > 0.05$) from a construct with complete deletion of the region (-347bp human and -340 canine promoters) (Figure 2.9). These results can also be seen with the canine cell lines (OSA8 and D17) transfected with the same mutant and wildtype constructs (Figure 2.10).

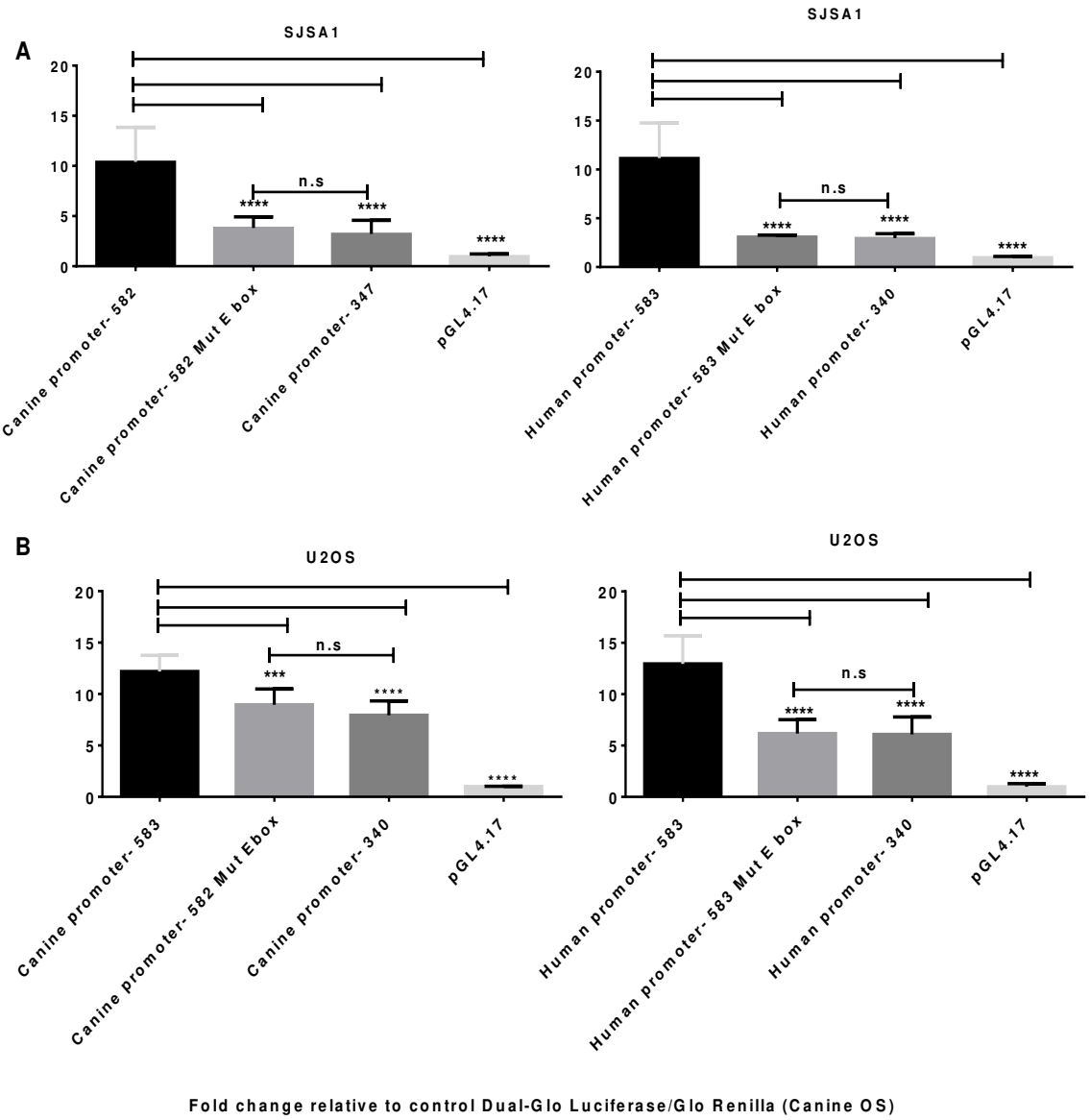

Figure 2. 9 Mutation of the E box in human -583bp and canine -582bp promoters (CACGTG » CAGCTG) significantly decreases activity relative to wildtype promoter in human OS cell lines.
Wildtype and mutant luciferase reporter constructs were cotransfected in human and canine OS lines with pRL-SV40 using Attractene as described in materials and methods. At 24 hours post-transfection cells were harvested and assayed for luciferase and Renilla luciferase activity. Luciferase activity was normalized to Renilla luciferase activity and expressed as fold increase over the empty pGL4.17 plasmid. A) SJSA1 human OS. B) U2OS human OS. Bars represent mean and standard deviation of transfection data from triplicate samples in three different experiments with two plasmids for each experiment. * = $p < 0.05$ using One-way ANOVA and Tukey's multiple comparisons.

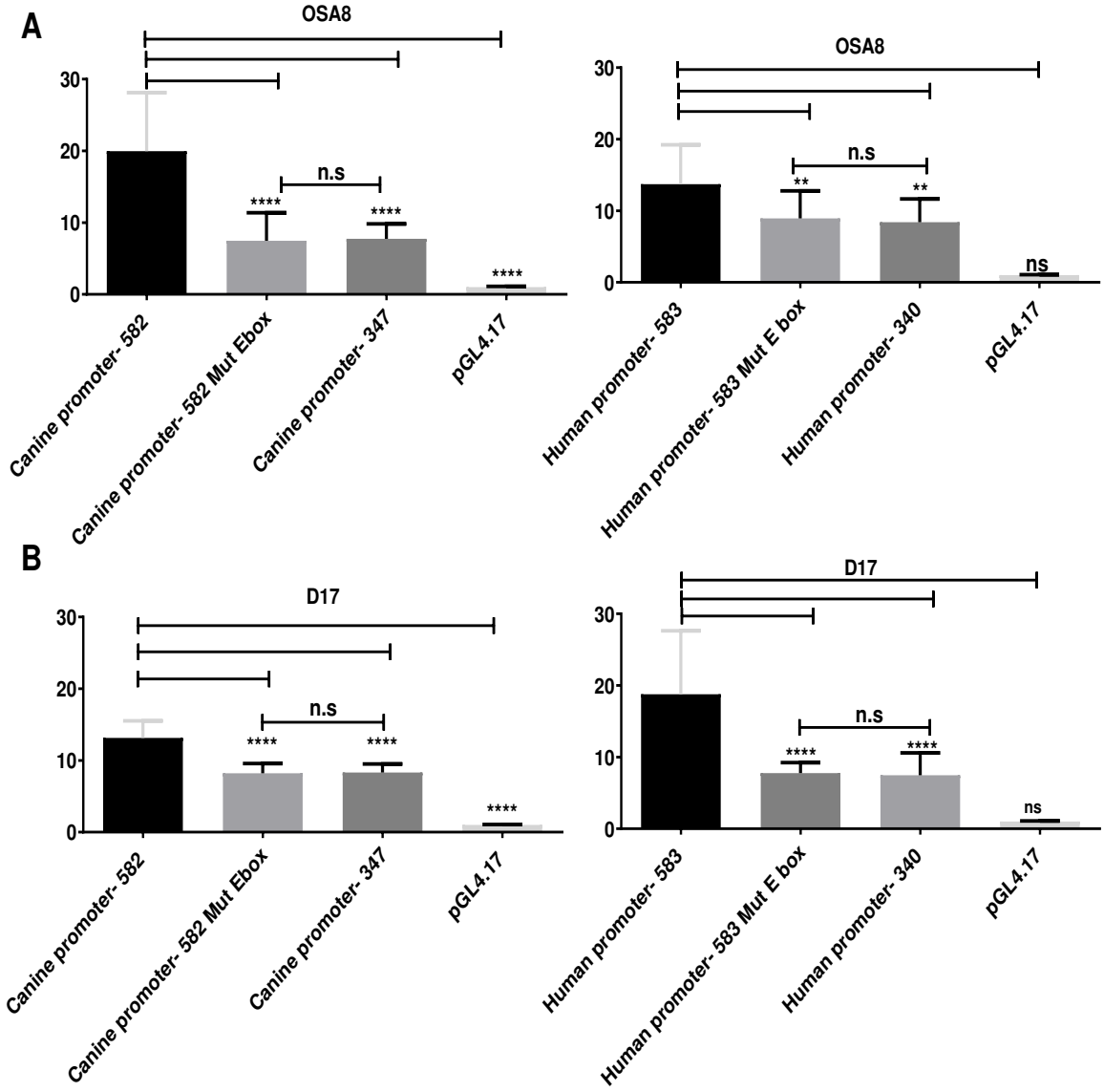

Figure 2. 10 Mutation of the E box in human -583bp and dog -582bp promoters (CACGTG » CAGCTG) significantly decreases activity relative to wildtype promoter in canine OS cell lines.
Wildtype and mutant luciferase reporter constructs were cotransfected in human and canine OS lines with pRL-SV40 using Attractene as described in materials and methods. At 24 hours post-transfection cells were harvested and assayed for luciferase and Renilla luciferase activity. Luciferase activity was normalized to Renilla luciferase activity and expressed as fold increase over the empty pGL4.17 plasmid. A) OSA8 canine OS B) D17 canine OS. Bars represent mean and standard deviation of transfection data from triplicate samples in three different experiments with two plasmids for each experiment. * = $p < 0.05$ using One-way ANOVA and Tukey's multiple comparison.

In conclusion, the *IGF2BP1* promoter appears to be activated through the input of multiple proto-oncogenes (MYC, TCF:β-catenin, AP-1 or NF-Kappa B), as well as repressors such as KLF6. Mutation of the MYC binding site in the E-box resulted in a consistent loss of activity in all cell lines tested.

2.3.3 Significant loss of the distal 3'UTR in canine, but not human, OS cell lines

Another mechanism of gene regulation is microRNA (miRNA) regulation of transcript stability and translation. MicroRNAs are small noncoding oligonucleotides (20 to 25 nt) that play a critical role in regulating gene expression. By targeting the mRNA of many different genes via complementarity between a miRNA and its targets, miRNAs can induce mRNA decay or inhibit translation [33]. The IGF2BP1 transcript has a large 3' untranslated region (3'UTR) of approximately 6.7 kb with three polyadenylation signals and multiple microRNA binding sites that may regulate gene expression [9]. Expression of the let-7 family of miRNAs which are tumor suppressor miRNAs correlates negatively with IGF2BP1 expression. Previous data from our lab compared loss of the 3'UTR with the IGF2BP1 (CDS) in 20 dog tumors. Dogs with the highest IGF2BP1 expression and shortest disease-free interval had the most significant 3'UTR loss [8].

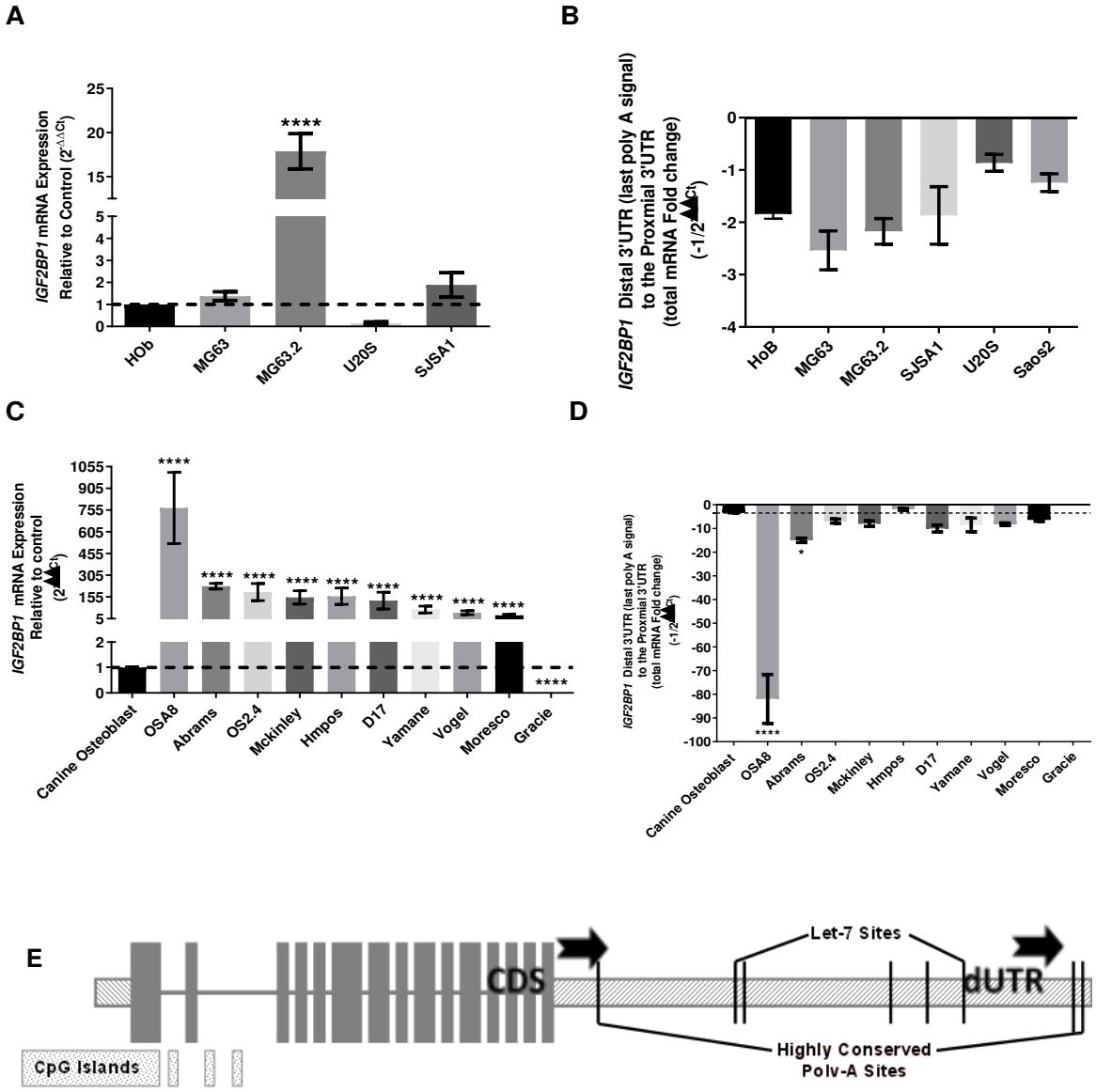

Figure 2. 11 Significant loss of the distal 3'UTR in canine, but not human, OS cell lines. A) RT-qPCR analysis as described in materials and methods reveals elevated mRNA expression of IGF2BP1 in the Mg63.2 OS cell line relative to human osteoblasts (HOB). B) RT-qPCR analysis reveals no significant differences in the ratio of IGF2BP1 mRNAs containing the distal 3'UTR (dUTR) relative to the coding sequence (CDS) in human OS cell lines compared to human osteoblasts (HOB). C)RT-qPCR analysis reveals elevated mRNA expression of IGF2BP1 in canine OS cell lines relative to canine osteoblasts. D) The ratio of distal 3'UTR transcripts relative to CDS transcripts is significantly lower in two of the canine OS cell lines (OSA8 and Abrams). E) Pictoral representation of the IGF2BP1 gene showing the relative sites of amplicons used to evaluate dUTR and CDS expression. Bars represent mean and standard deviation. One-way ANOVA and Dunnett's range test (* = $p < 0.05$).

To determine if this same phenomenon was present in our human and canine cell lines, we used RT-qPCR and selected two sets of primers to amplify both the *IGF2BP1* coding sequences and the distal 3'UTR near the site of the last two poly A signals, in both species. We assessed the loss of the distal 3'UTR by calculating the ratio of the distal 3'UTR compared to the coding sequences (Figure 2.9). While normal osteoblasts had fewer transcripts containing the distal 3'UTR relative to the CDS, the number of long transcripts was far lower in several cell lines. The results show a significant loss of the distal 3'UTR in the OSA8 and Abrams cell lines with an approximately 80-fold difference in transcripts expressing the 3'UTR for the OSA8 line and an 18-fold reduction in long transcripts in the Abrams cell line. These two cell lines also represented the highest levels of IGF2BP1 transcripts and had observable protein expression. However, there was no evidence of the loss of the 3'UTR using the human OS cell lines compared to normal human osteoblasts (Figure 2.9).

Next, to test the IGF2BP1 3'UTR for regulatory activity, we measured the Luciferase activity of pmir-Glo reporter constructs containing three different fragments isolated from the IGF2BP1 3'UTR in the canine OS cell lines (as shown in Figure 2.12.A). As predicted, constructs containing fragments of the distal 3'UTR, 3495 and 2981, each had lower luciferase activity than the fragment containing the proximal 3'UTR (614 bp) or the reporter with no 3'UTR, which had the highest activity (Figure 2.12, panels B-F). However, for those cell lines with the highest IGF2BP1 expression (Abrams and OSA8), even the proximal 3'UTR (614 bp) had less negative regulatory activity (Figure 2.12 B and C).

