ALPHA-CaMKII-INDUCED VEGF EXPRESSION IS CRITICAL FOR THE GROWTH OF HUMAN OSTEOSARCOMA

by

PAUL G. DAFT

MAJD ZAYZAFOON, MENTOR
RALPH D. SANDERSON, COMMITTEE CHAIR
DOUGLAS R. HURST
JOSEPH G. PRESSEY
KURT R. ZINN

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ALPHA-CaMKII IS CRITICAL FOR THE GROWTH AND METASTASIS OF
HUMAN OSTEOSARCOMA

PAUL G. DAFT

MOLECULAR AND CELLULAR PATHOLOGY

ABSTRACT

Osteosarcoma (OS) is among the most frequently occurring primary bone tumors, primarily affecting adolescents and young adults. Despite improvements in OS treatment, more specific molecular targets are needed. One target of interest is alpha-Ca\(^{2+}\)/calmodulin-dependent protein kinase II (α-CaMKII), a ubiquitous mediator of Ca\(^{2+}\)-linked signaling, which has been shown to regulate tumor cell proliferation.

Here, we show that α-CaMKII is highly expressed in primary OS tissue, and α-CaMKII deletion in human OS cell lines significantly reduces tumor burden in vivo. This inhibition of α-CaMKII results in decreased vascular endothelial growth factor (VEGF) protein secretion. Highly aggressive OS cells express VEGF receptor 2 (VEGFR-2), and respond to exogenous VEGF by increasing intracellular calcium. This response is ameliorated by CBO-P11 (VEGFR inhibitor), suggesting that secreted VEGF results in autocrine stimulated α-CaMKII activation. Moreover, CBO-P11 and the selective CaMKII inhibitor, KN-93, significantly reduce tumor burden in vivo, suggesting that OS tumor growth is controlled by CaMKII-regulated VEGF.

We expound on these initial studies and show that the FDA approved drugs tamoxifen and bevacizumab inhibit CaMKII, and decrease in vivo tumor growth.
Furthermore, we developed a novel preclinical mouse model to examine the metastasis of human OS. After amputation of the tumor containing hind limb, the incidence of pulmonary metastasis in saline treated mice was 100%, with no detectable metastases when mice are subcutaneously injected daily with tamoxifen (10 mg/kg/day) and twice weekly with bevacizumab (2 mg/kg/day). These results suggest that tamoxifen and bevacizumab may be effective at inhibiting primary tumor growth and preventing metastasis.

OS cancer stem cells (CSCs) have been shown to express high levels of c-kit and Stro-1. Using fluorescence-activated cell sorting, we isolated CSCs (CD117+, Stro-1+) and double negative cells (DNCs) (CD117− and Stro-1−) from 143B OS cells. We show that 100% of mice injected with CSCs develop tumors and only 25% of mice injected with DNCs did so. Taken together, our results show a critical role of CaMKII in primary and metastatic tumor growth of human OS.
DEDICATION

I dedicate this dissertation to my brother Joe. You helped spark my interest in science and are the best role model a younger brother could have.
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INTRODUCTION

Osteosarcoma

Human osteosarcomas (OS) are the most common primary malignant bone tumors (1). These highly aggressive neoplasms are composed of spindle shaped cells, which produce excessive amounts of immature tumor osteoid (2). OS are the sixth most common cancer in children and adolescents, and have only a 65% 5-year event free survival rate (3). The current management of OS is still associated with radical surgery involving excision of the primary tumor and/or amputation of the affected area, followed by painful and expensive chemotherapy (4). This dissertation will explore emerging targets for OS treatment, and examine the therapeutic potential of Ca$^{2+}$/Calmodulin dependent protein kinase two (CaMKII) inhibition for the treatment of this devastating disease.

Diagnosis and Staging

The incidence of OS in the general population is 1 in 400,000, but is higher in adolescence, in which the annual incidence peaks at 1 in 100,000 at 15–19 years of age. Because bone growth is most rapid in this age group, it is speculated that a relationship exists between OS development and the adolescent growth spurt. The skeletal growth period is longer in males than in females; hence, OS occurs 1.4 times more frequently in
males than in females. Additionally, males also have a slightly later disease onset, which may coincide with a later growth spurt (5).

Local pain, followed by localized swelling and limitation of joint movement, are the most common signs and symptoms of OS. In rare cases, particularly in patients with osteolytic tumors, a pathological fracture can be the first sign of disease (6).

Approximately 15% of patients present with radiographic metastases, most commonly to the lung. Furthermore, 80% of patients will develop pulmonary metastases after surgery if multiagent chemotherapy is not administered. Thus, it is assumed that all patients have undetectable microscopic metastases (7). Although OS can occur in any bone, it is most common in the metaphyses of long bones. The most common primary sites are the distal femur, the proximal tibia, with 50% originating around the knee. The evaluation of a patient with suspected OS begins with a full history, physical examination, and radiographs. Radiography is helpful to describe osseous changes: OS can present with osteoblastic, osteolytic or mixed appearance. They often have a soft tissue component in which patchy calcifications resulting from new bone formation may be observed (8). A triangular area of periosteal calcification in the border region of tumor and healthy tissue is known as a Codman triangle, and is commonly observed in OS. Magnetic resonance imaging (MRI) is the best modality to assess the soft tissue component, its relationship to surrounding tissues, vessels and nerves, and its intramedullary extension, which is essential for safe definitive surgery. MRI has to include the whole involved bone as well as the neighboring joints. This is done to avoid synchronous regional bone metastasis, which is often referred to as skip metastasis (9).
There are two main grading systems used for OS. The Musculoskeletal Tumor Society staging system is based on tumor grade (I = low-grade; II = high-grade), extension (A = intra-compartmental; B = extra-compartmental), and metastatic lesions (grade III). The presence of metastatic disease, results in a grade III diagnosis. The American Joint Committee subdivides stages I and II into categories A and B based on tumor size being greater or less than 8 cm, instead of tumor extension. Stage III is used for any tumor with skip metastases. In addition to stages I, II and III, the American Joint Committee system also uses an extra stage IV, which is divided into IVA for pulmonary metastases, and IVB for other metastases (3).