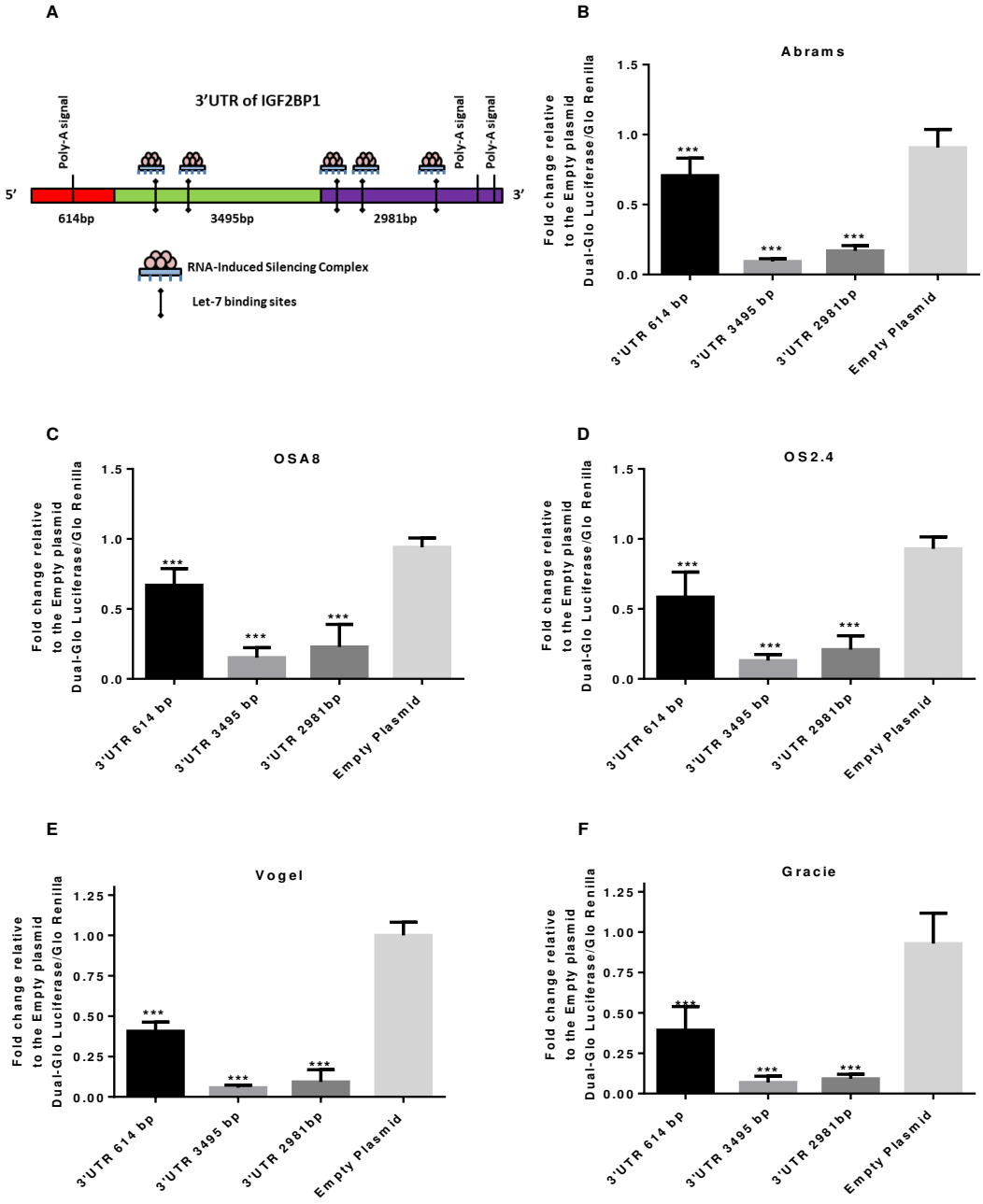

Figure 2. 12 Luciferase expression relative to empty plasmid (pmir-Glo) with three different fragments of the 3'UTR in canine cell lines. A Luciferase reporter constructs were transfected in human and canine OS lines using Attractene as described in materials and methods. At 24 hours post-transfection cells were harvested and assayed for luciferase and Renilla luciferase activity. Luciferase activity was normalized to Renilla luciferase activity and expressed as fold increase over the empty pmiRGlo plasmid.) Pictorial representation of IGF2BP1 3'UTR fragment sizes, location, and Let-7 miRNA binding sites. The 614bp fragment (short mRNA) had higher Luciferase activity than the two large fragments 3495bp and 2981bp from the distal 3'UTR (long) (* = $p < 0.05$). Bars represent mean and standard deviation. One way ANOVA and Tukey's range test. This graph represents data from three different experiments, with two plasmids per experiment.

To address the possibility that the larger size of the distal 3'UTR inserts is causing the lower luciferase activity, we generated smaller (approximately 1000bp) constructs across the distal 3'UTR and evaluated their luciferase activity in the OSA8, Abrams, and Gracie cell lines (Figure 2.11 A).

Even though we generated smaller constructs from the large 3'UTR, the luciferase activity was significantly lower in these constructs than the version generated using the proximal 3'UTR (614 bp). We found a significant negative correlation between the luciferase signal and the number of miRNA binding sites in each fragment of the 3'UTR in the OSA8 cell line, but not in the Gracie cell line ($p = 0.016$, Pearson's correlation, $r = -0.076$). The fragment containing the let-7, mir-196, and mir-23 binding sites had the lowest luciferase activity.

We also investigated the miRNA expression within the OS canine cell lines and found in general no loss of let-7 expresstion family these cell lines (Figure 2.13). The luciferase assay results and the let-7 levels indicated that post-transcriptional regulation by miRNAs that target the 3'UTR may limit IGF2BP1 expression and that some cell lines such as OSA8 escape from the miRNA regulation through 3'UTR truncation (Figure 2.14).

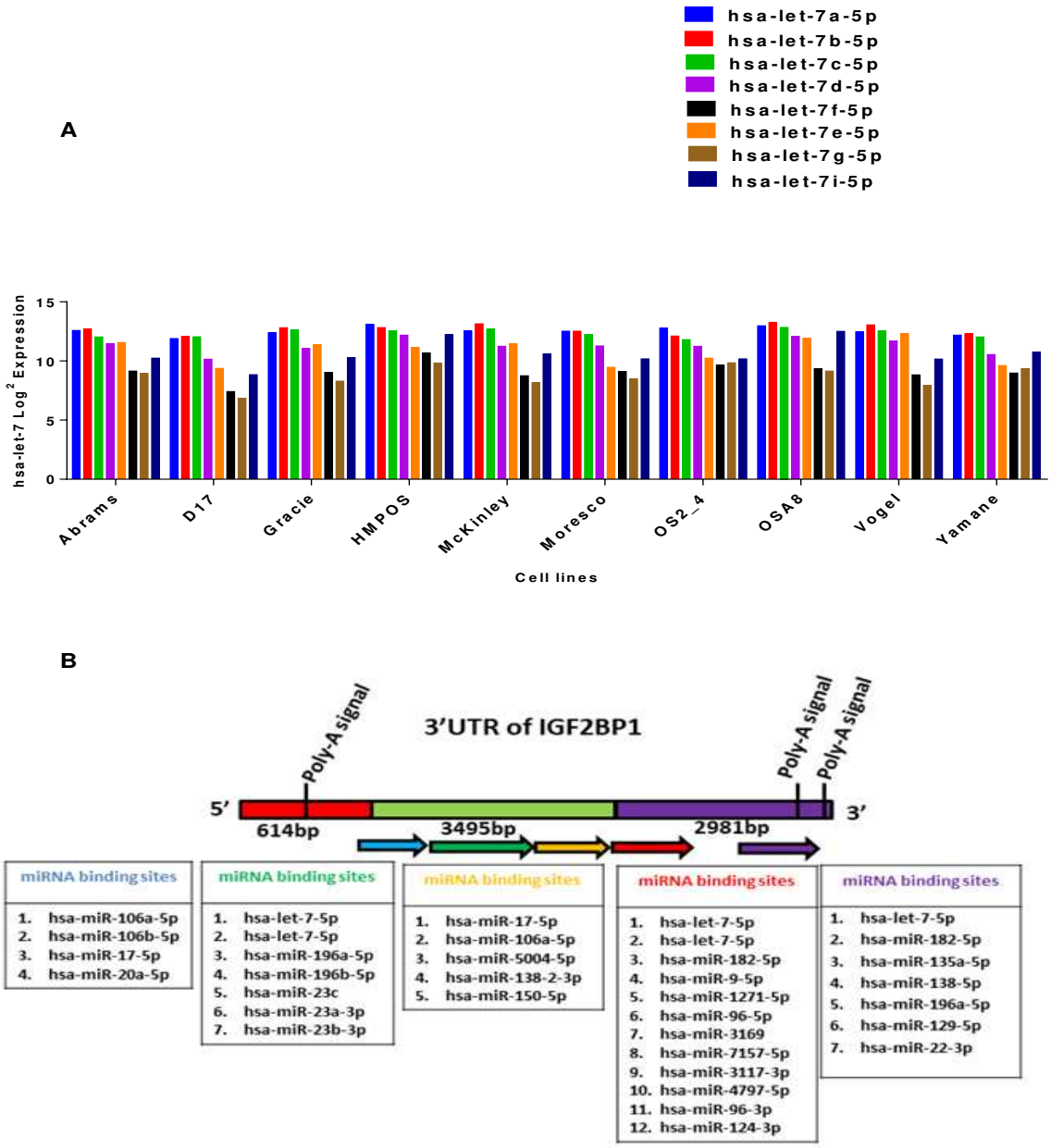

Figure 2. 13 Let 7 family (tumor suppresser) levels in the canine OS cell lines and miRNA binding sites at 3'UTR of IGF2BP1. A) In general, no loss for let-7 family expression in all the canine OS cell lines using Affymetrix Canine 2.0 microarrays (canine OS). B) IGF2BP1 3'UTR pictorial representation with fragment sizes, location, and miRNA binding sites.

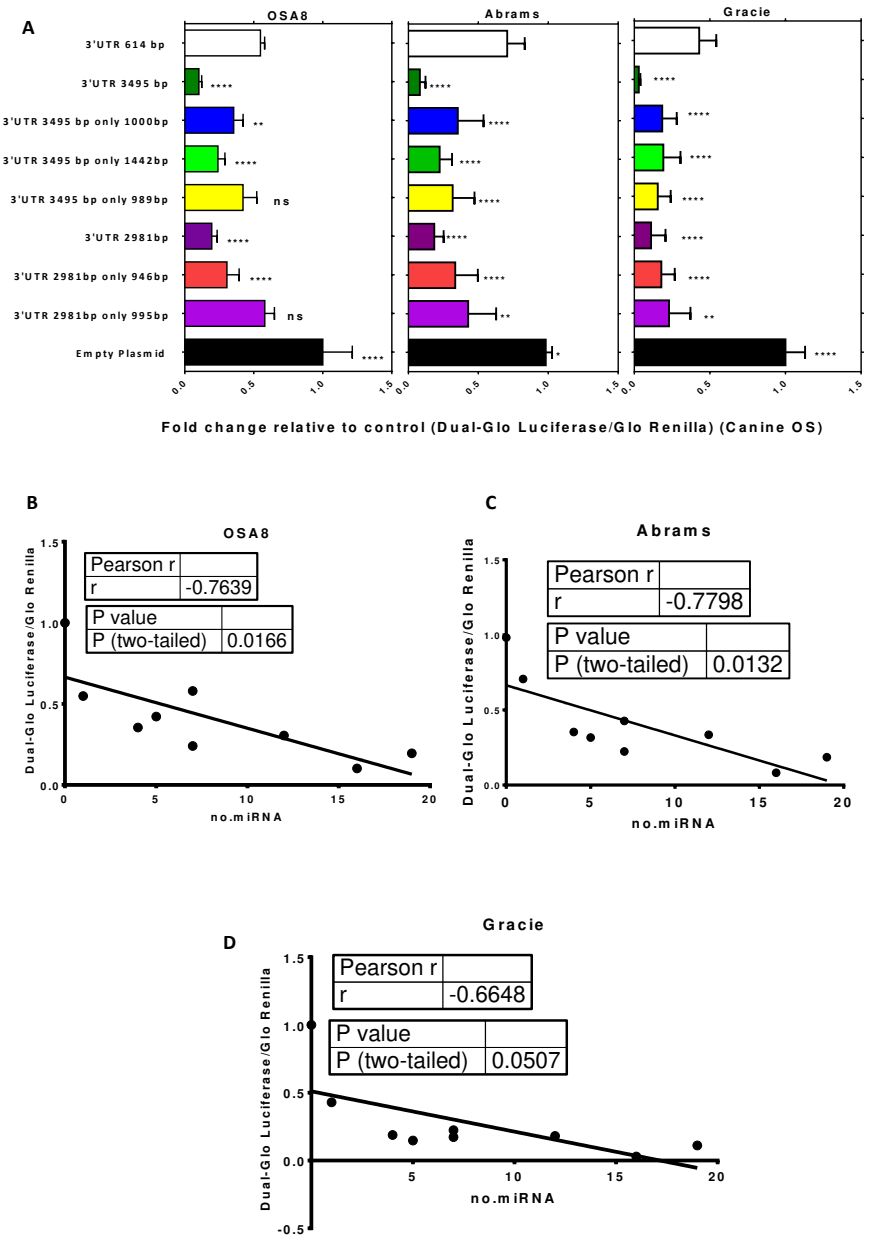

Figure 2. 14 Luciferase expression relative to empty plasmid (pmir-Glo) correlates significantly with the numbers of miRNA binding sites. Luciferase reporter constructs were transfected in canine OS lines using Attractene as described in materials and methods. At 24 hours post-transfection cells were harvested and assayed for luciferase and Renilla luciferase activity. Luciferase activity was normalized to Renilla luciferase activity and expressed as fold increase over the empty pmir-Glo plasmid. A) The 614bp fragment (short mRNA) had higher luciferase activity than any of smaller fragments of the large 3'UTR (3495bp and 2981bp). The bar colors represent the fragment from Figure 2.13 B. B) A significant correlation between let-7 and IGF2BP1 gene expression. C) significant and negative correlation between luciferase expression and miRNA binding sites in OSA8 and Abrams, but not D) Gracie cell line. Bars represent mean and standard deviation. Using one-way ANOVA and Dunnett's range test (* = $p < 0.05$). Using Pearson correlation coefficient and Linear regression test (* = $p < 0.05$). This graph represents data from three different experiments with two plasmids for each per experiment.

2.3.4 Demethylation of the IGF2BP1 promoter induces endogenous IGF2BP1 in a canine OS cell line

Several studies have demonstrated the effect of epigenetic regulation on IGF2BP1 expression [34-36]. As mentioned before, the promoter of IGF2BP1 contains CpG islands that may play a significant role in regulating the gene expression of this oncofetal protein. This idea is further supported by the observation that despite a complete lack of IGF2BP1 expression, the Gracie cell line was able to activate an IGF2BP1 promoter luciferase reporter construct, suggesting that epigenetic regulation through CpG island methylation could be maintained in this cell line. Human and canine cell lines with different levels of *IGF2BP1* expression were treated with the demethylating agent *5′-Azacytidine (5 and 10μM)* for three days. *Remarkably, 5′-Azacytidine* increased the expression of IGF2BP1 transcripts as measured using RT-qPCR in the Gracie canine cell line, indicating that in this cell line, which fails to express IGF2BP1, promoter methylation is a critical factor (Figure 2.14). Conversely, the cell lines that usually express IGF2BP1 did not show any response to *5′-Azacytidine, indicating that* CpG island methylation had already been lost in these cell lines to allow for the expression of this oncofetal gene. Among the human cell lines, U2OS also failed to express IGF2BP1, but had a normal copy number and exhibited activation of human and canine IGF2BP1 reporter constructs. However, neither U2OS nor any of the other human cell lines responded to 5′-Azacytidine treatment with increased IGF2BP1 expression (Figure 2.14).

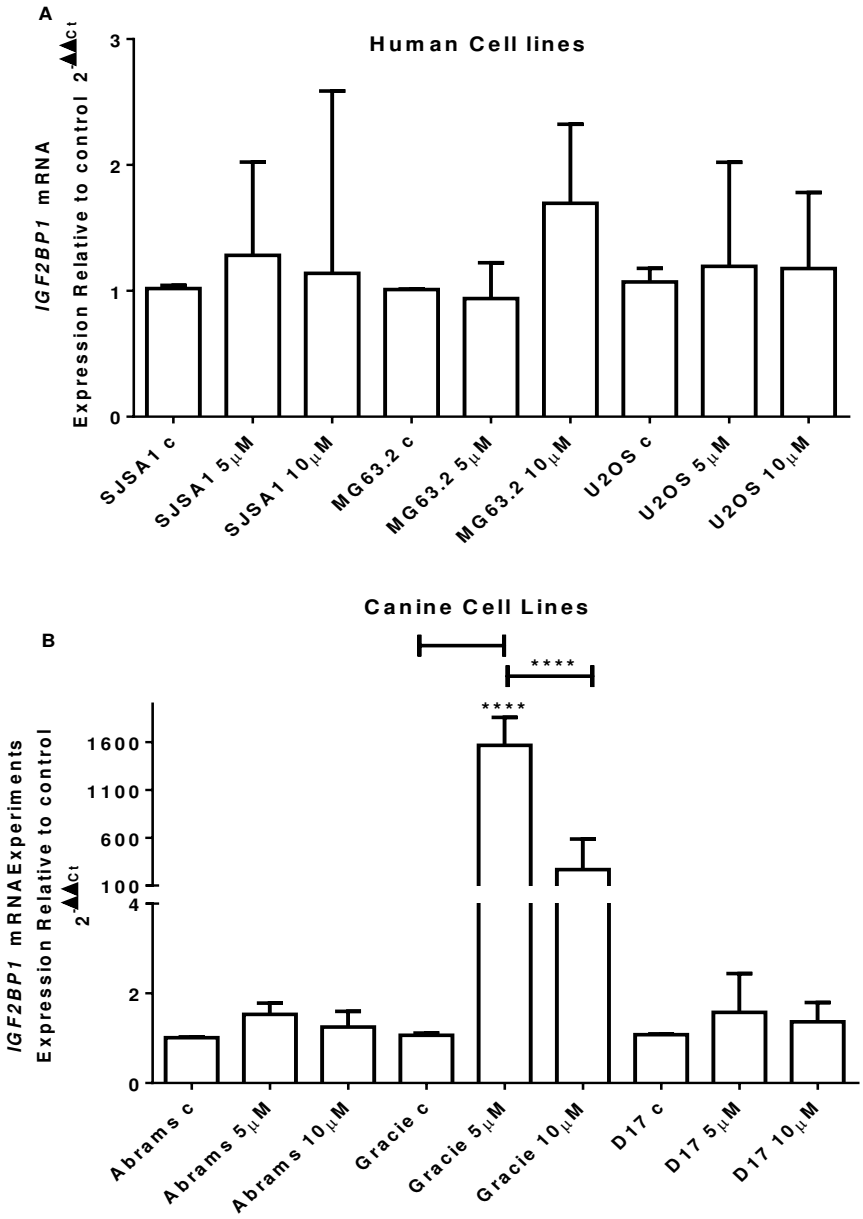

Figure 2. 11 qRT-PCR analysis reveals elevated mRNA expression of IGF2BP1 after treating the cell with 5 and 10μM of 5-azacytidine for three days.

A) Treating the human cell lines (SJSA1, MG63.2, and U2OS) with 5-azacytidine for three days showed no significant changes in the mRNA expression for IGF2BP1, as compared to controls. cDNA synthesis and qRT-PCR analysis were conducted using total RNA isolated from treated cells. Data was normalized to HPRT expression(c). B) The canine cell line Gracie treated with 5-azacytidine (5-az) (5μM) showed significantly increased expression of IGF2BP1 mRNA levels, as compared to the untreated control (c). These results show that methylation of IGF2BP1 is one of the mechanisms that the canine OS escape to activate IGF2BP1. Bars represent the mean and standard deviation of the averages of three individual experiments. One-way ANOVA and Dunnett's range test (* = $p < 0.05$). 5-azacytidine is a nucleoside analog that serves as a DNA methyltransferase inhibitor.

2.4 Discussion

This investigation started with the analysis of gene expression in 16 chemotherapy-naïve, primary canine OS tumors to identify a gene signature of aggressive metastasis or resistance to chemotherapy. Tumors taken from dogs with a disease from interval (DFI) greater than 300 days (good responders) were compared to those with a DFI less than 100 days (poor responders) following treatment limb amputation and adjuvant chemotherapy with doxorubicin and/or a platinum drug. We also utilized RT-qPCR to confirm the differential expression of eleven genes between the two groups [8]. One of the genes identified as having significantly different expression between these two groups in the microarray data and confirmed by RT-qPCR was *IGF2BP1*.

In the current stud, using a panel of human OS cell lines, canine OS cell lines, and canine tumors, we conducted experiments to determine the mechanisms and elements that increase the transcriptional expression of *IGF2BP1*, as well as the functions of the IGF2BP1 protein that contribute to OS metastasis and chemoresistance.

Many studies have shown that IGF2BP1 expression plays a contributing role in cancer progression [12, 25, 26]. For example, IGF2BP1 expression promotes proliferation, migration, invasion, and drug resistance [26]. We proposed four mechanisms that may increase *IGF2BP1* expression in OS: amplification, promoter regulatory elements, polyadenylation signals, and demethylation.

In many types of cancer, gene amplification is a fundamental mechanism of oncogene activation and cancer development and progression [26, 37]. In neuroblastoma, increases in *IGF2BP1* copy number and protein expression predicted disease aggressiveness and stage [26]. Doyle et al. (2000) identified amplification of the gene copy number of IGF2BP1 in approximately

one-third of human breast cancers, which helps to stabilize and to maintain *c-MYC* mRNA from degradation [12].

Here we show that there is no significant correlation between the gene expression and genomic gain in the human OS cell lines. However, we identified a positive correlation between the genomic amplification and gene expression using the canine OS cells, with significant gains in IGF2BP1 copy number in 5 of the 10 cell lines ($p < 0.05$, Pearson $r = 0.89$). In addition, 35% of the tumor samples collected from dogs with OS shown genomic amplification, and 62.5% of the tumors with genomic amplification shown an increase in *IGF2BP1* transcription. Of the remaining 13 tumors (65%) without IGF2BP1 genomic amplification, 3 tumors (23%) had above average levels of *IGF2BP1* mRNA, including one tumor with genomic deletion of *IGF2BP1*. Although gene amplification is an important factor contributing to increased expression of IGF2BP1 in canine osteosarcoma, these results show that additional mechanisms such as regulatory elements in the promoter and 3'UTR can lead to the elevated expression of *IGF2BP1*.

To explore transcriptional regulation of IGF2BP1, we generated a series of deletion constructs of the proximal promoter of *IGF2BP1* in a luciferase reporter system. We found in general that in OS cell lines, the -proximal 800bp promoter contains the essential TFs that are required to transcribe *IGF2BP1* in both species with significant and consistent losses of activity observed with deletion of a region of the promoter (approximately -582 to -340) containing binding sites for TCF4, MYC:MAX, NFKB, and E2F. Using signaling pathway luciferase reporters containing tandem repeats of binding sites for: TCF4, NF-Kappa B, AP-1, CREB, E2F, E box, and LEF/TCF, we found significant TF activity for MYC (E box), NF-Kappa B, AP-1, and TCF4 β-catenin (E box).

Previous studies have suggested that IGF2BP1 is part of a feedback loop in which MYC and β-catenin are both regulated by IGF2BP1 and can activate the transcription of IGF2BP1 (REFS). Since an E-box which could bind either MYC or TCF: β-catenin complexes were located within the region from -582 to -340, we generated a mutation construct that disrupted MYC binding in the E-box, but not TCF binding in the context of the -582bp canine and -583bp human promoters. We found each of the cell lines tested exhibited a significant loss in activity with these point mutations confirming that this E-box element is an important activator of IGF2BP1 transcription. Interestingly, despite the loss of activity with the E-box mutation in the U2OS cell line, MYC activity was not significantly activated in this cell line and the deletion constructs of the canine promoter did not exhibit significant losses in activity with deletion of this region. This may suggest that other factors are capable of binding to this E box or that analysis across this region is not sufficiently sensitive to identify significant changes in a larger group of constructs.

One alternative factor that could bind to the E-box and would also be impacted by the point mutation we generated in that site is USF-1 (Upstream Transcription Factor 1). USF-1 is upregulated in cancer cells and also promotes the expression of Interleukin-like EMT inducer (ILEI) which is a secreted factor that contributes to melanoma metastasis [38]. Additional studies would be necessary to confirm this hypothesis.

Since the E box mutation doesn't affect TCF4: β-catenin binding efficiency this indicates that TCF4: β-catenin is not a critical factor binding to this site. However, the cell lines that have the highest expression of IGF2BP1 showed both activation of MYC and LEF/TCF reporters, so both of these factors may contribute to IGF2BP1 expression. We also detected significant activity of the AP-1 reporter in the OSA8, D17, SJSA1, and U2OS cell lines although deletion of the AP-1 site only resulted in significant loss of promoter activity in the D17 and U2OS cell lines for the

canine promoter and in the D17 cell line for the human promoter. AP-1 motifs which bind dimers from the JUN and FOS proto-oncogene transcription factor families, have also shown the ability to activate the *IGF2BP1* promoter in melanoma [39]. AP-1 transcription also regulates cell proliferation differentiation and bone metabolism [40, 41]. Inhibiting AP-1 caused a reduction in cellular migration, invasion and the formation of metastasis in murine models for OS [42].

NF-KB reporter activity was also detected in each of the cell lines with several potential promoter binding sites in both the canine and human promoters. However, with the exception of a site adjacent to the E-box, consistent losses in activity were not observed with deletion of these sites making it difficult to assess their role in IGF2BP1 promoter activity. A recent study in 2019 identified increased IGF2BP1 mRNA expression and protein levels in response to periodontal infection in THP-1 human macrophages and primary human peripheral blood mononuclear cells (PBMCs). In mediating the pro-inflammation response, NF-Kappa B translocates to the nucleus during transcriptional activation. They found that IGF2BP1 co-immunoprecipitated with the NF-Kappa B complex and that knockout of IGF2BP1 inhibits NF-Kappa B complex activation and translocation in response periodontal infection [43]. Another study shows NF-Kappa B can recruit HMGA2 to the bind *IGF2BP2* genomic DNA at an intronic region and can have a massive impact in IGF2BP2 expression [44]. We also predicted binding sites for NF-Kappa B at the first intronic region in *IGF2BP1* that might also help in recruiting HMGA2. As transcription regulators, HMGA2 is well established to have a positive feedback loop between IGF2BP1 and LIN28 [45].

Not only is *IGF2BP1* under strict regulation at the nuclear level, but also exhibits significant regulation by miRNA at the cytoplasmic level. *IGF2BP1* has a 3'UTR of approximately 6,000 bp that contains an many miRNA binding sites. Further, a study by Mayr et al. (2009) has shown a general shortening of the 3'UTR by alternative polyadenylation. Alternative

polyadenylation (APA) activates oncogenes in cancer cells to escape post-transcriptional regulation by miRNA [46].The most investigated miRNA regulating IGF2BP1 is the let7 family [47, 48]. It has also been reported that the let-7 family of miRNA correlates negatively with IGF2BP1 mRNA [48, 49]. Peter et al. (2008) generated point mutations at the binding sites of let-7 on *IGF2BP1* mRNA and illustrated significantly higher luciferase expression compared to reporters that were unmutated [48]. Studies have also shown multiple miRNAs including: miR-873,miR-423-5p, miR-491-5p, miR-372 miR-150,miR-708, miR-506, miR-4500, and miR-98-5p that suppress IGF2BP1 mRNA to inhibit carcinogenesis and metastasis [50-57].

Our investigations have identified significant loss of the extensive 3'UTR in the canine OS cell lines with the highest expression of IGF2BP1 (OSA8 and Abrams). When we compare the regulatory activity of the remaining 614 bp proximal 3'UTR to fragments of the distal 3'UTR (3495 and 2981bp) we find that these fragments have significantly greater negative regulatory activity. These fragments of the distal 3'UTR are rich with binding sites for miRNAs. We found a significant negative correlation between the number of miRNA binding sites in 3'UTR fragments and luciferase activity of pmirGlo reporter constructs indicating that truncation of the 3'UTR would be an effective method to escape post-transcriptional regulation. We also found that cell lines with 3'UTR truncations (OSA8 and Abrams) also have high expression of Let 7 miRNA to support our hypothesis.

IGF2BP1 also plays as central role in a feedback loop implicated in the cancer stem cell phenotype. In this loop, LIN28B (Lin-28 homolog B) promotes the stem cell phenotype by impairing let-7 family miRNA biogenesis to block their regulation of *IGF2BP1* mRNA. IGF2BP1 protein enhances the expression of LIN28B and HMGA2 creating, as Busch et al. (2016) describe, an oncogenic triangle feedback loop [45].

While we didn't identify shortening of the 3'UTR in any of the human cell lines, regulation of the let-7 family through the LIN28B feedback loop could represent any alternative way to impact post-transcriptional regulation without altering the 3'UTR [45].

The canine Gracie cell line and human cell line U2OS have no transcript or protein expression of IGF2BP1, yet have no genomic loss of IGF2BP1 and exhibit activity of IGF2BP1 promoter constructs. We treated the OS cell lines with (*5'-Azacytidine*), a chemical reagent that competes with cellular Cytosine during replication and prevents DNA methyltransferase from functioning. The treatment was able to activate the transcription of IGF2BP1 mRNA in the Gracie cell line. This treatment had no impact on any of the other OS cell lines, including the U2OS line. We believe histone modifications maybe responsible for blocking IGF2BP1 transcription in the U2OS cell line.

2.5 Conclusions

In conclusion, our study highlights the cellular regulation of IGF2BP1 in canine and human OS. Amplification, truncation of the 3'UTR, Transcriptional regulation, and DNA methylation can alter IGF2BP1 expression in human and canine OS. This gene shows adverse prognostic significance at the DNA, mRNA, and protein levels. Additionally, the high expression of the protein draws a parallel with many known oncogenes. While more research is required to identify the primary targets of IGF2BP1 in OS, it serves as a biomarker of aggressive osteosarcoma and we propose that IGF2BP1 identifies RNA binding family members as potential drug targets for the treatment of OS. Although further studies will be necessary to solidify the role of these genes and pathways in OS, which will be discussed in chapter 3.

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CHAPTER 3: ILLUMINATE THE FUNCTIONAL ROLE AND TRANSCRIPT TARGETS OF IGF2BP1 IN OSTEOSARCOMA PROGRESSION

SYNOPSIS

Background: Osteosarcoma (OS) is a malignant bone tumor that afflicts over 10,000 dogs. Most dogs and approximately 30-40% of children with OS succumb to metastatic disease. We identified elevated insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1) as one of the biomarkers of poor prognosis in canine OS. IGF2BP1 is an oncofetal protein that regulates mRNA subcellular localization, nuclear export, stability, and translation. IGF2BP1 controls the expression of oncogene targets and correlates with poor outcome in a variety of human cancers. Our preliminary data show that IGF2BP1 knockdown (shRNA) in a human OS cell line increased sensitivity to doxorubicin by ≥ tenfold compared to control. Significant reductions in cellular migration, invasion, proliferation, and tumor growth in nude mice were also observed ($p < 0.05$).

Methods: Using the McKinley canine OS cell line we generated and validated stable overexpression of IGF2BP1 (IGF2BP1-pLVX-Puro, Clontech). The stable OS cell line pool and individual clones with a corresponding empty vector control were analyzed and tested for migration, invasion, proliferation, and resistance to standard chemotherapeutic agents. We analyzed migration and invasion using a scratch wound assay and measured cellular proliferation as a surface confluence for 90 hours on an IncuCyte Zoom. We also assessed the clones' sensitivity to doxorubicin over 48 hours using a bioreductive resazurin-based fluorometric assay. We assessed changes in transcript expression in response to IGF2BP1 from isolated total RNA analyzed on

Affymetrix Canine 1.0ST microarrays (University of Colorado Cancer Center Genomic and Microarray Shared Resource).

Results: The overexpressing IGF2BP1 clones had increased resistance to doxorubicin compared to the control, and the IC50 levels correlated with IGF2BP1 mRNA levels ($p < 0.05$, $r2 = 0.89$). For cellular proliferation, we found that only the IGF2BP1-expressing pool, that represents random insertion of the plasmid without selecting isolated clones, exhibited a significantly higher rate of proliferation relative to the empty vector control ($p < 0.05$). However, one of the highest expressing IGF2BP1 isolated clones had significantly greater cellular mobility and invasion than this pool, and both the pool and isolated clone had significantly higher rates of migration and invasion that cells transfected with the empty plasmid ($p < 0.05$).

Microarray analysis of control and overexpressing cells was used to detect global changes in gene expression and to identify potential targets of IGF2BP1. Differentially expressed genes were cross referenced to the RNA Binding Protein Immunoprecipitation database, published by Conway et al. (2016) using human stem cells, to identify direct mRNA targets bound by IGF2BP1. We identified 162 genes that were differentially expressed between control and overexpressing cells (FC ≥ 2, FDR< 0.05), and 13 of those genes have been previously reported to bind IGF2BP1 directly. Pathway analysis of these 13 genes identified enrichment for genes involved in the regulation of cell adhesion, migration, and the extracellular matrix. Altered expression and IGF2BP1 binding of a subset of these transcripts was confirmed using RNA immunoprecipitation and RT-qPCR.

Conclusions: Our data suggest that IGF2BP1 plays a significant role in human and canine osteosarcoma. This study revealed the functional relevance of IGF2BP1 and identified it as a biomarker for aggressiveness in osteosarcoma. With this knowledge, new treatment strategies can

be developed that target IGF2BP1 or it is signaling pathways for osteosarcoma, or any cancer that expresses high levels of IGF2BP1. This treatment may have a high impact on the cell's ability to metastasize.

3.1 Introduction

Osteosarcoma is a highly aggressive malignant bone tumor, commonly found in young adults (under the age of 20 years) or children. Sadly, patients with metastases (micrometastases) have five-year survival rates of only 30-40%. One of the challenges of studying osteosarcoma is the limited availability of samples; notably, canines spontaneously develop osteosarcoma at a higher rate than humans (10 times more), and canine osteosarcoma shares many key features with human osteosarcoma, including histological and morphologic features, as well as development of chemotherapy-resistant metastases [1]. Furthermore, there is increasing evidence of significant parallels between the genomic landscape in dog and human cancers. Cross-species analysis indicates that these diseases are similar at the molecular level making dog breeds powerful and ideal subjects for genetic mapping of disease alleles [2-5].

Recently research has focused on personalizing therapy based on biomarkers unique to the cancer cells through genomic analysis [6]. OS cells exhibit high genomic instability that affects multiple genes and miRNAs. This instability creates alterations in numerous pathways that the cells can use to resist chemotherapy [7]. However, by understanding the biomarkers associated with metastasis and chemotherapy-resistance at the molecular level we can use these biomarkers to predict patient responses, human or dogs, and develop therapeutic approaches that inhibit these pathways and lead to longer survival [6, 8, 9].

We first identified IGF2BP1 as a potential biomarker through microarray analysis of tumor samples from dogs with different disease-free intervals [9]. We analyzed gene expression profiles using Affymetrix Canine 2.0 microarrays in two groups of canine OS tumors. The first group had a disease-free interval (DFI) of <100 days following amputation and chemotherapy, and the second

had a DFI >300 days. Remarkably, the tumors taken from the patients with the shortest disease-free interval had higher expression levels (7-fold, p=0.047) of IGF2BP1 than long DFI patients. We also found that IGF2BP1 transcript levels were extremely low via microarray analysis of normal canine bone, similar to the transcript level in human osteoblasts. In a comparison of mRNA levels between normal canine bone and the two groups of tumor samples, we found significant elevation of IGF2BP1 in the tumor samples—132-fold in DFI >300 days and 915-fold in DFI <100 days [10]. Thus, we chose to further analyze IGF2BP1 as a candidate biomarker of OS.

Insulin-Like Growth Factor 2 mRNA Binding Protein 1 (*IGF2BP1*), also known as *IMP, VICKZ, ZBP,* and *CRD-BP,* is located on chromosome 17q21 in the human genome and on chromosome 9 in the dog genome. It is only expressed during embryonic development and is an oncofetal protein, as it is not detected in adult cells except in certain types of cancer [11, 12]. The *IGF2BP1* promoter region contains CpG islands that may allow transcription to be blocked via methylation in normal adult cells [12]. Mice with IGF2BP1 knockdown were deficient in gut development, had increased mortality, and had symptoms of dwarfism. Conversely, transgenic mice expressing IGF2BP1 in mammary tissue developed tumors [11, 12]. These findings illustrate the critical role of *IGF2BP1* during development. IGF2BP1 functions include binding to mRNA targets and regulating their stability, translation, subcellular localization, and nuclear export. With those functions in mind, the IGF2BP1 protein can interact with the mRNAs of various genes that have a high impact on cancer progression [11]. These genes include c-MYC, KRAS, and β-catenin, which promote migration, invasion, proliferation, and drug resistance, as well as the tumor suppressor PTEN gene, which promotes cell migration [13-15]. Furthermore, IGF2BP1 can interact with other proteins involved in transduction signaling pathways, such as PI3K, mTOR,

and MAPKs [16-18]. IGF2BP1 expression also correlates with poor outcomes in a variety of human cancers, including melanoma, breast, ovary, liver, and colorectal cancers [19-23].