Current Treatment Protocol

In OS the primary objective of surgery is complete tumor excision; conservation of the limb is the secondary objective, while amputation is a last resort. Limb salvage success continues to increase with better surgical methods and technologies. This is accomplished by careful resection of the tumor, followed by the reconstruction of a functional limb. With the development of modern surgical procedures and chemotherapy regimens, the disease-free survival rate substantially increased and limb removal continues to be more infrequent (10).

Before the 1970’s, when the treatment for OS was mainly limb amputation, the 5-year survival rate of patients was 10–20%. However, the combination of surgery and modern multi-agent, dose-intensive chemotherapy drastically increased the 5-year survival rates of patients to roughly 65% (11). The current chemotherapeutic protocols for OS typically include neoadjuvant (preoperative) therapy, followed by adjuvant (postoperative) therapy. The most commonly administered chemotherapy drugs include
cisplatin, doxorubicin, ifosfamide, and high-dose methotrexate. Unfortunately, the use of multiagent chemotherapy is associated with acute and long term toxicities. These side effects include but are not limited to: hearing loss, hypomagnesemia, cardiomyopathy and sterility. In addition to the many side effects, patients who relapse after treatments have a low probability of survival due to the lack of second line chemotherapeutics (12). These issues have led many researchers to try and find effective alternative treatments to fight this devastating disease.

Emerging Targets

Efficient molecular target drugs are promising from the viewpoint of maximizing the treatment potential for cancer, while also limiting toxicity. The promise of these targeted drugs is their ability to more selectively target tumor cells without harming healthy cells. The following targets are just a few that have been proposed by OS researchers and may warrant further investigation.

Interferon-γ is known to enhance the sensitivity of OS to a number of chemotherapeutic drugs, and it plays an important role in angiogenesis (13, 14). Studies have demonstrated that a combination of interferon-γ and conventional chemotherapeutic agents such as doxorubicin may be used for the management of OS with functional p53. It has also been demonstrated that interferon-γ sensitizes OS cell lines via the upregulation of Fas receptors and caspase-8, and that combined immunotherapy with IFN-gamma and either anti-Fas monoclonal antibody or cytotoxic T cells that express Fas ligand might be a useful adjunctive therapy for patients with OS. Interferon-γ 1b is approved by the U.S. Food and Drug Administration (FDA) for treatment of chronic
granulomatous disease and osteopetrosis, and may be worth examining for OS treatment (15).

Early *in-vitro* studies show that the Notch pathway downregulates osteoclastogenesis/osteoblastogenesis and plays a critical role in OS pathogenesis. In 2010, it was reported that Deltex1 blocks OS invasiveness by binding the intracellular domain of Notch and ubiquitinating the Notch receptor (16). This mechanism may have important clinical significance for targeting the Notch signaling pathway in OS.

Curcumin, an inhibitor of Notch, induces G2/M arrest in OS cells. This inhibitory effect is accompanied by downregulation of Notch-1 and its targets, including several matrix metalloproteinases (MMPs), thereby preventing cell invasion. Thus, using curcumin to decrease Notch-1 signaling may be a useful strategy for OS treatment (17).

Insulin-like growth factors (IGFs) are involved in cell differentiation and growth, and their association to OS’s rapid bone growth has been clearly defined (18, 19). Overexpression of IGF-1 and IGF-2 was found to be present in a number of cancer cell lines, including primary sarcomas. The usefulness of the molecules that target the IGF-1 receptor is currently being investigated in a number of preclinical and clinical studies. OSI-906, which specifically targets the IGF-1 receptor, has shown success in clinical trials (20). The molecule exhibited inhibitory activity in an IGF-1 receptor-dependent xenograft model in preclinical studies (19). Cixutumumab, a fully human IgG1 monoclonal antibody directed against the human insulin-like growth factor-1 receptor, is well tolerated in children with refractory solid tumors. Limited single-agent activity of cixutumumab was observed in phase 2 clinical trials; however, prolonged stable disease was observed in only 15% of patients (21). Ongoing studies evaluating the toxicity and
benefit of cixutumumab in combination with other agents that inhibit the IGF pathway are underway.

Another promising approach may be the use of the mammalian target of rapamycin (mTOR) inhibitors in OS (22, 23). mTOR is a key component of the AKT signaling pathway, which plays an important role in cell growth and proliferation. The inhibitory activity of rapamycin against tumor cells has been demonstrated in murine OS models and in pediatric clinical studies. Research has shown that rapamycin strongly inhibits cell growth by increasing the number of cells in the G1-phase and decreasing the number of cells in the S-phase of the cell cycle, which also results in the downregulation of cyclin D1 and mTOR (24).

The anti-VEGF antibody bevacizumab is FDA approved for colon, lung, renal, breast, ovarian and glioblastoma (25-29). VEGFA is abundantly expressed in 74.1% of OS cases, and patients with VEGFA-positive OS had significantly worse tumor-free survival rates than patients with VEGFA-negative OS (30). Thus, antivascular therapy might be an effective treatment for OS patients. Endosialin, a protein highly expressed during angiogenesis in the embryo, uterus and in tumor development and growth, is highly expressed in HOS OS cell lines, suggesting it may play a role in OS angiogenesis (31).

Though OS is a relatively rare disease, it continues to be extensively studied due to limited advances in treatment over the past 25 years. The molecular targets mentioned above are just a handful of the proteins that could lead to more effective treatments. Some other proteins being examined include but are not limited to: human epidermal growth factor receptor two, tyrosine-protein kinase transmembrane receptor two, nuclear factor
kappa-light-chain-enhancer of activated B cells, and signal transducer and activator of transcription 3. Our lab has been particularly interested in the role of CaMKII in the regulation of the growth and metastasis of human OS.

**Calcium/Calmodulin-dependent Protein Kinase Two**

Calcium (Ca$^{2+}$) is a universal second messenger in eukaryotic cells. Ca$^{2+}$ plays an essential role in vesicle fusion, muscle physiology, glycogenolysis, mitochondrial respiration, endocytosis, neurotransmitter synthesis, and gene transcription (32). The predominant intracellular receptor for Ca$^{2+}$ is calmodulin (CaM), a highly conserved Ca$^{2+}$ sensor that is ubiquitously expressed in mammalian cells and belongs to the EF-hand family of Ca$^{2+}$-binding proteins (33). Among the numerous Ca$^{2+}$/CaM-activated enzymes, protein kinases are a prominent class of proteins that alter the function of key cellular proteins by phosphorylation. CaM, has been found to activate a novel membrane-bound kinase that phosphorylates numerous proteins in brain and other tissues (34). This membrane-bound enzyme is also found in the cytosolic fraction and is now recognized as the multifunctional CaMKII.

The designation II in CaMKII comes from the fact that it was the second Ca$^{2+}$/CaM-dependent protein kinase peak eluted from a sizing column used to fractionate rat brain cytosol (35); it is also the Ca$^{2+}$/CaM-dependent protein kinase that phosphorylates site II on synapsin (36). The CaM kinases purified from cytosol of brain (37), liver, and muscle cells using a variety of substrates, including synapsin I, myosin light chains, tryptophan hydroxylase, tubulin (38), microtubule-associated protein 2 (39),
and casein (40), all turned out to be various isoforms of the same enzyme (CaMKII).

Unlike the other CaMK family members, CaMKII does not appear to require activation loop phosphorylation for activation. CaMKII undergoes autophosphorylation, a process that regulates its dependence on and affinity for its activator, Ca\(^{2+}/\text{CaM}\), as well as its intracellular targeting (41).

**CaMKII in Cancer**

The biologic function of CaMKII is cell type and disease state dependent. In neuronal cells, it is involved in regulating postsynaptic signaling complexes, neurotransmission and memory. In fibroblasts and myocytes, CaMKII has been shown to modulate insulin signaling (42). Much like the biologic function of CaMKII is cell type dependent; it also appears that its biologic function varies greatly from cancer to cancer. In medullary thyroid cancer CaMKII is activated by RET mutants where it mediates the oncogenic pathway leading to cell proliferation. The mRNA expression of its endogenous inhibitor hCaKIINα inversely correlates with the severity of disease. Therefore, CaMKII might represent a new target for medullary thyroid cancer therapy (43).

It has been demonstrated that increases in Anoctamin-1 (ANO1) contributes to breast cancer, head and neck squamous cell carcinomas (HNSCC), and epithelial squamous cell carcinoma (ESCC) by activating EGF receptor (EGFR) and CAMKII, subsequently inducing activation of AKT and mitogen-activated protein kinase 1 (MAPK) signaling. Moreover, knockdown of ANO1 in ANO1-amplified breast cancer, HNSCC, and ESCC cell lines inhibited cell proliferation, induced apoptosis, and reduced tumor growth in established xenografts. Thus, inhibiting ANO1 results in decreased
CaMKII activation, which leads to decreased cell survival and proliferation in HNSCC, ESCC, and breast cancer (44).

Store-operated Ca\(^{2+}\) entry (SOCE) is a major mechanism of Ca\(^{2+}\) import from extracellular to intracellular space, involving detection of Ca\(^{2+}\) store depletion in the endoplasmic reticulum (ER) by stromal interaction molecule (STIM) proteins, which then translocate to plasma membrane and activate the Ca\(^{2+}\) release-activated Ca\(^{2+}\) channel protein (ORAI). It has been shown that STIM1 and ORAI1 isoforms are abundantly expressed in human melanoma tissues and multiple melanoma/melanocyte cell lines. Induction of SOCE was associated with activation of extracellular-signal-regulated kinase (ERK), and was inhibited by inhibitors of CaMKII, suggesting that SOCE-mediated cellular functions are controlled by the CaMKII/ERK signaling pathway. (45).