In support of a role in the progression and chemotherapeutic resistance of human osteosarcoma, we identified elevated IGF2BP1 expression in the human OS MG63.2 cell line, which was selected due to its ability to metastasize to the lungs in a mouse model and because it expressed *IGF2BP1* transcripts at 17.8-fold higher levels as compared to cultured human osteoblasts and the parental cell line MG63 [24].

In order to study the functional role of IGF2BP1 in human OS, we knocked down IGF2BP1 expression levels in the metastatic MG63.2 cell line using five different Mission® shRNA constructs and assessed reduction in *IGF2BP1* transcript levels by RT-qPCR. We moved forward with the two most effective shRNA constructs and selected clonal isolates with greater than 50% *IGF2BP1* knockdown, as determined by RT-qPCR. The five clonal isolates chosen exhibited between 50 and 80% reduction in *IGF2BP1* expression, resulting in transcript levels like the parental, non-metastatic MG63 cell line. These IGF2BP1 knockdown studies in the MG63.2 cell line suggested that IGF2BP1 expression is essential for maintaining the cancer phenotype, as knockdown of *IGF2BP1* reduced cellular proliferation, migration, and invasion. Knockdown of IGF2BP1 in human OS cells also increased doxorubicin sensitivity and reduced tumor growth in athymic nude mice. Previous studies have proposed and tested various mechanisms of IGF2BP1 function including: stabilizing c-MYC or N-MYC mRNA to sustain the oncogenic phenotype, directly stabilizing the mRNA for multi-drug resistance 1 (MDR1) that encodes an ATP binding transport membrane to pump drugs across the cell membrane into the extracellular space [22, 25, 26], or by manipulating actin dynamics and cell junction proteins, such as Tau Tubulin Kinase

1(TAU), Integrin Subunit Alpha 6 (ITGA6) and Actin Related Protein 2/3 Complex Subunit 2 (Arp2/3) to regulate cellular adhesion, migration, and invasion [27-30].

However, it is currently unclear exactly what is the mechanism or the pathways that IGF2BP1 manipulates in OS development and metastatic progression. In this study, we tested the significance of IGF2BP1 overexpression and identified mRNA targets of IGF2BP1 in OS.

3.2 Materials and methods

3.2.1 Cell culture

All the cell lines were validated for species and genetically identified using short-tandem-repeat analysis [31]. The human OS cell lines MG63, SJSA-1, and U-2OS were purchased from ATCC (Manassas, Virginia). MG63.2 human osteosarcoma cells are a metastatic subline of the MG63 cell line developed through Fiddler selection of MG63 cells metastasizing to the lung [32]. These cells were a gift from Dr. Hue Luu (University of Chicago, Illinois). Canine cell lines Abrams, Gracie, D17, OSA8, OS2.4, McKinley, HMPOS, Yamane, Moresco, and Vogel. Abrams cells were derived from metastatic OSA nodules whereas McKinley, Vogel, and Yamane derived from primary tumors (Supplemental Table 1). Canine cell lines were generated by the referenced originators and provided by Dr. Douglas Thamm. All cells were cultured in DMEM High Glucose (Thermo Scientific, Cat#SH30022.01) supplemented with 2X MEM vitamins, 100 mM sodium pyruvate, 1X MEM non-essential amino acids, and 10% fetal bovine serum (FBS, Atlas Biological, Fort Collins, Colorado). For the methylation experiment, we used a demethylating agent (*5′-Azacytidine*) that inhibits DNA methyltransferase from (Sigma, Cat#A2385). We dissolved the drug for each experiment in phosphate buffered saline (1X PBS, Corning cellgro, Cat#45000-446) and stored at -80ºC as a 1000x or 500x stock solution. Cells were cultured to ~70% confluence, and separate plates were treated with *5- Azacytidine* at (5 and 10µM) for 72 hours or with only PBS for the control. We changed the media and gently washed with PBS each time we added the drug, after 24 hours. After 72 hours of treatment, the cells were collected for RNA extraction and cDNA was prepared for RT-qPCR analysis.

3.2.2 RNA Isolation, cDNA, DNA, Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-qPCR) and Quantitative Polymerase Chain Reaction (q-PCR) Analysis

Quantitative RT-qPCR was performed on total RNA from human and canine OSA cell lines. cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) with 1µg input RNA. Total RNA was extracted using RNeasy kit (Qiagen) with on-column DNase I (Qiagen) based on the manufacturer's protocol (San Diego, California). Primers were designed to be intron-spanning using Primer-BLAST, to amplify all possible isoforms noted in *National Center for Biotechnology Information* (NCBI). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) expression was performed using the iQ SYBR Green Supermix and Stratagene Mx3000P instrument. The mixture contained cDNA from 25ng of RNA and 100nM of forward primer and 300nM of the reverse in a 25µL volume.

In all cases, the amplification efficiencies were equal or greater than 90%, and both amplicon size (70 to 200 bp) and sequence were confirmed. Furthermore, the ΔΔCt method used for data analysis of gene expression levels as described by Livak et al. (2001) [33]. To normalize the data, we used hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) as a housekeeping gene. *HPRT1* was shown to be expressed consistently across all cell lines cultured in-house. We used *HPRT1* to normalize the data in both RT-qPCR and qPCR.

Thermal cycling was performed on the Mx3000p instrument with the following parameters: 95°C for 10m followed by 40 cycles of 95°C for 30s and 60°C for one minute. Data collection was performed at the end of the 60°C step. Dissociation curve ramps were presented at the end of the cycle to verify that only a single product was generated. Data analysis was performed with the Mx3000p software. Primers (Table 3.1) were designed based upon NCBI

RefSeq mRNA sequences (Integrated DNA Technologies, Coralville, IA, USA) and checked for specificity using NCBI blast and UCSC In-Silica PCR [34, 35].

Table 3. 1 Primer sequences and amplicon sizes for RT-qPCR for selected genes.

Primer	Species	Sequence (5' to 3')	Amplicon Size
HPRT1 Forward	Human & Canine	TGCTCGAGATGTGATGAAGG	192 base pairs (bp)
HPRT1 Reverse	Human & Canine	TCCCCTGTTGACTGGTCATT	
IGF2BP1 Forward	Human	CATCAGCAGCTGGCCGGGTC	240 bp
IGF2BP1 Reverse	Human	TCCGTGCCTGGGCCTGGTTA	
IGF2BP1 Forward	Canine	AAGGACAACGGGCTGAAATCG	109 bp
IGF2BP1 Reverse	Canine	CAAGCAAGTGGGCAAACCTGA	

3.2.3 Western blot analysis

Cells were harvested for western blots from ~70% confluent plates after washing with PBS buffer twice on ice. Then the cells were lysed for 5 minutes on ice with Radioimmunoprecipitation assay buffer (RIPA buffer) that contains: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Disodium EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate with Complete, EDTA-free Protease Inhibitor Cocktail Tablets (Roche). The lysates were collected in 1.5ml tubes and repeatedly passed through a 26-gauge needle or were sonicated three times for 10sec at power 6. Finally, the lysed cells were centrifuged for 15 minutes at 13,000 x g, and the supernatant was transferred to a new tube and stored at -80°C before analysis. Total protein was quantified with the BCA Assay Kit (Pierce, Cat# 23227) and 10 to 20 µg of complete protein was denatured in 2 or 6X Laemli Buffer and run on a (4 -20%) Mini-PROTEAN TGX Stain-Free Gel (BIO-RAD,

Cat#456-8095) at 50V for 5 minutes then 250V for 25-30 minutes, prior to transfer to a PVDF membrane using the semi-dry transfer unit Trans-Blot Turbo (BioRad).

The membrane was dried, blocked for 1 hour in 10% nonfat dry milk in PBS at room temperature and washed three times using Tris-buffered saline containing 0.05% Tween-20 (PBS-T) for 5 minutes. The blots were incubated with the anti-IGF2BP1 mouse monoclonal antibody (1:5000, MPL code no. RN001M) in 1% milk/PBS overnight at 4 °C. The membrane was washed three times for five minutes each in PBS-T and incubated with goat-anti-mouse-horseradish peroxidase (HRP) conjugate (1:10000, BioRad Cat# 170-6516) in 5% milk in PBS for 1 hour.

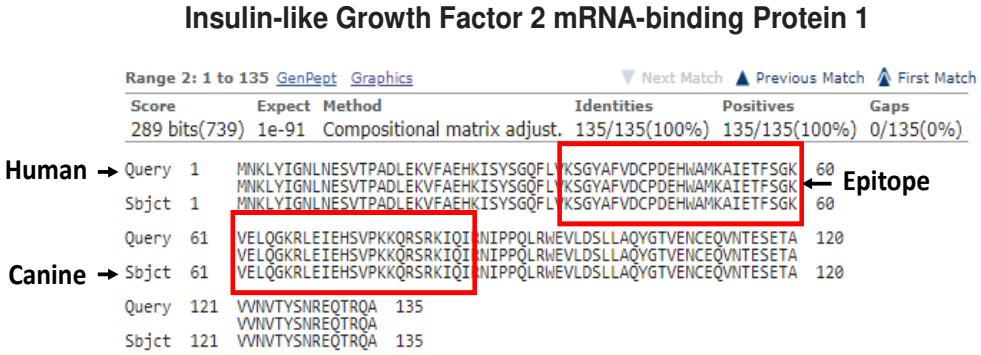


Figure 3. 1 Alignment of the IGF2BP1 antibody epitope and canine IGF2BP1.
The region probed by the antibody has 100% identity between the canine and human proteins. The 576 AA canine protein is 99% identical to the human protein. The matching blocking peptide is identical in sequence to the epitope.

3.2.4 Preparation and transformation of IGF2BP1 expression construct

The mammalian expression construct pLVX-Puro-IGF2BP1 was generated from a human IGF2BP1-GFP fusion construct (construct provided by Stephen Hűttelmaier). This construct was digested to excise the IGF2BP1 cDNA and remove the GFP fusion. The digested fragment was ligated into pLVX-Puro. Ligation reactions were transformed in 50µl DH5α competent E. coli cells mixed with 2 µl of the ligation reaction and incubated on ice for 10 minutes. After that, we

heat- shocked the mixture for 20 seconds at 42°C and incubated again on ice for 2 minutes. We added Super Optimal broth with Catabolite repression (SOC media, 100µl 2M Glucose, 100µl 2M MgCl, and LB media up to 10ml), and incubated with shaking for 1 hour (250 rpm) at 37°C. Transformations were plated on Agar LB media plates (Agar, BD Cat# 214050, LB Broth, Miller, Fisher) with 100 µg/ml Ampicillin for selection (Ampicillin Trihydrate 50mg/ml, Fisher, Cat# BP902-25) and incubated for 18 hours at 37°C.

Using the empty digested vector as a negative control, we selected 5 to 10 colonies from the plate for growth into 3ml of LB media, 6µl of Ampicillin (Trihydrate 50mg/ml) and incubated them with shaking (250 rpm) at 37°C for 18 hours. Plasmid DNA was isolated from the bacterial cultures using alkaline lysis purification and diagnostic digests were conducted as mentioned above.

Plasmid inserts were confirmed by diagnostic digest with EcoRI and XhoI to excise an insert of 1733bp without GFP sequences (Table 3.2). Plasmids with confirmed inserts by diagnostic digest and sequencing were prepared using QIAprep Spin Midiprep or Megaprep Kits and transfected into the cells.

3.2.5 Plasmids and Reagents

We generated and validated stable overexpression of human IGF2BP1 (pLVX-IGF2BP1) in the McKinley canine OS cell line. We electroporated $2x10^6$ cells (200ul) with 10ug of plasmid using 4mm sterile gap cuvettes (BTX, Cat#45-1026) and 220V for 20msec (BTX, ECM 830). We added 1 mL of media to the cuvette to cool down the cells, then transferred the cells into 10mL of media on a 10 cm plate and incubated for 24 hours at 5% CO_2.

After 24 hours, we treated the cells with 4 µg/mL puromycin acetyltransferase. Every 2 to 3 days we washed the plate with 1XPBS and retreated with a new drug. We collected pools of stably transfected cells and thinly plated cells to obtain individual colonies. Following colony formation, we transferred the clones into individual wells using Trypsin-EDTA. Multiple aliquots of these stable clones were stored in 10% Dimethyl sulfoxide (DMSO, Sigma) in FBS, and tested for IGF2BP1 expression using western blot and RT-qPCR.

3.2.6 Growth Inhibition by Doxorubicin

Growth inhibition assays were conducted using serial dilutions of doxorubicin. 2000 cells/well were seeded in 96 well plates in 100µl media and allowed to attach overnight. The following day, cells were treated with serial dilutions (1:3) starting with 77.8µg/mL (30µM) of doxorubicin in replicates of three, and then the cells were incubated for 48 hours. Then Resazurin was added to each well (10µl for every 100 µl media, 200mg/mL in PBS) and incubated at 37 degrees for 3 hours. Relative cell number was assessed at both time zero control (Tzc), and after 48 hours we incubated the controls with a resazurin-based bioreductive fluorometric assay for 3 hours. Fluorescence (530 nm excitation and 590 nm emission, sensitivity = 45) values of each tested dilution (Ti) and untreated controls (C) were collected. The data was blanked against wells containing only media and resazurin. The data was normalized by subtracting the time zero (Tzc) values, then dividing the fluorescence of the tested cells by the fluorescence of the untreated control cells: (Ti-Tzc)/(C-Tzc) if the tested value Ti $\geq$ Tzc, or (Ti-Tzc)/Tzc if the tested value Ti $<$ Tzc. Cellular survival data were expressed as a percentage of the control, and GI50 values were calculated by fitting curves to log(inhibitor) vs. the averaged triplicate responses in 3 separate experiments using nonlinear regression (three parameters) on GraphPad Prism 8.

3.2.7 Scratch Wound Migration and Invasion Assay

3.2.7.1 Migration Assay

We assessed cellular migration using Incucyte live cell imaging (Essen Biosciences). Cellular migration was assessed in stable IGF2BP1 overexpressing clones and pools using a scratch wound assay on IncuCyte ImageLock 96 well plates (Cat. #4379). Based on the manufacturer's protocol, when the plate reached ~70% confluence, we split the plate and seeded the cells at 30,000 cell/well in 100µl media on ImageLock 96 well plates. After 24 hours in the incubator, the scratch wound maker was used to create the wound across each well and then washed three times with 100µl media. The plate was monitored for 24 hours in the IncuCyte, which evaluated relative wound density at 3-hour intervals. This data was expressed as the Relative Wound Density (percent) average for each experiment and analysis the data using a two-way ANOVA with Tukey's posttest analysis ($p < 0.05$) using GraphPad Prism 8.

3.2.7.2 Invasion Assay

Following the IncuCyte protocol, we coated an ImageLock 96 well plate with BD Matrigel (Cat.354234, 100µg/ml) at a concentration of 9.6 mg/mL and mixed with media (50µl/well). After incubating the plate for 24 hours with Matrigel, we removed any unsolidified Matrigel and seeded the plate for the cellular invasion assay. After 4 hours of plating the cells on Matrigel, wounds were created, washed with PBS, and then layered with another Matrigel layer. After 15 minutes, we added 8 mg/mL of Matrigel (50µl/well). We performed the experiments three times and analyzed the plates as we did in the migration assay.

3.2.7.3 Proliferation Assay

To assess cellular proliferation rates, we used the Incucyte Zoom to measure surface confluence for 90 hours. Using a 96-well plate, we seeded the wells with 2000 cells/100µl; we performed the experiments three times and analyzed the data as we did in the migration assay.

3.2.8 Affymetrix Canine 1.0ST Genome Array Analysis

Microarray analysis of control (pLVX-Puro) and overexpressing clones (pLVX-Puro-IGF2BP1; Pool and Clone 4) was used to detect and identify potential targets for IGF2BP1. Using Transcriptome Analysis Console 4.0 and R program , we cross referenced the resulting gene list to the RNA Binding Protein Immunoprecipitation database (RIP), that is available online, to identify direct mRNA targets bound by IGF2BP1[36]. *Gene Set Enrichment Analysis* (*GSEA*) was used to identify pathways. We validated the microarray results using RT-qPCR [37].

3.2.9 RNA immunoprecipitation (RIP)

An anti-IGF2BP1 mouse monoclonal antibody (15µg/500µL of cell extract from 1.2x107 cells, MPL code no. RN001M) was used to pull down the RNA binding protein (IGF2BP1) and the RNA complex using RIP assay kit (MBL International Cat# RN1001) and Dynabeads™ Protein A (Cat # 10001D, Invitrogen). We followed the kit instructions but replaced the centrifugation of the agarose beads with magnetic Dynabeads™ Protein A (100µL/lysis) and used a magnet stand to pull down the beads. RNA was isolated from the IGF2BP1 complexes and converted to cDNA using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA) with 200ng input RNA. We performed RT-qPCR using 25 ng of relative RNA input per reaction

(2.5µL). Using this method, we were able to confirm the direct binding by IGF2BP1 of 2 differentially expressed genes predicted to be bound by IGF2BP1. Mapping binding sites of RNA binding motif (http://rbpmap.technion.ac.il/) is a software program that we used to identify IGF2BP1 binding sites on the mRNA (the binding sequences we used were; acaccc, cwuu, cauh, uaca, aaca, and caua) that was predicted from microarray results and then cross referenced with RIP database. Binding sites for IGF2BP1 with the highest predicted Z score and p value were selected and we designed primers to amplify regions containing those sites as shown below we also used c-MYC.CDS primers as a positive control (Table 3.2).

Table 3. 2 Primer sequences and amplicon sizes for RIP assay RT-qPCR for selected genes.