**CaMKII in Osteosarcoma**

\(\alpha\)-CaMKII was once thought to be exclusively expressed in neuronal tissue, but over the past 15 years several labs have shown its expression in various extraneural cells. One of the earlier studies to show its importance in bone examined the expression of CaMKII in osteoblast-like cells (MC4) during osteoblast differentiation. These studies showed that CaMKII, specifically the alpha isoform, is expressed in osteoblasts both in vitro and in vivo. Inhibition of CaMKII by trifluoperazine or KN93 resulted in decreased alkaline phosphatase activity and mineralization, as well as decreases in osteocalcin gene expression (46). Using the newborn mouse calvaria in vivo model, a significant decrease in osteoblast number and decrease in mineralization was observed. Genetic inhibition of alpha-CaMKII by small interfering RNA decreased the expression of c-fos, AP-1 transactivation, and AP-1 DNA binding activity. These findings demonstrated that \(\alpha\)-
CaMKII is expressed in osteoblasts and α-CaMKII inhibition results in decreased c-fos expression and AP-1 activation, leading to inhibition of osteoblast differentiation (46). Others have since shown that CaMKII regulates osteoblast differentiation through osterix and Dlx5 (47, 48).

The clear establishment of α-CaMKII as a critical regulator of osteoblast differentiation led our lab to examine its expression in rapidly proliferating OS tumors. While there have been several papers published to date on CaMKII’s essential role in other cancers, our lab was the first to examine its role in human OS. The alpha isoform (α-CaMKII) was shown to be the most predominantly expressed splice variant in OS cell lines and primary human OS tissue. It was then observed that the pharmacologic inhibition of CaMKII by KN-93 arrested OS cells in G0/G1 phase of the cell cycle. Furthermore, the *in vivo* inhibition of CaMKII by KN-93 significantly reduced the size of OS tumors. This first paper briefly showed that CaMKII regulates the growth of OS, and may have important implications in the development of therapeutic intervention strategies to treat OS (49).

We later expounded on these original observations by showing that p-α-CaMKII is critical in the growth and tumorigenesis of human OS *in vitro* and *in vivo*. We showed that p-α-CaMKII levels are increased in aggressive human OS cell lines. Furthermore, the knockdown of α-CaMKII decreases proliferation, motility and invasion, while α-CaMKII overexpression increases the aggressive phenotype of human OS cell lines *in vitro*. Finally, we showed that the disruption of α-CaMKII positively controls OS growth *in vivo*. These results suggested that α-CaMKII plays a critical role in the growth and aggressiveness of human OS (50).
In order to further elucidate the mechanism by which CaMKII was acting we went on to investigate the roles of both the paracrine and autocrine effects of α-CaMKII-induced VEGF on human OS \textit{in vitro} and \textit{in vivo}. We discovered that VEGF_{165} levels are positively correlated with OS cell line aggressiveness. Furthermore, we determined that α-CaMKII positively regulates the levels of VEGF RNA and protein in OS cells. We demonstrated that the inhibition of both α-CaMKII and VEGF dramatically decreases the aggressiveness of OS \textit{in vitro} and \textit{in vivo}. Our findings show that α-CaMKII-induced VEGF is critical for the growth and aggressiveness of human OS and compounds that inhibit both CaMKII and VEGF might provide novel therapies for treatment.

While our lab’s previous work shows a vital importance for CaMKII in the growth and aggressiveness of human OS, we were using a laboratory drug to inhibit CaMKII (KN-93). Additionally, we were using outdated animal models that failed to examine metastasis in a manner that faithfully mimics that which is seen in the clinic. We showed that inhibiting CaMKII through Tamoxifen resulted in similar decreases in growth as KN-93. Additionally, by injecting mice with human OS and removing the tumor containing hind limb we were able to more accurately study metastatic progression in our mice. Our results show that Tamoxifen treatment results in both decreases in the primary tumor mass and decreases in detectable pulmonary metastasis \textit{in vivo}.

\textit{CaMKII inhibitors}

The most widely used and published inhibitor of CaMKII has been KN-93 (51). KN-93 was originally developed by Hidaka and his colleagues, which are also well known for developing a line of inhibitors for protein kinase A (PKA), protein kinase C (PKC), and myosin light chain kinase (MLCK), many of which have become
commercially available and widely used (52). Unfortunately, these drugs were developed for laboratory purposes and are therefore not optimized for potency, selectivity, or pharmacokinetics. With its inception, KN-93 supplanted KN-62 as the most widely used CaMKII inhibitor. Though different, these two drugs share structural elements and have highly similar mechanisms of action. KN-62 binds to the holoenzyme of CaMKII and interferes with the ability of $\text{Ca}^{2+}/\text{CaM}$ to activate the protein, but does not directly bind to CaM (53). Though KN-93 has a similar structure and function, unlike KN-62, it has been shown to be a CaM antagonist (54). It is thought that KN-93 (and KN-62) likely blocks the ability of $\text{Ca}^{2+}/\text{CaM}$ to wrap around the CaM-binding segment and free it from the catalytic domain. Though these drugs have been shown to be useful tools in the laboratory, there are several caveats that need to be accounted for when using them. The initial characterization of KN-93 and KN-62 showed them to be selective for CaMKII relative to PKA, PKC and MLCK (52), but they were later shown to potently inhibit CaMKI and CaMKIV (55, 56), meaning that KN-93 is more of a general CaMK inhibitor and not CaMKII specific. Additionally, KN-93 is not a very potent inhibitor of CaMKII with an IC50 of 1–4 $\mu$M depending on experimental conditions (52). A recent screen against 234 protein kinases shows that KN-93 also targets Haspin kinase, Tyrosine-protein kinase HCK, lymphocyte-specific protein tyrosine kinase, MLCK, and neurotrophic tyrosine kinase receptor type 1 (57). While useful in the laboratory, the growing body of literature suggests KN-93 has limited potential as a clinical drug due to general lack of potency and selectivity.

CaMKIIN or CaM-KIIN are small endogenous proteins that inhibit CaMKII with high affinity and can be applied pharmacologically. CaMKIIN was discovered by use of a
yeast two-hybrid screen whereby the catalytic domain of CaMKIIβ served as bait to clone interacting proteins from a rat neuronal library. Two small proteins sharing high homology were identified and termed CaM-KIINβ (79 amino acids) and CaM-KIINα (78 amino acids) which are often referred to as simply CaMKIN. It is important to note that the α and β in their names are unrelated to the CaMKII isoform. Both of these proteins have been shown to inhibit all CaMKII isoforms with an IC50 of 50 nM (58). Identification of the core inhibitory domain of CaMKIN led to generation of a 28 amino acid peptide inhibitor termed CaMKINtide, which was subsequently shortened and modified to improve potency (59). CaMKIN and CaMKINtide only bind to the activated conformation of CaMKII (60). The application of these proteins as therapeutic agents is still being explored.

Though specific CaMKII inhibitors do exist, none have been approved for treatment in the clinic. Tamoxifen, an estrogen receptor (ER) antagonist, was proven to reduce the incidence of breast cancer by 49% in at risk women in the Breast Cancer Prevention Trial (61). To examine tamoxifen’s mechanism of action in these patients, normal breast tissue obtained from 42 fibroadenoma patients, randomly assigned to receive placebo or tamoxifen were analyzed. Total RNA was obtained from normal breast tissue from patients with clinical, cytological and ultrasound diagnosis of fibroadenoma. After a 50-day treatment with tamoxifen (10 or 20 mg/day) or placebo, normal breast tissue adjacent to the tumor was collected during lumpectomy with local anesthesia. CaMKII, was found to be down-regulated during tamoxifen treatment (62). These results are extremely exciting because tamoxifen is already FDA approved.
for early and advanced ER positive breast cancers, and also indicates the potential benefit of CaMKII inhibition as preventative medicine.

Berbamine, a natural bis-benzylisoquinoline alkaloid, was identified from the traditional Chinese herbal medicine *Berberis amurensis* and is used for cancer treatment. Berbamine has been shown to have potent antitumor activities with low toxicity in various cancer types, including hepatoma, breast cancer and imatinib-resistant chronic myelogenous leukemia. However, the mechanism of action of berbamine in human cancers remains largely unknown (63). Within the past year there have been multiple publications describing the anti-CaMKII effects of berbamine in liver cancer (64), hepatoma (65), and chronic myeloid leukemia stem cells (66).

**CaMKII inhibition as a therapy**

The inhibitory agents and approaches described above have been essential in identifying key roles of CaMKII in health and disease and make a compelling case for targeting CaMKII for its potential anti-cancer effects. Recently there has been a large increase in the number of protein kinases targeted in oncology, with hundreds in clinical development and many more being currently utilized in the clinic (67).

One of the early programs aimed at developing clinical CaMKII inhibitors started at Scios, and expanded following its acquisition by Johnson and Johnson. When Johnson and Johnson ultimately closed Scios, their efforts towards clinical CaMKII inhibitors were discontinued. Even though these drugs were in early stage development, the program did provide some potent ATP competitive inhibitors, along with structure – activity relationships that enabled an understanding of how to more effectively inhibit CaMKII (68). Their lead compound, a pyrimidine (Scios 15b) with IC50 of 9 nM in
vitro and 320 nM in situ, was a promising candidate. Additionally, Bosutinib which was developed as an ATP competitive inhibitor has shown surprising potency at inhibiting CaMKII. A co-crystal of CaMKII with bosutinib has been published (69).

Dainippon Sumitomo Pharma had the most advanced CaMKII program and developed Rimacalib (SMP-114) for treatment of rheumatoid arthritis (70). It passed the Phase I safety trial, but did not show significant efficacy in a 24-week Phase II trial. Sanofi and Dainippon also developed compounds that were potent CaMKII inhibitors (2–60 nM) but failed to reach the market. Currently, Allosteros Therapeutics is developing both ATP-competitive and ATP non-competitive inhibitors for cardiovascular disease and other indications. A CaMKII program at Myogen (with Novartis) was part of an acquisition by Gilead, but no publications of structures have been released. In addition to the ones mentioned above, there are several other early stage CaMKII inhibitor programs, which will hopefully lead to both tool inhibitors for academic research as well as new chemical entities for treating cancer.