Primer	Binding site	Species	Sequence (5' to 3')	Amplicon Size
FLRT2 Forward	3'UTR	Canine	TGCTGGCAACAAGGGATGCT	158 bp
FLRT2 Reverse		Canine	TGCGATGGCCTTTGTGCTCT	
DSC2 Forward	3'UTR	Canine	ATCTAAAATTCACAAGTTTGGCTTG	160 bp
DSC2 Reverse		Canine	GTAATACACACATTGTGGACATGAT	
c-MYC Forward	CDS	Canine	CTGGGAGGAGACATGGTGAAC	85 bp
c-MYC Reverse	Positive control	Canine	TGCAGTCCTGGATGATGATGT	

3.3 Results

3.3.1 IGF2BP1 Expression is increased in OS compared to normal osteoblast Cell lines

Previously we found that *IGF2BP1* expression was elevated in canine OS relative to healthy bone and was associated with poor prognosis [10]. We also observed a 17.8-fold increased level of expression of *IGF2BP1* in a highly metastatic cell line (MG63.2) relative to normal human osteoblasts and the parental cell line (MG63) [38]. Moreover, when *IGF2BP1* expression was reduced in these cells, they showed a significant reduction in cellular migration, invasion, proliferation, and tumor growth in nude mice (Kalet *et. al*, unpublished data).

In this investigation, we assessed *IGF2BP1* expression in canine OS cell lines using RT-qPCR and found a significant elevation in 9 of the 10 canine OS cell lines relative to normal osteoblasts (Figure 3.2). These results support our early finding that IGF2BP1 is elevated in the development and progression of osteosarcoma.

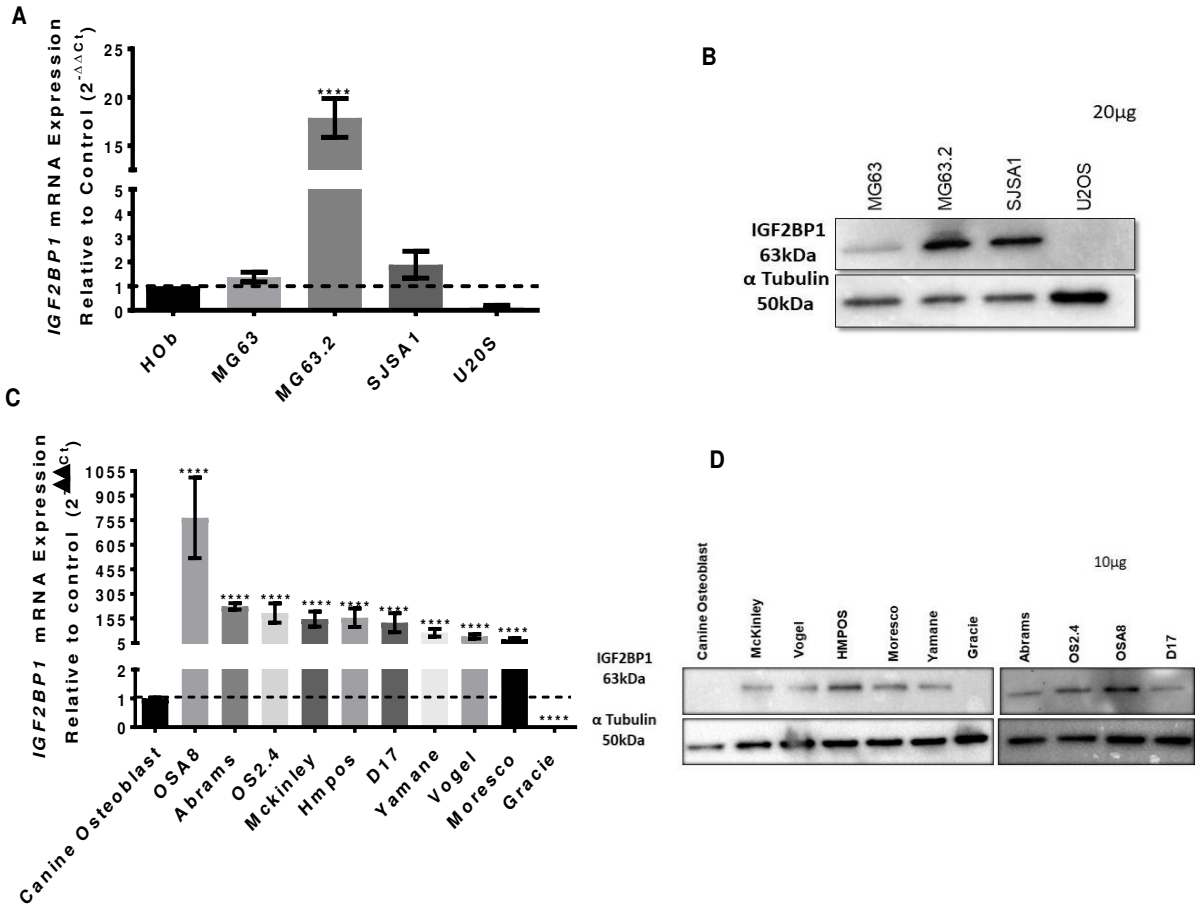


Figure 3. 2 IGF2BP1 expression increased in OS cells as compared to normal osteoblast cell lines. RT-qPCR analysis and western blot revealed elevated mRNA expression of IGF2BP1 in OS cell lines.
A and B) IGF2BP1 expression is highest in the MG63.2 metastatic variant of the MG63 cell line at both the mRNA and protein levels, as assessed by RT-qPCR and western blot analysis. C and D) RT-qPCR analysis reveals elevated mRNA expression of IGF2BP1 in canine OS cell lines relative to canine osteoblasts. This result also holds for protein levels. Bars represent mean and standard deviation. Statistical significance was determined using a one-way ANOVA and Dunnett's range test (* = $p < 0.05$).

3.3.2 Overexpression of IGF2BP1 in canine OS

To thoroughly understand the functional role of IGF2BP1 in OS, we selected the McKinley cell line which expresses moderate levels of IGF2BP1, for further overexpression, as we were able to generate a stable cell line overexpressing IGF2BP1 to evaluate the impact of high IGF2BP1 on cell function. We used a mammalian expression vector for IGF2BP1 (pLVX-Puro, Clontech). Using RT-qPCR and western blot analysis, we confirmed the elevated mRNA and protein in a stably expressing pool and three isolated clones relative to the empty plasmid and non-transfected cell line.

These clones and the IGF2BP1 pool exhibited between 2.16- to 4.65-fold elevation in *IGF2BP1* mRNA expression and a 6.32-to 9.58-folds increase in protein levels. Expression level increases were determined using a western blot and quantified using ImageJ relative to the McKinley control (Figure 3.3).

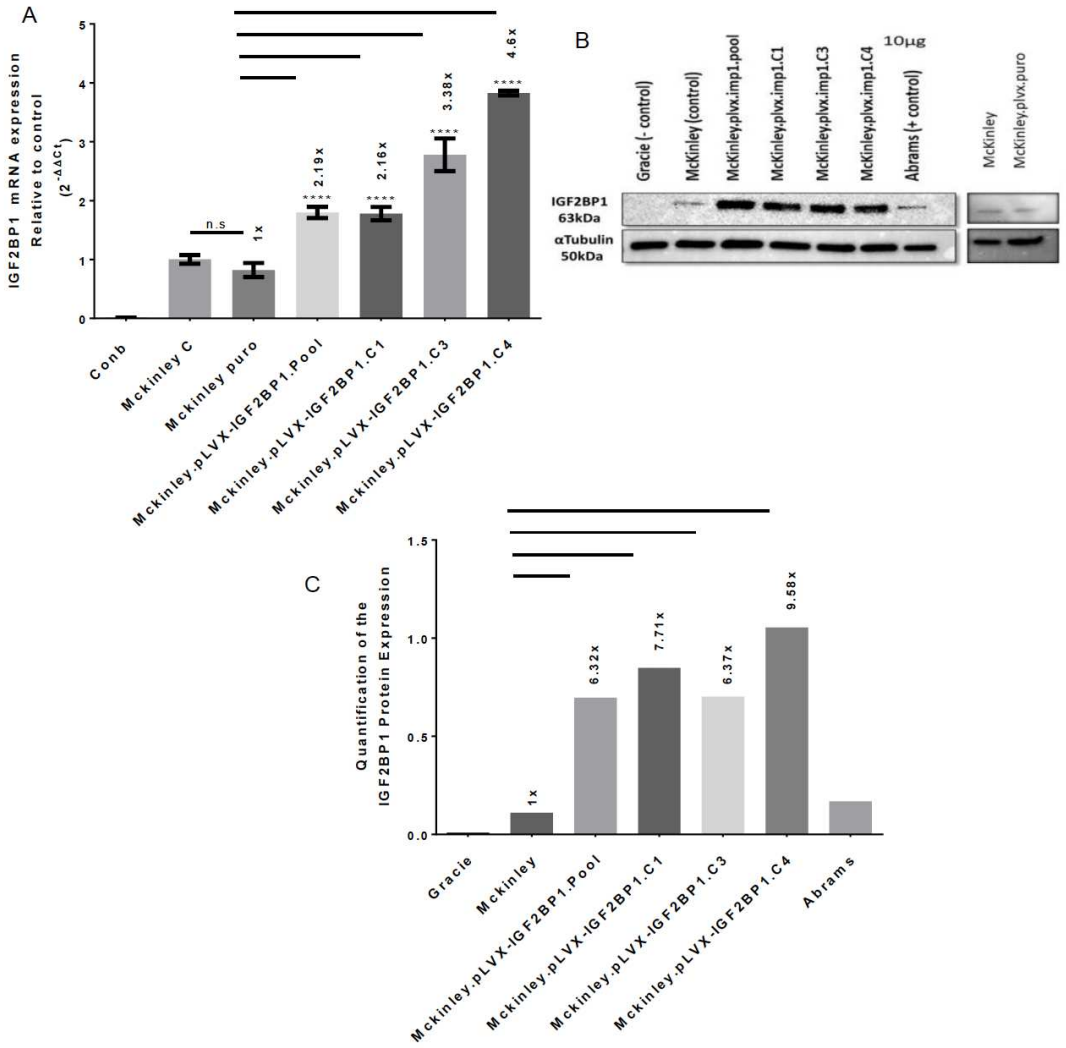


Figure 3. 3 Overexpression of IGF2BP1 in canine OS.

A) RT-qPCR analysis and western blot reveals elevated mRNA expression of IGF2BP1 relative to McKinley control OS cell line A) The analysis revealed elevated expression of IGF2BP1 in McKinley cell line pool and clonal isolates C1, C3, C4, relative to the empty plasmid pool (pLVX-Puro) and the control cell line (Conb, canine osteoblast cell lines). Bars represent mean and standard deviation. Statistical significance was determined using one-way ANOVA and Dunnett's range test (* = $p < 0.05$). B) western blot analysis revealed elevated expression of IGF2BP1 in McKinley colonies (clones 1 and 4, Pool) relative to the empty plasmid pool (pLVX-Puro) and control cell lines (Gracie, Abrams, McKinley). C) Quantification of the protein expression from figure B using ImageJ.

3.3.3 Overexpression of IGF2BP1 in Canine OS Clones correlates with reduced doxorubicin sensitivity

As doxorubicin is a standard chemotherapeutic agent used in the treatment of OS in both humans and dogs [10, 39], and *IGF2BP1* was highest in tumors with an inadequate clinical response following treatment (DFI<100 days), we hypothesized that IGF2BP1 may play a role in chemoresistance to these cytotoxic therapies.

We measured the chemosensitivity of the IGF2BP1 stable overexpressing clones to doxorubicin by treating cells for 48 hours with serial dilutions of the drug. All four stable overexpressing cell lines displayed significantly higher doxorubicin IC_{50} values, with increases from 2-8-fold relative to the empty plasmid cell line (pLVX-Puro) (Figure 3.4).

Additionally, these doxorubicin IC_{50} values significantly correlate with the relative expression of IGF2BP1 (Figure 3.4; $p = 0.0027$, $r = 0.91$). These data support the hypothesis that elevated expression of IGF2BP1 in the poor responders (DFI<100 days) may contribute to a low therapeutic response. Similarly, our previous data in the human cell line (MG63.2) clones exhibited increased sensitivity to doxorubicin in response to IGF2BP1 knockdown ($p = 0.0049$, $r = -0.9901$).

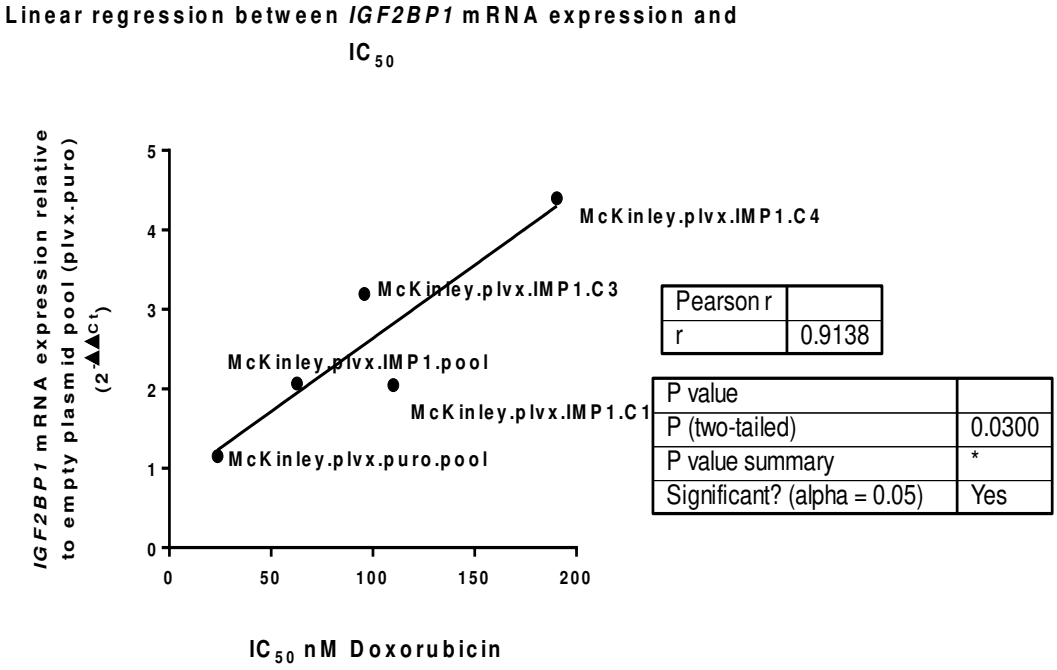


Figure 3. 4 Expression levels of IGF2BP1 in canine OS clones are correlated with doxorubicin sensitivity. A significant and a positive correlation between the IC50 and the relative IGF2BP1 expression by qRT-PCR was identified in the overexpressed IGF2BP1 McKinley cell lines. The analysis reveals that increased IGF2BP1 expression in McKinley cell line clones 1 and 3, 4, pool) was correlated with DOX (doxorubicin) resistance, relative to the empty plasmid pool (pLVX-Puro). Using linear regression (Pearson r) (* = $p < 0.05$).

3.3.4 Overexpression of IGF2BP1 in canine OS clones increased cellular proliferation, migration, and invasion

Multiple studies have suggested that IGF2BP1 regulates cellular adhesion, migration, and even invasion by manipulating actin dynamics and cell junction proteins, such as TAU, ITGA6 and Arp2/3 [27-30].

We first evaluated the rates of cellular proliferation in the stable IGF2BP1 overexpressing clones using the Incucyte Zoom to measure the surface confluence over 90 hours. Only the pool exhibited a significant increase in proliferation compared to the empty plasmid, while the proliferation rate of clones 1 and 3 were not significantly different (Figure 3.5). Oddly, clone 4 (C4) was characterized by a significant reduction in proliferation rate compared to the control (Figure 3.5).

Cellular migration and invasion were assessed in the stable IGF2BP1 overexpressing pool, the C4, and the control. The results illustrate a significant elevation in the migration compared to the control empty plasmid cell line, with the C4 clone exhibiting the highest levels of migration. These results indicate that increased IGF2BP1 expression made cells more mobile (Figure 3.6).

We then analyzed the same stable clones, but added Matrigel to the scratch wound and incubated for 24 hours to test the cells' ability to invade through the Matrigel (Figure 3.6). Again, the pool and the C4 clone were able to mobilize through the Matrigel to fill the wound, supporting our hypothesis that IGF2BP1 overexpression increased the metastatic phenotype, as seen in the DFI>100-day tumors.

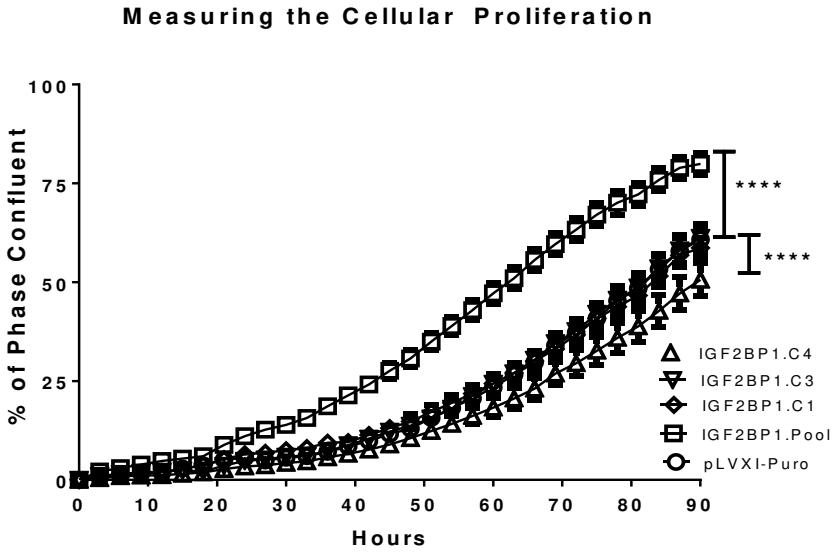


Figure 3. 5 Overexpressing IGF2BP1 canine OS pool exhibited increased cellular proliferation.
Our stable clones were seeded (2000cells/100µl media) for 90 hours in the IncuCyte. We collected the data (% of phase confluent) and corrected for time zero. We found that the pool multiplies at a faster rate than the control. Bars represent mean and standard error; this figure is a result of 3 average experiments. Statistical analyses were conducted using two-way ANOVA tests and Tukey's posttest (* = $p < 0.05$).