CaMKII is starting to be accepted as a key target in cancer, but the creation of selective inhibitors that are safe and effective for therapeutic use is still a key need. The global market for kinase inhibitors is over $30B, mostly targeting protein tyrosine kinases with both biologics and small molecules. Structure-guided drug design and virtual library and fragment screening are likely to benefit from the recent availability of high resolution crystal structures of CaMKII in various conformations. Targeting the ATP-binding site is the most common approach with small molecule inhibitors; however, specificity becomes a challenge because there are over 500 kinases whose active conformations of the site have a similar shape and amino acid composition. The first generation of CaMKII
therapeutics will likely target the ATP-binding site because of the large body of structural information and medicinal chemistry experience that facilitates the design of relatively selectivity of ATP competitive inhibitors.

**Vascular Endothelial Growth Factor**

Vascular endothelial growth factor (VEGF) is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth, and reproductive functions. In addition to its normal physiological functions, VEGF has also been implicated in pathological angiogenesis associated with tumors and intraocular disorders (71). The biological effects of VEGF are mediated by two receptor tyrosine kinases, VEGF receptor 1 (VEGFR-1) and VEGFR-2, with VEGFR-2 being the main bioactive receptor. Additionally, non-signaling co-receptors such as Neuropilin-1 and Neuropilin-2 (NRP-1/2) also modulate VEGF signaling (72). Currently several VEGF inhibitors are undergoing clinical testing to treat several malignancies. In addition to its potential as an anti-cancer drug, VEGF inhibition is also being tested as a strategy for the prevention of vascular leakage and resulting vision loss in age-related macular degeneration (73, 74).

In addition to promoting endothelial cell proliferation and migration, VEGF has several other pro-angiogenic activities. It induces endothelial expression of proteases such as interstitial collagenase and the urokinase-type and tissue-type plasminogen activators. These proteases release cells from anchorage, allowing migration, which may
promote vascular leakage (75). This vascular leakage is thought to increase tumor cell extravasation, which is a step in the metastatic cascade. Additionally, VEGF promotes the continued survival of nascent endothelial cells (76).

Hypoxia, a state of low oxygen, induces a rapid and strong increase in VEGF mRNA levels, which is particularly noticeable around necrotic areas of tumors (77). Interestingly, other VEGF family members and basic fibroblast growth factor are not induced by hypoxia; therefore VEGF might be the main mediator of hypoxia-induced neovascularization (78). A hypoxia response element (HRE) acts upstream of the VEGF gene as an enhancer. This HRE contains a consensus binding site for hypoxia-inducible factor 1 (HIF-1), a heterodimer of the transcription factors HIF-1α and HIF-1β. The HIF signaling cascade mediates the effects of hypoxia on the cell. HIF-1, when stabilized by hypoxic conditions, upregulates several proteins to promote survival in low-oxygen conditions. One of these proteins is VEGF. HIF-1 acts by binding to HRE on the human VEGF promoter.

The von Hippel-Lindau tumour suppressor (VHL) negatively regulates hypoxia-induced genes, including VEGF. VHL sequesters PKCζ and PKCδ, preventing their translocation to the cell membrane, and subsequent MAPK activation and induction of VEGF. Changes in cell signaling through differentiation might also influence VEGF expression through control of PKC and cAMP/PKA pathways. The VEGF promoter contains binding sites for the transcription factors Sp1, AP-1 and AP-2, through which PKC and PKA can influence gene expression (79).

VEGF in cancer
During embryonic vasculogenesis, blood vessels are formed *de novo*, from endothelial-cell precursors (angioblasts) that assemble into a primary capillary plexus. This primitive network then differentiates, and new blood vessels sprout and branch from pre-existing capillaries (angiogenesis) (80). The vasculature is usually quiescent in the adult, and endothelial cells are among the longest-lived cells outside the nervous system. The few adult tissues that do require ongoing angiogenesis include female reproductive organs and injured tissues (81).

The point at which these ‘normal’ processes differ from pathological angiogenesis is when the tightly regulated balance of pro and anti-angiogenic signals is lost. During normal physiological angiogenesis, new vessels rapidly mature and become stable. By contrast, tumors lose the appropriate balances between positive and negative controls. Tumor blood vessels fail to become quiescent, enabling the constant growth of new tumor blood vessels. Consequently, the tumor vasculature develops unique characteristics and becomes distinct from normal vasculature. Tumor blood vessels are architecturally different from their normal counterparts, and are not organized into definitive venules (82).

**VEGF and CaMKII**

It is well known that many OS are large and highly vascularized tumors. We are the first to propose an autocrine feedback loop in human OS between CaMKII and VEGF, where CaMKII regulates VEGF secretion and in return secreted VEGF causes increases in CaMKII activity. While the supporting literature remains sparse there is a slowly growing amount of interest in the relationship between CaMKII and VEGF.
The barrier function of endothelial cells and tumor angiogenesis are partially dependent upon increases of intracellular Ca\(^{2+}\) through G-protein coupled receptor agonists such as thrombin and VEGF. It has been shown that CaMKII responds to VEGF-stimulated Ca\(^{2+}\) signals and regulates endothelial cell barrier function and angiogenesis. CaMKII has been shown to be highly expressed in cultured human umbilical vein endothelial cells (HUVEC). When CaMKII protein expression was inhibited by siRNA VEGF-induced endothelial cell permeability and migration was greatly decreased. These results suggest that CaMKII may play a role in mediating changes in barrier function in response to physiological relevant doses of VEGF (83).

Additionally, the expression of HIF-1\(\alpha\) in macrophages is regulated by CaMKII pathways. Inhibition of HIF-1\(\alpha\) protein expression and significant inhibition of VEGF production in macrophages was found using the CaMKII inhibitor SMP-114, which in 2006 was in clinical trials as a disease modifying arthritic drug for the treatment of rheumatoid arthritis. This effect may contribute to the anti-arthritic effects of SMP-114 (84).

The relationship between CaMKII and VEGF in cancer is just starting to be investigated. It has been shown that the CaMKII inhibitor KN-62 effectively suppresses the hypoxic expression and activation of HIF-1\(\alpha\), specifically in hepatocellular carcinoma cells, and this HIF-1\(\alpha\) suppression by KN-62 may be attributed to impaired translation of HIF-1\(\alpha\) due to Akt inactivation (84). Though the authors did not measure VEGF expression it is likely that the observed decreases in HIF-1\(\alpha\) would result in decreased hepatocellular carcinoma VEGF expression.

*Anti-VEGF therapy as a treatment for cancer*
Given the requirement of angiogenesis for growth and progression of dormant tumors, it would be assumed that inhibition of the angiogenic switch could prevent progression of tumors and their metastases. Preventative anti-angiogenic strategies could be especially useful in patients who are at high risk for developing cancer or recurrence of cancer. Several experimental studies in animals, as well as in clinical trials, have already shown promising results (85, 86).

The anti-angiogenic drugs field has focused on developing VEGF and VEGFR inhibitors (VEGFIs and VEGFRIs, respectively) (87). While low autocrine VEGF signaling maintains quiescent vessel integrity, increased VEGF/VEGFR2 signaling induces angiogenesis, thereby creating a therapeutic window for anti-angiogenic therapy. Current VEGFI/VEGFRI-based therapies prolong progression free survival and/or overall survival in a fraction of cancer patients. The most commonly used VEGFI, bevacizumab, has shown efficacy as a neoadjuvant to chemotherapeutics in breast and colorectal cancers (88).

Despite the success of anti-angiogenic drugs, several questions warrant further research to improve cancer treatments. Even in responsive patients, anti-angiogenic drugs generally prolong survival by only a few months. Additionally, the FDA recently revoked the approval of Bevacizumab for metastatic breast cancer, because its risks were deemed to outweigh its benefits. In general, clinical efficacy is lower than that observed in preclinical cancer models, and the exact reasons remain to be elucidated. One possible explanation is that these models often represent rapidly growing ectopic tumors that do not reflect the heterogeneous human cancers which develop over years in situ. Even transgenic models do not fully reflect the multistep carcinogenesis that occurs in humans.
Another concern is that the majority of preclinical studies were undertaken in the neoadjuvant setting, which is a poor model for human metastatic cancer (89).

One mechanism underlying resistance is that tumors produce multiple pro-angiogenic molecules in addition to VEGF, including Phosphatidylinositol-glycan biosynthesis class F protein (PIGF), fibroblast growth factors (FGFs), interleukin-8, and others. Several pro-angiogenic molecules become upregulated under selective pressure by VEGFIs/VEGFRIs. PIGF and FGF2 plasma levels increase prior to progression of colorectal cancer in patients treated with bevacizumab and chemotherapy. A phase III trial reported the efficacy of Aflibercept, which blocks VEGF and PIGF, in patients who progressed while on Bevacizumab therapy (90).

Another hypothesis to explain the lower than expected efficacy of VEGF-targeted anti-angiogenic drugs is that these treatments increase, rather than reduce, tumor malignancy. Certain preclinical studies show enhanced metastasis in tumor-bearing mice treated with VEGF-blocking drugs, such as Sunitinib (91, 92). However, these findings remain debated because the majority of preclinical studies have not detected increased metastasis, and large meta-analyses have not shown more metastatic dissemination in patients treated with VEGFIs (93). Strategies combining anti-angiogenesis with inhibition of metastasis might be useful to increase therapeutic efficacy. VEGF inhibitors can also cause class-specific side effects thromboembolic events, hypertension, gastrointestinal perforation, impaired wound healing, renal toxicity, and congestive heart failure by depriving quiescent endothelial cells of VEGF’s pro-survival effects (94).

There still appears to be great promise in anti-VEGF therapy becoming a frontline defense against cancer, but we must first have a greater understanding of why our efforts
are failing or succeeding. Numerous areas of clinical research are of high priority, including the optimization of drug regimes, the use of predictive biomarkers to identify putative responders versus non-responders, the development of anti-angiogenic treatment of pediatric tumors, the development of vessel normalization drugs, and the development of VEGF-independent anti-angiogenic drugs that can be used in combination with existing anti-angiogenic therapies. The development of allosteric anti-angiogenic inhibitors, which offer a superior advantage of safety, specificity, and efficacy over current orthosteric anti-angiogenic antagonists, is also commendable (95). Finally, more bedside-to-bench studies are needed to provide the necessary feedback needed to further improve the overall efficiency of anti-angiogenesis therapy.

**Cancer stem cells**

In the cancer stem cell (CSC) model of tumors, it is hypothesized that there is a small subset of cancer cells, which constitute a reservoir of self-sustaining cells with the ability to self-renew and maintain the tumor (96). These CSCs have the capacity to both divide and expand the cancer stem cell pool and to differentiate into the heterogeneous cancer cell types that in most cases appear to constitute the bulk of the tumor. CSCs appear to be relatively refractory to therapies that have been developed to eradicate the rapidly dividing non-stem cell component of tumors. This would explain the common occurrence of relapse in patients that cannot effectively be treated by surgery. The CSC hypothesis requires that we rethink the way we diagnose and treat tumors, as our
objective would have to turn from eliminating the bulk of rapidly dividing but terminally differentiated components of the tumor to the minority stem cell population that fuels tumor growth.