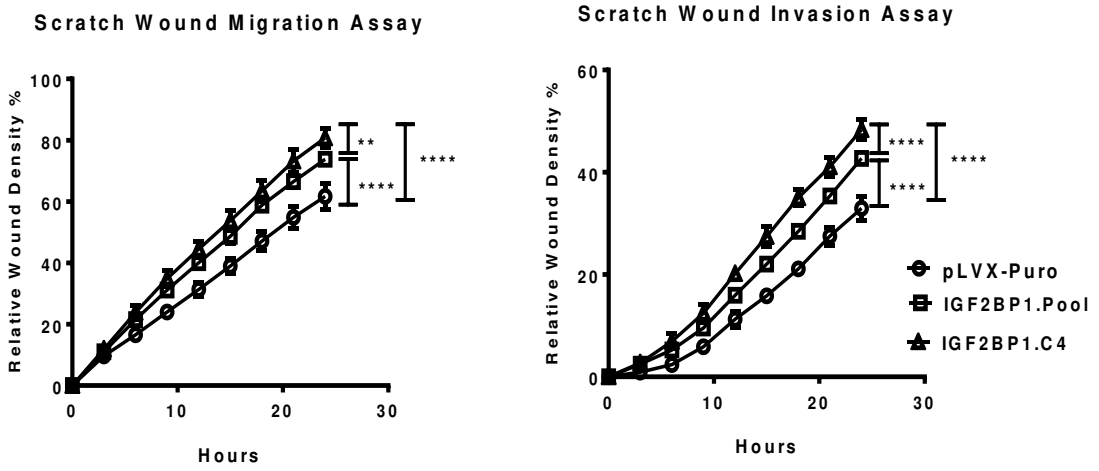


Figure 3. 6 IGF2BP1 Overexpression in canine OS clones increased cell migration/invasion.
Our stable clones were seeded (30000 cells/100µl media).Using the scratch assay, we collected the data (% of Relative Wound Density) and quantified the cell migration/invasion described in materials and methods. A significant increase in migration and invasion relative to empty plasmid control (pLVX Puro) was observed. Bars represent mean and standard error; this figure is a result of 3 experiments. Statistical analyses were conducted using two-way ANOVA tests and Tukey's posttest (* = $p < 0.05$).

3.3.5 Affymetrix Canine 1.0ST gene array analysis reveals enrichment for genes involved in the regulation of cell adhesion, migration, and the extracellular matrix

To evaluate the processes that contribute to the increased tumorigenicity of cells expressing IGF2BP1, we evaluated changes in gene expression between the pLVX-Puro control, the IGF2BP1 overexpressing pool, and the highest IGF2BP1 expressing clone, Clone 4. Previous studies have identified mRNA target genes for IGF2BP1 using RNA Binding Protein Immunoprecipitation, or RNA co-immunoprecipitations (RIP) methods. For example, using RIP, Muller and his lab found that IGF2BP1 enhances tumorigenic phenotypes by impairing microRNA that targets oncogenes [40]. In this study, we cross-referenced these and other previously published studies with our Affymetrix results to identify direct mRNA targets bound by IGF2BP1 [36]. We used the Transcriptome Analysis Console 4.0 and R program to analyze and arrange the data.

We extracted RNA from three different passages of the highest IGF2BP1 overexpressing clone (C4), the pool, and the empty plasmid control. We identified 162 differentially expressed genes (FC $\geq$2, FDR$<$ 0.05) (Figure 3.7). Using Gene Set Enrichment Analysis (GSEA), we analyzed these 162 genes and found enrichment in epithelial-mesenchymal transition (EMT) markers (Figure 3. 9). Using these gene lists and direction of differential expression (up- or downregulated), we were able to define the genes related to EMT using the published data by Stylianou *et al.* (2019) [41]. We also validated the array results using RT-qPCR for BDNF (Brain Derived Neurotrophic Factor), DCN (Decorin), FAD (Flavin Adenine Dinucleotide Synthetase 1), and GLIPR1 (GLI Pathogenesis Related 1) gene transcripts that show significant elevation in mRNA expression based on our analysis (Figure 3.8). We also validated MDR1 (Multidrug Resistance Protein 1, also known ABCB1) that was upregulated 2.17-fold between clone 4 and the empty control, but was only upregulated 1.68-fold between the pool and empty vector control so

was not listed within the 162 differentially expressed genes despite being a putative IGF2BP1 target. Gene Ontology pathways enriched in these 162 differentially expressed genes are regulating the cell extracellular matrix, space, cytoskeleton, cell junction, cell neuron projection parts and finally intrinsic components of the cellular plasma membrane (Figure 3.9).

Of the 162 differentially expressed genes, we identified 13 as being direct targets of IGF2BP1. For these 13 (Table 3.2) 7 of these genes are upregulated by overexpression of IGF2BP1 and the other 6 genes are downregulated. *Gene Set Enrichment Analysis* (*GSEA*) identified enrichment in regulation of cell adhesion, cell migration, and kinase activity [37]. RT-qPCR analysis confirmed the upregulation of transcripts for FLRT2 and DSC2 in the IGF2BP1 overexpressing pool and Clone 4 (Figure 3.11A). We also observed a significant increase in c-MYC expression using primers directed against an IGF2BP1 binding site in the coding sequence of c-MYC as a positive control. Moreover, a RIP assay using an anti-IGF2BP1 antibody revealed enrichment of mRNA in clone 4 and the IGF2BP1 expressing pool compared to control by RT-qPCR through direct IGF2BP1 binding of FLRT2 and DSC2, two of the 13 predicted targets (Figure 3.11).

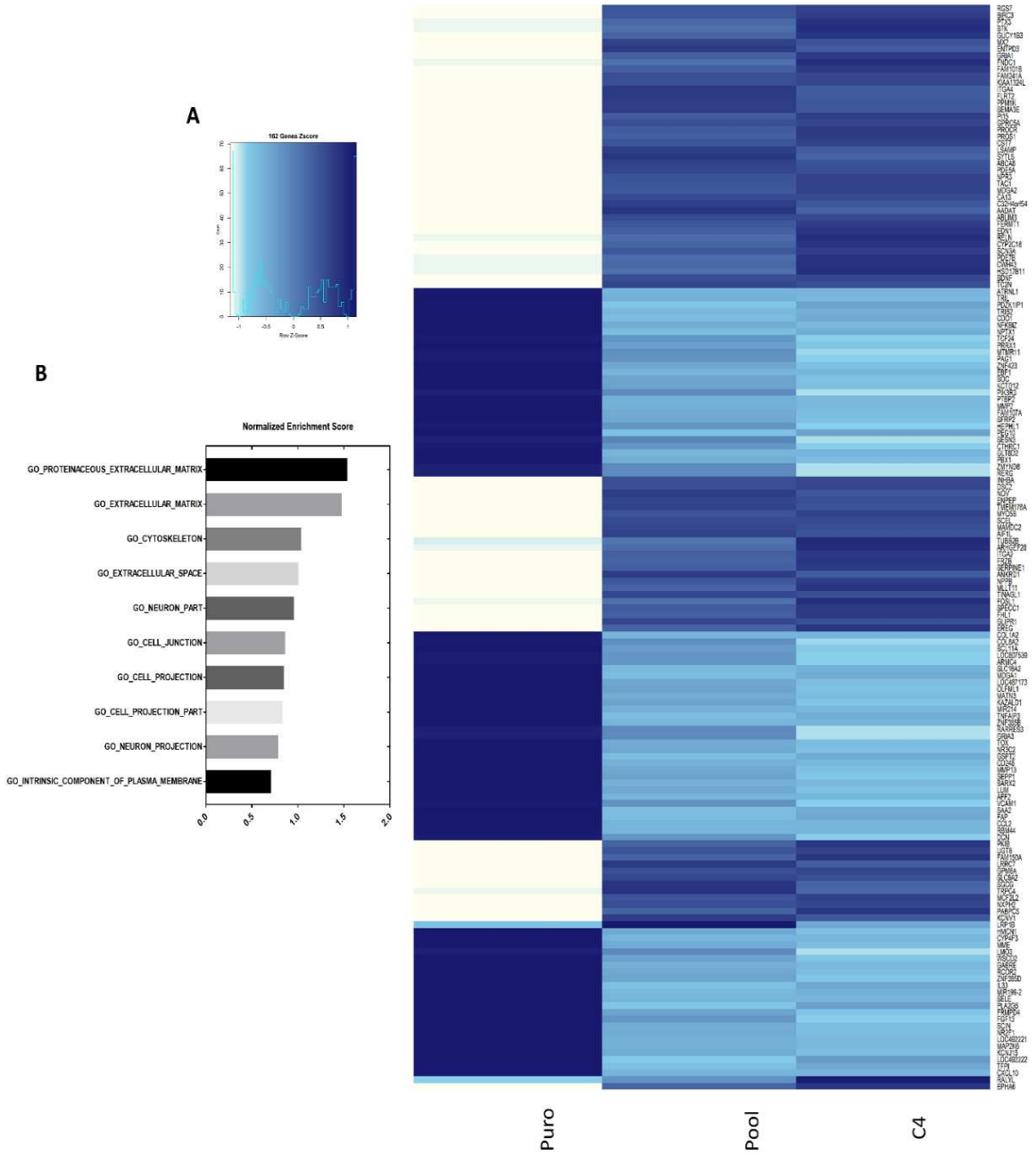
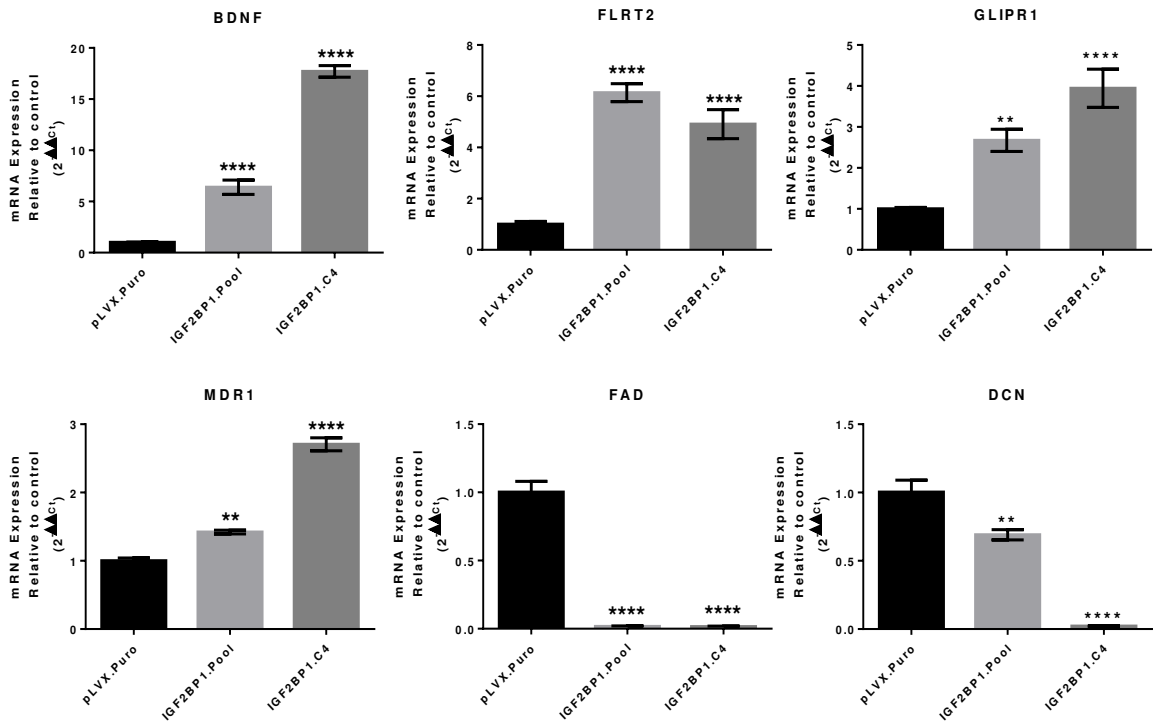


Figure 3. 7 Heatmap of Z scores for the 162 differentially expressed genes when comparing Pool and C4 to pLVX-Puro (control).

We identified 162 genes that were differentially expressed (FC ≥2, FDR< 0.05). B) Gene Ontology (GO) analysis identified pathways and regulation of cell functions, such as adhesion, migration, and the extracellular matrix. For pathway analysis, we used Gene Set Enrichment Analysis (GSEA). We used Transcriptome Analysis Console 4.0 and R program to identify and arrange the data.



Affymetrix Canine 1.0ST gene array for Canis lupus familiaris (dog)_Microarray Data Analysis				
Gene Symb	IGF2BP1.Pool Avg (log 2)	IGF2BP1.C4 Avg (log 2)	Pool vs Puro P-val	C4 vs Puro P-val
BDNF	7.46	7.56	4.00E-04	3.00E-04
FLRT2	8.6	8.21	2.51E-08	1.70E-07
GLIPR1	11.23	11.17	2.00E-04	6.00E-04
MDR1	11.1	11.47	8.45E-05	4.56E-06
FAD	3.23	4.03	1.49E-07	7.74E-07
DCN	6.31	4.34	3.80E-05	6.03E-06

Figure 3. 8 Validating the array results using RT-qPCR.

We selected four genes with significant elevations in gene expression using Affymetrix Canine 1.0ST (BDNF, FLRT2, GLIPR1, and MDRI) and two genes with significantly lower gene expression (FAD and DCN) for validation using RT-qPCR as described in materials and methods. Bars represent mean and standard deviation of triplicate samples. Statistical analyses were conducted using one-way ANOVA tests and Dunnett's range test (* = $p < 0.05$). We used Transcriptome Analysis Console 4.0 to evaluate changes in gene expression.

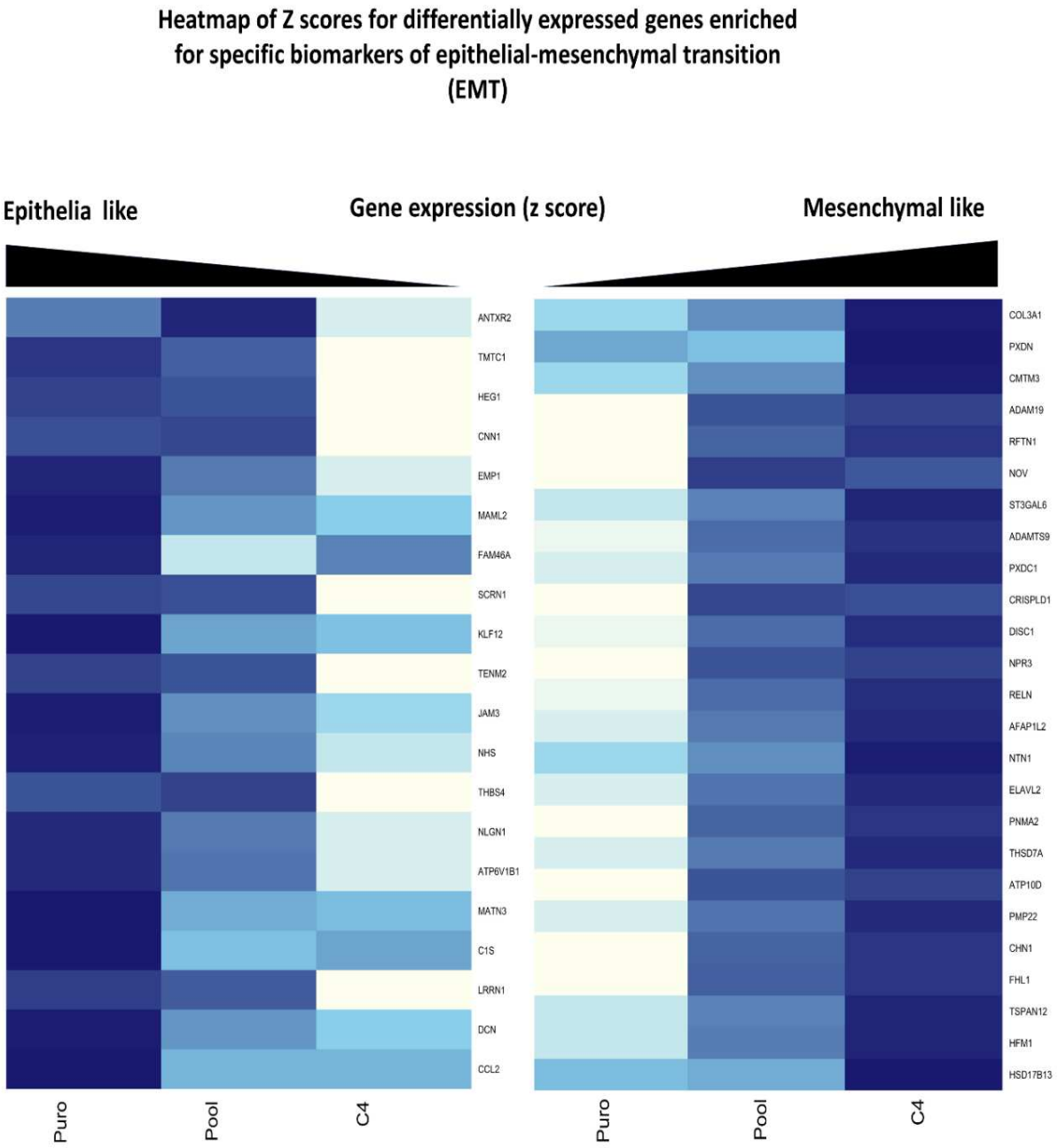


Figure 3. 9 Heatmap of Z scores for differentially expressed genes enriched for specific biomarkers of epithelial-mesenchymal transition (EMT) between Pool and C4 compared to Puro (control).
Heat map showing the Z score for these EMT genes that are up- or downregulated as EMT markers. EMT is an indicator of aggressiveness and poorly differentiated cells. We used the Transcriptome Analysis Console and R program to identify and arrange the data.

Table 3. 3 List of genes that are predicted to be bound to IGF2BP1.

Gene Name	Annotation	C4 vs Puro Fold change (log2)	Function
Mitogen-Activated Protein Kinase Kinase 6	MAP2K6	-3.22	This protein activates p38 MAP kinase in response to inflammatory cytokines or environmental stress [42].
Integrin Subunit Alpha 2	ITGA2	4.95	Plays a role in connecting cells with collagen, as well as collagenase gene expression, synthesizing the extracellular matrix, the actin dynamics signaling pathway, axon gaudiness, and activation of the MAP-Erk pathway [43]
Brother of CDO	BOC	-3.85	Plays a significant role in modulating cell-to-cell interactions. It also promotes myogenic differentiation and the axon guidance pathway by positively regulating the Hedgehog signaling pathway [44].
Matrix Metallopeptidase 2	MMP2	-2.83	Zinc-dependent enzyme that cleaves the extracellular matrix and signal transduction molecules. Involved in many pathways especially in the nervous system [45].
AF4/FMR2 Family Member 2	AFF2	-14.58	A transcriptional activator that is a member of the AF4\FMR2 gene family and an important regulator for brain development [46].
Paternally Expressed 10	PEG10	-2.06	Paternally Expressed ten gene is adapted from retrotransposons and is carried in the mammalian genome where it plays a role during pregnancy for the formation of the placenta [47].
Desmosome junction 2	DSC2	2.92	DSC2 is important for maintaining tissue integrity and has been found to promote the invasive phenotype of esophageal squamous cell carcinoma (ESCC) by remodeling cell-to-cell attachments and cytoskeleton rearrangements [48].

Fibronectin Leucine Rich Transmembrane Protein 2	FLRT2	3.23	Functions as a cell-to-cell adhesion protein, aids in cellular migration and axon guidance, regulates the development of the embryonic vascular system, and is involved in fibroblast growth factor-mediated signaling cascades [49].
KIAA1324L	KIAA1324 Like	2.13	KIAA1324L is conserved in mammalian genomes, but the function is still unclear [50].
cAMP-dependent protein kinase inhibitor-β	PKIB	2.6	It is believed that PKIB promotes metastasis through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway that is a key player in OS progression, breast cancer, and non-small lung cancer [51, 52].
UDP Glycosyltransferase 8	UGT8	2.43	Catalyzes the transfer of galactose to ceramide. Overexpression of UGT8 is an indicator of tumor aggressiveness, and a potential marker of lung metastases in breast cancer [53, 54].
Tribbles Pseudokinase 2	TRIB2	-2.42	Member of the Tribbles family that interacts with and regulates the activity of MAP kinases [55].
Tandem C2 Domains, Nuclear	TC2N	5.01	TC2N is a protein containing tandem C2 domains similar to protein kinase C. These domains are associated with calcium ion binding and protein-protein interactions for signal transduction [57-59].

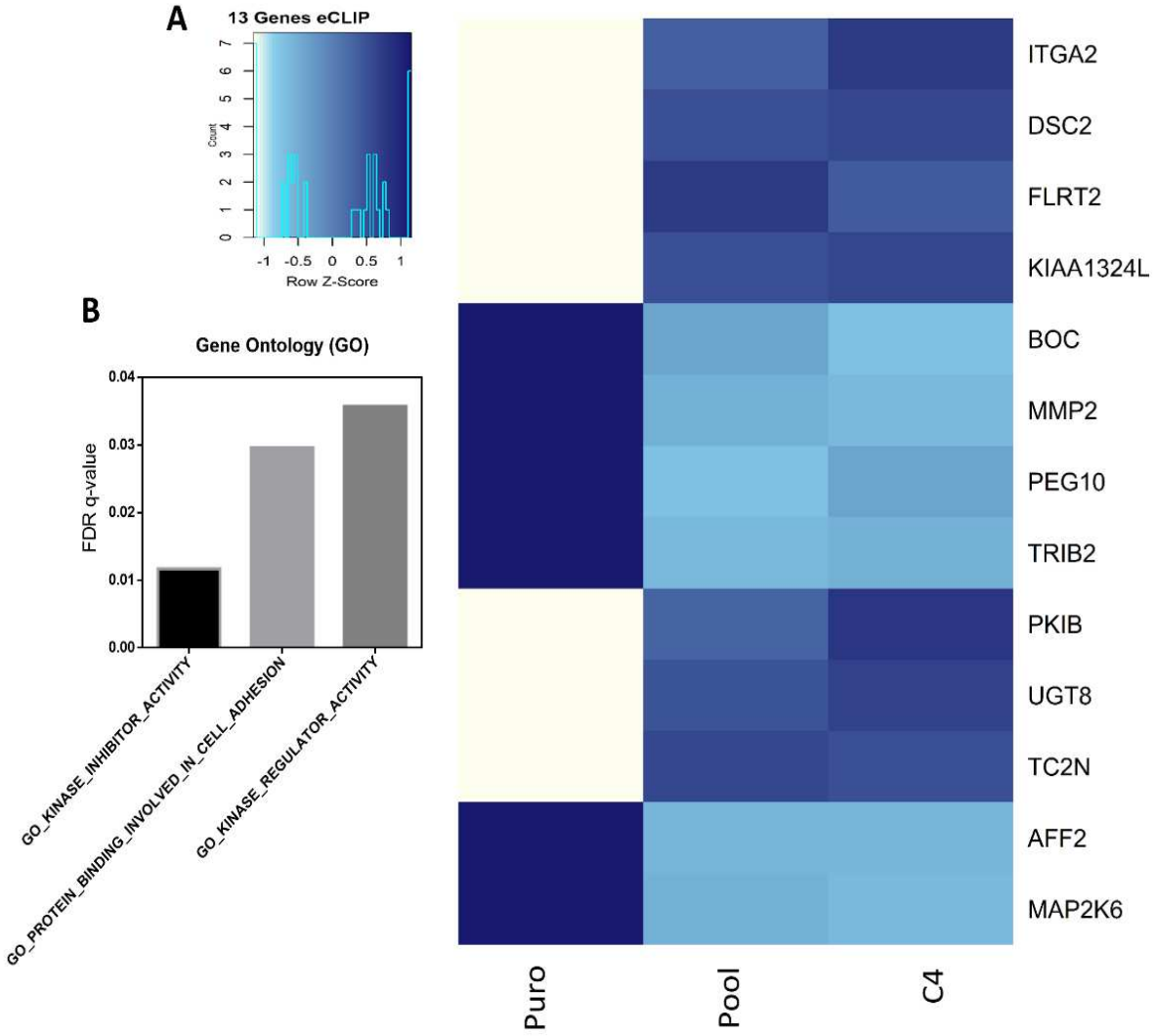


Figure 3. 10 Heatmap showing the Z scores for the 13 differentially expressed genes that we identified as direct targets for IGF2BP1 binding.
A) We identified 13 genes that are mRNA targets bound by IGF2BP1 by cross-referencing the RIP database with the 162 genes that were differentially expressed (FC ≥2, FDR< 0.05). B) Gene Ontology (GO) shows the function of these genes. We used the Transcriptome Analysis Console and R program to identify and arrange the data.

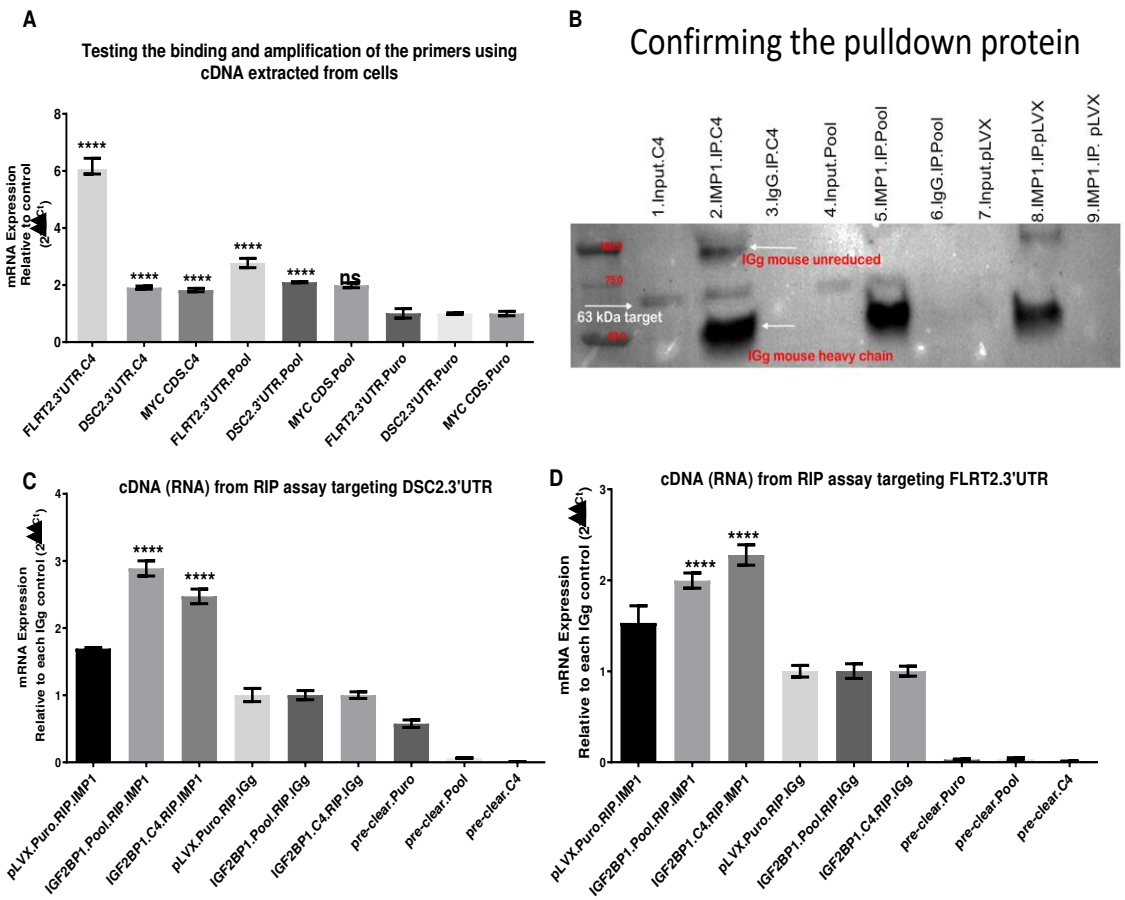


Figure 3. 11 RNA immunoprecipitation (RIP) confirmed the microarray results revealing enrichment of transcripts for two direct mRNA targets for IGF2BP1.

A) Using the mRNA extracted from cell lines C4, Pool and empty plasmid we were able to detect and amplify c-Myc CDS, FLRT2 3'UTR and DSC2 3'UTR mRNA. B) Western blot analysis for IGF2BP1 showing the IGF2BP1 input quality and IP using either of IMP1 (IGF2BP1) or IgG antibody. C and D) RNA was isolated from anti-IMP1 (IGF2BP1), anti-IgG, and Dynabead precleared total cell lysates. RT-qPCR analysis, normalized to the IgG control, identified a significant increase of DSC2.3'UTR mRNA (C) and FLRT2.3'UTR (D) in clone 4 and Pool compared to the empty plasmid (pLVX-Puro). D) Bars represent mean and standard deviation of 3 replicates. Statistical significance was determined using one-way ANOVA and Dunnett's range test (* = $p < 0.05$).

3.4 Discussion

The present study explores the role of the mRNA binding protein IGF2BP1 in the development and progression of OS. Previously, our lab identified IGF2BP1 as a biomarker associated with poor outcome in canine OS tumors with a short disease-free interval (DFI) relative to tumors with a long DFI. Based on both microarray analysis and RT-qPCR, IGF2BP1 expression levels were upregulated in poor responders (DFI<100 days) relative to tumors with a good response by 7-fold [10].

Using four human OS cell lines and isolated human osteoblasts, we measured *IGF2BP1* mRNA transcripts by RT-qPCR and found that the OS cell lines expressed higher levels of *IGF2BP1* compared to human osteoblasts. Of particular interest was the MG63.2 cell line, a metastatic variant of the MG63 cell line, which displayed 17.8-fold higher levels of *IGF2BP1* compared to its parental, low-metastatic line [59].

Our preliminary data used shRNA technology to knock down IGF2BP1 expression levels in the MG63.2 cell line (Kalet *et al*., unpublished data). Using these stable knockdown cell lines, we measured decreased cellular proliferation. In support of these results, IGF2BP1 knockdown has also been shown to reduce cellular proliferation in melanoma cells [19]. Since IGF2BP1 is known to stabilize c-MYC, and KRAS mRNAs [60, 61], decreased expression of these factors upon the loss of IGF2BP1 may account for the reduced cellular proliferation and induced apoptosis [30]. We also found that stable *IGF2BP1* knockdown decreased cellular migration. These data agree with many studies demonstrating that IGF2BP1 directs lamellipodia formation and cell migration, and enhances cellular polarization suggesting mechanisms through which knockdown *IGF2BP1* may reduce OS cellular migration [59, 62, 63]. The decrease in cellular invasion and

migration in the stable *IGF2BP1* knockdown lines suggests that these two abilities are enabled by IGF2BP1. However, the reduction of invasion was only observed in one of the IGF2BP1 knockdown clones suggesting that the role of IGF2BP1 in tumor invasion is likely due to cytoskeleton remodeling rather than protease secretion. Moreover, a recent study published in 2019 explored the impact of knockdown or overexpression of IGF2BP on the microenvironment and the development of melanoma lung metastases through extracellular vesicle (EV) secretion, also known as exosome formation [64]. Interestingly, the overexpressed IGF2BP1 melanoma cell lines released exosomes that had a significant role in the formation of distant metastases [64, 65]. Another publication describes reduction in OS tumor growth and pulmonary metastasis in nude mice upon antagonism of the Wnt signaling pathway [66]. Since IGF2BP1 expression was elevated by Wnt/β-catenin signaling [67], the authors' observed reduction in OS growth and metastasis could be at least partially due to a reduction in IGF2BP1 expression levels.

We previously observed greater than 10-fold increase in chemosensitivity to doxorubicin in IGF2BP1 stable knockdown cell lines compared to the control (Kalet et al., unpublished data) Similarly, knockdown of IGF2BP1 in melanoma cell lines increases sensitivity to Vemurafenib, an inhibitor of an oncogenic driver mutation (BRAFV600E), by 6-fold. This combination induces apoptosis and significantly reduces tumorigenic characteristics of the melanoma cell lines. Even in a Vemurafenib-resistant cell line, which is a useful model because most patients will develop resistant cells after treatment, knocking down IGF2BP1 is sufficient to reduce cell proliferation, cell cycle progression, and resensitizes the cells to Vemurafenib [68].

Since a wide variety of transcripts are targeted by IGF2BP1, other mechanism(s) of action may also play a role in cell survival and resistance to therapy. Craig et al. and others found that IGF2BP1 can bind and stabilize MDR1. MDR1, Multi-Drug-Resistance Factor 1, is an efflux

pump that recognizes and exports doxorubicin, taxol, and vinblastine, but fails to recognize carboplatin [25, 69]. Boyerinas et al. explain the altered chemosensitivity data in their ovarian carcinoma model by suggesting that IGF2BP1 stabilizes MDR1 mRNA transcripts, thus increasing the expression levels of MDR1 [25]. MDR1 stabilization by IGF2BP1 was also documented by Sparanese et al. [70]. In support of a role for MDR1 in the chemoresistance of canine osteosarcoma, we found that overexpressed IGF2BP1 increased the expression of MDR1 in the McKinley cell line and reduced their sensitivity to doxorubicin. Since MDR1 does not recognize carboplatin, this may explain why IGF2BP1 knockdown in our previous study increased sensitivity to doxorubicin, but not carboplatin.

In this study, we overexpressed IGF2BP1 in canine OS (McKinley) and showed opposing results from the knockdowns. Specifically, IGF2BP1 overexpression increased cellular proliferation in McKinley IGF2BP1 overexpressing pool cells, but not in individual clones. Clones 3 and 4 each express a higher level of IGF2BP1 than the pool, but do not exhibit increased proliferation. One potential explanation is that in the single clonal isolates, we may have selected for one possible insertion of the plasmid in the genome that may interfere with other genes that play a role in cellular proliferation. We did not design the plasmid with any specific instruction on where the vector (DNA) should be integrated which is one downfall of using this method.

Another explanation is that IGF2BP1 overexpression in the clones restricted progression through the cell cycle or forced the cells into dormancy. One study found that epithelial-mesenchymal transition (EMT) programming induces tumor movement and invasion, cancer progression, treatment resistance, and restricts the cell cycle [41]. Arresting the cells at G1/G0 results in a reduction in cellular proliferation and decreases the cell's response to chemotherapy, as well as increasing metastatic ability and cancer progression [41]. IGF2BP1 further promotes

mesenchymal phenotypes by enhancing LEF1 mRNA stability. LEF1 interacts with SNAI2 (SLUG) transcription factors to directly repress E-cadherin by binding to the specific E-boxes of its proximal promoter. Loss of E-cadherin serves as a marker of EMT. EMT is characterized by loss of differentiation, reduced cellular proliferation, and thus highly metastatic cancer cells [41, 71].This programming to be more chemotherapy-resistant can be seen clearly with the *IGF2BP1* overexpression clones, which show a significant positive correlation between *IGF2BP1* transcript levels with doxorubicin resistance.

We confirmed these results by showing that the highest overexpressing IGF2BP1 clone (IGF2BP1.pLVX-C4) was 1.6-fold more resistant to doxorubicin and had up to 3-fold augmented MDR1 transcript expression compared to the empty plasmid. These data suggest that IGF2BP1 may play a role in chemoresistance to doxorubicin in both human and canine OS by stabilizing MDR1 mRNA transcripts. Since IGF2BP1 expression levels inversely correlate with DFI in OS patients receiving platinum- and/or doxorubicin-based drugs as the standard of care [10, 39], these results help to establish potential mechanisms by which overexpression of IGF2BP1 may play a role in the decreased DFI of OS patients.

Previous work has shown that IGF2BP1 can bind to mRNAs that are involved in cytoskeletal formation. For instance, IGF2BP1 binds to the mRNAs of the Arp2/3 protein complex, which is responsible for actin polymerization, and that of the TAU, which maintains and stabilizes the microtubule and directs cell polarity [30]. In the current study, overexpressing IGF2BP1 increased cell mobility, as illustrated by the increased ability to close the wound in the scratch assay in the presence (to assess invasion) and absence of Matrigel.

Using Affymetrix Canine 1.0ST microarrays to assess changes in gene expression upon the overexpression of IGF2BP1, we found 162 differentially-expressed genes (FC $\geq$2, FDR$<$ 0.05)

between C4, pool and the empty plasmid (control). Our pathway analysis found that these genes were enriched for epithelial-mesenchymal transition, a hallmark of cancer and indicator of tumor aggressiveness and poorly differentiated cells. Moreover, the enriched pathways identified using Gene Ontology terms are: regulating the cell extracellular matrix, cell extracellular space, and cytoskeletal regulation, cell junction, neuron parts, projection and intrinsic components of the plasma membrane. Moving forward with the differentially expressed genes, we cross-referenced them with the RIP database to identify direct mRNA targets bound by IGF2BP1 [41]. Out of the 162 genes, 13 genes directly bind to IGF2BP1. Pathway analysis for these 13 genes (Table 3.2) was similarly enriched for processes involved in the regulation of cell adhesion, migration, and extracellular matrix.

Increased tumorigenesis is associated with the ability to remodel cytoskeletal architecture, detach from the primary tumor, and, through increased cell motility and invasiveness, migrate into different locations in the body [72]. The function of IGF2BP1, during fetal development, is to regulate the migration of cells in the neural crest, branchial arches, cranial neural crest (CNC) and axonal guidance [73]. With those functions IGF2BP1 protein can interact with the mRNAs of various oncogenes that have a great impact on cancer progression [11]. Also, IGF2BP1 protein induces EMT characteristics, such as, cell migration, invasion, and drug resistance, resulting in metastasis and cancer progression [41, 71]. Functional evaluation of the 13 direct targets of IGF2BP1 highlights these areas.

Elevated expression of the following direct IGF2BP1 target genes was associated with overexpression of IGF2BP1. Integrin subunit alpha 2 (ITGA2), an EMT marker, was significantly upregulated in the overexpressing clones. This gene encodes the alpha subunit of a transmembrane receptor for laminin, collagen, fibronectin, and E-cadherin. ITGA2 plays a role in synthesizing the

extracellular matrix, regulating actin dynamics, axon guidance, and activation of the MAP-ERK pathway [43]. We believe that IGF2BP1 may induce cell migration by protecting ITGA2 mRNA in OS as ITGA6 has also been shown to be a target for IGF2BP1and regulates cell motility [74].

Furthermore, a component of intercellular desmosome junction 2 (DSC2) is another target for IGF2BP1 that is significantly increased in expression in the clones as compared to the control. DSC2 is important to maintaining tissue integrity and promotes an invasive phenotype by remodeling the cell-to-cell attachment and cytoskeleton rearrangement [48] and is one of the top markers of EMT.

FLRT2 (Fibronectin Leucine Rich Transmembrane Protein 2) is also upregulated in the overexpressed IGF2BP1 clones compared to the empty plasmid. FLRT2 also functions in cell-to-cell adhesion, aids in cellular migration and axon guidance, and regulates the development of the embryonic vascular system [49, 75].

Another mRNA that interacts with the nervous system that is also upregulated by IGF2BP1 overexpression is UGT8. (UDP Glycosyltransferase 8) is a key enzymatic regulator of ceramide. Overexpression of UGT8 is an indicator of tumor aggressiveness, and a potential marker for lung metastases in breast cancer, which is also the main site of metastasis in OS [53, 54].

Tandem C2 Domains, Nuclear (TC2N) was also targeted by IGF2BP1 as this gene is extremely overexpressed in our clones. TC2N contains a C2 domain similar to protein kinase C which is a calcium binding domain involved in protein-protein interactions and signal transduction [56-58]. In 2018, a study identified the function of TC2N as an oncogene that was overexpressed in lung cancer, and almost 90% of human and dogs with OS develop lung metastases. Moreover, when TC2N knockdown cells were treated with doxorubicin cell cycle arrest and cell death were induced [76]. Doxorubicin is also an OS treatment, and we have observed increased sensitivity to

doxorubicin with IGF2BP1 knockdown. TC2N also antagonizes the p53 pathway by preventing Cdk5 (Cyclin-Dependent Kinase 5) from phosphorylation and degradation by p53 to arrest the cell cycle, and vice versa p53 can also inhibit TC2N [76]. So, we hypothesize that the ability of IGF2BP1 to sustain TC2N mRNA, as well as MDR1 mRNA, may serve to maintain drug resistance and prevent apoptosis in response to p53.

Lastly, PKIB mRNA that is also overexpressed in IGF2BP1 clones but not the control. Cyclic AMP-dependent protein kinase inhibitor-β is a protein kinase inhibitor that is strongly correlated with breast cancer and promotes tumor aggressiveness in prostate and non–small cell lung cancers [52]. It is believed that PKIB promotes metastasis through the phosphatidylinositol 3-kinase (PI3K)/Akt pathway that is also a key player in OS progression [51].

We think that as IGF2BP1 increases the transcripts of FLRT2, UGT8, DSC2, TC2N and PKIB there is a resulting increase in cellular metastasis, cancer progression, and poor response to treatment through epithelial-mesenchymal transition (EMT) programming

One of the targets decreased by IGF2BP1 that we identified was MAP2K6 (Mitogen-Activated Protein Kinase Kinase 6, also known as MKK6. Also, another study found that the ability of IGF2BP1 to negatively regulate MAPK4 (Mitogen-Activated Protein Kinase 4), a paralog to MKK6, altered actin regulation to promote migration velocity [15, 42] . MKK6 activation also plays a significant role in regulation of the Rho family GTPases that are required for the formation of lamellipodia and filopodia, via actin polymerization [77].

Finally, using the *IGF2BP1* knockdown cell lines, we measured tumor growth in nude mice and found a dose-dependent reduction of palpable tumors and tumor growth with IGF2BP1 knockdown. While several manuscripts describe the effect of *IGF2BP1* knockdown on colony

formation and cellular proliferation, this is the first study describing reduced formation and growth of OS tumor xenografts with *IGF2BP1* knockdown.

In summary, we have identified elevated expression of IGF2BP1 as a biomarker for OS by comparing expression levels in OS tumor samples and normal bone via microarray and RT-qPCR. The increased elevation of IGF2BP1 mRNA transcripts in OS samples taken from patients with DFI>300 days as compared to samples taken from patients with DFI<100 days also points to IGF2BP1 expression as a prognostic indicator in OS. We identified similar patterns of IGF2BP1 expression in human OS cell lines compared to human osteoblasts, particularly in the highly metastatic line MG63.2. Knockdown of IGF2BP1 in MG63.2 resulted in reduced cellular proliferation, migration, and invasion, increased sensitivity to doxorubicin, and reduced OS tumor growth in a nude mouse model. Further, overexpression of IGF2BP1 in canine OS cells led to significant increases in cellular migration, invasion, and reduced drug sensitivity to doxorubicin.

Additionally, we found 162 genes that are overexpressed in response to IGF2BP1. Thirteen of these genes are directly bound to IGF2BP1 and are enriched for pathways (Gene Ontology) involved in cellular adhesion, migration, the extracellular matrix and space, and kinase activation and inhibition. Most interestingly, there was enrichment for epithelial to mesenchymal transition, one hallmark cancer pathway, which may be related to the observed cellular migration, invasion, and reduced drug sensitivity.

Taken together, these data suggest that IGF2BP1 drives tumor growth, metastasis, and chemoresistance. Future studies exploring the mechanisms of IGF2BP1 regulation, and its role in cancer progression will provide valuable new insights into the mechanisms that drive OS progression and lead to improved therapeutic strategies for the treatment of metastatic OS.

3.5 Conclusion

IGF2BP1 was previously identified as a poor prognostic biomarker in canine OS. The present study has explored the role of IGF2BP1 in the development and progression of human and canine osteosarcoma. IGF2BP1 expression has been associated with tumor growth and cellular migration, invasion, and therapeutic sensitivity. IGF2BP1 increased expression of transcripts associated with the extracellular matrix and epithelial to mesenchymal transition implicating IGF2BP1 as a driver and potential target for the prevention of OS metastasis.

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CHAPTER 4: GENERAL CONCLUSIONS

The studies presented in this dissertation assess the mechanisms of regulation and functional relevance of Insulin-like growth factor mRNA binding protein 1 (IGF2BP1), a factor that is elevated in canine osteosarcomas (OS) from patients with a short disease-free interval. OS is the most common primary bone tumor in both dogs and children. However, while the incidence of OS is very low in children with only 900 children diagnosed in the United States each year, it is much more common in dogs with an estimated 10,000 dogs diagnosed each year. The relative rarity of OS in humans has hampered extensive study due to the lack of available samples and a standard of care that implements intense chemotherapy to debulk the tumors prior to surgery. As a result, access to chemotherapy-naïve tumors for study is very limited. Non-surgical treatments for OS have not changed appreciably since the 1980s, with death from this disease due to lung metastasis occurring in approximately 30% of patients. On the other hand, dogs have a high incidence of histologically identical and genetically similar spontaneous OS and share many of the same environmental exposures that humans do. The benefits of using dogs as a model for naturally occurring human OS are documented extensively in the literature.

To identify factors in the primary tumor that could be used as a predictor of resistance to therapy and metastasis in canine patients, we previously evaluated Affymetrix Canine 2.0 microarrays to measure changes in gene expression between primary tumors taken from two cohorts of canine OS patients: those with good responses and those with poor responses following definitive treatment by amputation of the affected limb followed by adjuvant therapy with doxorubicin and/or a platinum-based drug. Patients were selected for these groups based on disease-free intervals (DFI, good responders = DFI>300 days, poor responders = DFI<100 days).

We identified IGF2BP1, Insulin-like Growth Factor mRNA binding protein, as a gene that is highly expressed in tumors taken from patients with a short DFI. This gene is highly conserved between species and is one of three members of this RNA binding protein family. As an oncofetal protein, IGF2BP1 is an essential protein for the embryonic development of nerves and vascular system and is overexpressed in many types of cancer relative to normal tissues. Additionally, it is documented that IGF2BP1 is a poor prognostic indicator for melanoma, breast, ovary, liver, and colorectal cancers. In our studies, we found that it was virtually unexpressed in normal bone, with a progressive increase in tumors from patients with long and short disease-free intervals and that it was overexpressed in a highly metastatic subline of the human MG63 osteosarcoma cell line.

In this dissertation (Chapter 2), we focused on identifying the mechanisms that drive the expression of this oncofetal protein in the human and canine OS, and the functional consequences of IGF2BP1 in canine OS (Chapter 3).

In Chapter 2, our study explored methods for cellular regulation of IGF2BP1, including gene amplification, truncation of the 3'UTR, transcriptional activation, and DNA methylation in human and canine OS.

Our studies of the regulation of IGF2BP1 in canine and human OS indicated that in dogs' tumors, up to 35% of tumors exhibit gene amplification comparing to the total average of IGF2BP1 mRNA expression in 20 tumors. However, only 71.4% of the amplified tumors show a significant elevation of IGF2BP1 mRNA. Moreover, of the 65% of tumors that show no genomic amplification, 23% still have increases in IGF2BP1 transcript. Tumors have been shown to have genomic rearrangements, including aneuploidy because of chromosomal segregation errors during cell division. We observed genomic amplification of in 5 of the 10 of the canine cell lines that correlate with mRNA expression ($p = 0.0006$, Pearson $r = 0.88$). While similar changes were not

observed in the human cell lines, our samples may not have been representative of highly metastatic tumors. Further analysis with a larger number of human tumors and cell lines would be needed to adequately compare findings from our canine studies.

Another method for elevating expression of IGF2BP1 is escaping the post-transcription regulation from the miRNAs by truncating the 6,000 bp 3'UTR of IGF2BP1 mRNA. We identified a loss of the 3'UTR in two of the highest IGF2BP1 expressing canine cell lines (OSA and Abrams) that also have a significant gain of IGF2BP1 protein. This directed us to inspect the functionality of the microRNA (miRNA) in these cell lines. We generated luciferase reporter fragments containing sections of the 3'UTR and found that the region between the end of the coding sequences and the first poly A signal (614bp) produced the highest luciferase activity as compared to the other pieces of 3'UTR, in all of the canine cell lines, especially in OSA and Abrams cell lines. These distal 3'UTR fragments had lower luciferase signal than 614bp, indicating a significant post-transcriptional regulation by miRNAs. We did not identify loss of the 3'UTR in any of the human cell lines, but this finding does not exclude the possibility that IGF2BP1 mRNA is still escaping miRNA regulation using a positive feedback loop. IGF2BP1 may upregulate LIN28B and HMGA2 which block let-7 and prevent it from downregulating IGF2BP1 mRNA in the human cell lines.

Next, we generated a series of luciferase reporter constructs bearing 5' deletions of the human and canine IGF2BP1 promoters and transcription factor-specific reporters. We mapped the critical elements that drive transcriptional activation of the IGF2BP1 proximal gene promoter in subsets of the human and canine cell lines. We found that the essential binding sites of both the canine and human promoters are localized to the proximal ~580 bp relative to the start site of

translation. The most consistent and dramatic loss of activity occurred with the deletion of a highly conserved E-box located within 582bp for the canine and 583bp using the human promoter.

Based on the transcription factor (TF) luciferase reporter assays, we also identified the common TFs in both human and dog promoters that may contribute to the elevation of IGF2BP1: MYC (E box), NF-Kappa B, AP-1, and TCF4: β-catenin (E box). Point mutations in the E box binding site resulted in the loss of activity in this region equivalent to the complete deletion of the area, illustrating the importance of TFs that are capable of binding to the E-box, such as MYC, in the regulation of the IGF2BP1 promoter.

These studies also led us to investigate another mechanism of regulation in our cell lines that failed to express IGF2BP1, did not exhibit any genomic loss of IGF2BP1, and were still capable of activating IGF2BP1 promoter-reporter constructs (canine Gracie and human U2OS). These characteristics suggested that CpG island methylation could play a critical role in suppressing gene expression. To test this mechanism, we treated both canine and human cell lines with 5′-Azacytidine, a chemical reagent that inhibits DNA methyltransferase activity. The treatment significantly increased IGF2BP1 transcripts in the Gracie cell line, but not in the U2OS cell line. Since there was no evidence of genomic deletion or lack of promoter activity in the U2OS line, other mechanisms may serve to suppress expression.

Regarding promoter activation, multiple mitogenic pathways including MYC, β-catenin, and the MAP kinase pathway may drive IGF2BP1 expression acting through E-boxes, TCF-4 binding sites, NF-kB, and AP-1 binding sites. Transcriptional activation may vary depending on the mitogenic driver that contributes to oncogenesis, given that we found higher levels of AP-1 activation in cell lines driven by oncogenic RAS (D17 cell lines). Each of our canine cell lines exhibited significant MYC reporter activity, while only one of the human cell lines did. While this

mitogenic signaling may contribute to IGF2BP1 expression, an important question that remained was, “What was the phenotypic consequence of elevated IGF2BP1 expression, and what targets did it act on to initiate those changes? These questions were addressed in Chapter 3.

In Chapter 3, we overexpressed IGF2BP1 in the canine McKinley cell line and assessed the chemosensitivity, proliferation, and metastatic phenotype in both individual and pooled stable clones. We found that overexpression of IGF2BP1 significantly increased doxorubicin resistance up to 1.67-fold increase and resistance correlated positively with IGF2BP1 expression ($p = 0.029$, Pearson $r = 0.91$). Furthermore, IGF2BP1 expression also increased the cells’ ability to migrate and invade through a layer of Matrigel. These results supported our hypothesis that IGF2BP1 increases the tumorigenic phenotype based on our finding that tumors from patients with the shortest disease-free interval show significantly higher levels of IGF2BP1 relative to tumors taken from patients with a long DFI (7-fold, $p =0.047$).

To explore the mRNA targets of IGF2BP1 that contribute to this phenotype, we collected RNA from the highest IGF2BP1 expressing clone (C4) and the stable Pool, and the empty control plasmid (Plvx-Puro) for Affymetrix Canine 1.0ST gene array analysis. In Chapter 3, we analyzed the microarray data and identified 162 genes that are significantly differentially expressed between the control and both the clone and the Pool. These genes show enrichment for pathways that are involved in cellular adhesion, cell junctions, migration, the extracellular matrix, and the extracellular space.

Furthermore, we identified 13 mRNAs that IGF2BP1 can directly bind to protect and enhance their expression or down regulate their expression by degradation. These 13 genes are enriched in the gene ontology pathways that regulate cell adhesion and kinase activity: MAP2K6, ITGA2, BOC, MMP2, AFF2, PEG10, DSC2, FLRT2, KIAA123L, PKIB, UGT8, TRIB2, and

TC2N. To confirm that these genes are direct targets of IGF2BP1, we performed an RNA immunoprecipitation (RIP) assay to pull down mRNAs binding to IGF2BP1 in the McKinley cells. This mRNA was converted to cDNA and analyzed by RT-qPCR for DSC2, and FLRT2 transcripts. We found significant enrichment of transcripts containing IGF2BP1 binding sites in the 3'UTR of both DSC2, and FLRT2 mRNA in the IGF2BP1 overexpressing clones. Based on the identified IGF2BP1 targets, we believe that IGF2BP1 induction promotes the mesenchymal phenotype or epithelial to mesenchymal transition (EMT).

This EMT phenotype may explain many of our conclusions regarding cellular proliferation, chemoresistance, and migration/invasion. EMT may cause the cell to dedifferentiate and become more mobile and invasive. Although as observed in the clone with high IGF2BP1 expression (C4), it may also be associated with decreased cellular proliferation. Reduced cellular proliferation has also been associated with decreased sensitivity of cancer stem cells to chemotherapy, which is compounded by the observation that IGF2BP1 increased the expression of the multi-drug resistance 1 (MDR1) drug efflux pump.

We believe that IGF2BP1 could serve as a valid target to prevent metastasis in human and canine OS in which this factor is overexpressed. In addition to serving as a poor prognostic biomarker, agents which block the expression or function of this factor might incapacitate the cancer cells by limiting their ability to migrate and invade distant tissues and reduce their drug resistance. Targeting this biomarker may decrease metastases in patients with OS or any cancer with IGF2BP1 elevation.

FUTURE WORK

In the future, I would propose to explore the ability long noncoding RNAs and IGF2BP1 inhibitor, BTYNB, to block IGF2BP1 function in OS. Studies have identified long noncoding RNAs (lncRNAs) that significantly impact IGF2BP1 mRNA levels, or directly interfere with the protein and suppressed its activity such as LINC01093 and HULC. We could also use BTYNB, a novel small molecule inhibitor that prevents IGF2BP1 from binding and stabilizing c-MYC mRNA and β-TrCP1 mRNA that induces NF-Kappa B activity. Using these inhibitors, we could interfere with the function of IGF2BP1, assess the impact on the 13 mRNA that we identified to be directly bound by IGF2BP1, and determine if these agents can decrease the cell resistance to chemotherapy and metastatic phenotype in OS.