The CSC concept has existed for over a century (97), but only emerged at the forefront of cancer research with the identification of molecular markers that allowed the isolation of leukemia CSCs (98). Over the past decade, several CSC markers were identified in a wide range of solid and hematopoietic malignancies. The requirement for such approaches to study CSCs ignited fierce controversy, as opponents argued that xenografts resulted in the unintentional selection of cells more fit to grow in a hostile environment (99, 100). Although these arguments remain valid, recent reports of work combining mouse models that spontaneously develop tumors with genetic tracing provided exciting support for the CSC theory (101).

**Osteosarcoma Cancer Stem Cells**

Following the success in hematological malignancies, FACS for stem cell surface markers including CD34, CD138, CD20, CD90, CD133, and CD44 have now been widely employed to identify CSCs in a number of cancers (102). However, the use of tissue-specific stem cell markers to identify CSCs is limited by the lack of knowledge of these markers for every tissue type. Other methods to isolate CSCs are based on common characteristics of normal stem cells. These include growth of cells in serum-free media, non-adherent sphere assays, serial colony-forming unit assays, and sorting of cells for aldehyde dehydrogenase (ALDH) activity.

Using the above mentioned assays, the presence of CSCs has now been identified not just in hematological malignancies but also in a number of solid tumors including
breast, brain, skin, lung, colon, pancreatic, liver, head and neck, and prostate cancers (103). Overall, the identified CSCs are a subpopulation (< 10%) of the overall tumor cell population and have high tumorigenic potential, requiring much lower numbers of cells to form tumors in mice. These cells not only regrow tumors when transplanted into mice, but, re-form the whole heterogeneous population of tumor cells within these xenograft models (104). They also have upregulation of genes associated with stem cell maintenance of self-renewal and pluripotency such as Oct4, Nanog and Sox2.

CD117(c-kit) is the receptor for stem cell factor and a known proto-oncoprotein. It is also one of the markers used to isolate CSCs from ovarian cancer (105). Stro-1 is a cell surface marker for mesenchymal stem cells (106). It has been shown that sphere cells generated from the mouse OS cell lines K7M2, 318-1, and P932 possessed characteristics of CSCs such as having increased tumorigenicity when injected subcutaneously into mice, and an ability to differentiate into multiple lineages. These mouse sphere cells also had increased expression of double positive (DP) c-Kit and Stro-1 cells. These DP cells were shown to be more resistant to doxorubicin than both double negative (DN) and parental cells. DP human HOS, and MNNG/HOS OS cells have increased tumorigenicity when subcutaneously injected into nude mice compared to DN cells derived from the same cell lines. It was also shown that lung metastases had more positive CD117 and Stro-1 cells when compared to the primary bone tumor (106), suggesting that the OS CSCs have an increased ability to metastasize to lung.

**CaMKII regulation of CSCs**

While it has yet to be investigated if CaMKII plays a role in maintaining stem cell pluripotency, there is strong evidence that constitutively active Wnt/β-catenin signaling
may confer a stem/progenitor cell phenotype to cancer cells (107). Inhibition of this signaling pathway in colon cancer cell lines induced the expression of the cell-cycle inhibitor p21, and also induced the cells to stop proliferating and to acquire a more differentiated phenotype. Interestingly, CaMKII has previously been shown as a downstream target of the Wnt signaling pathway, and our lab previously showed that CaMKII inhibition results in increased p21 expression in human OS. We show that CaMKII inhibition results in decreased expression and protein levels of genes associated with stem cell maintenance of self-renewal, and pluripotency such as Oct4, Nanog and Sox2 [chapter 4].

Future of CSC Therapy

In order to develop CSC-targeted therapy, it is important to be able to specifically isolate CSCs. Although OS CSCs have been investigated, no specific markers for the OS CSC have been widely agreed upon. Although the CSC may be a great target for cancer therapy, one major problem with the CSC as a therapeutic target is that it has many similar properties to normal stem cells. It will be important to monitor the amount of non-specific toxicity with treatments.

Exosomes

Exosomes are small (50-120 nm) membrane bound vesicles of endocytic origin that are released into the extracellular environment by fusion of multivesicular bodies (MVB) with the plasma membrane (108). Many cells have the capacity to release exosomes, including reticulocytes (109), dendritic cells (110), B cells, T cells (111),
epithelial cells (112), and tumor cells (108), but the functions of these secreted exosomes are not completely understood. Several mechanisms have been hypothesized describing the interactions of exosomes and recipient cells. Exosomes can bind to cells through receptor-ligand interactions, similar to cell-to-cell communication. Alternatively, exosomes could putatively attach or fuse with the target-cell membrane, delivering exosomal surface proteins and perhaps cytoplasm to the recipient cell. Finally, exosomes may also be internalized by the recipient cells by endocytosis (113).

Exosomes from different cell types contain a core set of identical proteins. These include members of the tetraspanin family (CD9, CD63, CD81, and CD82), members of the endosomal sorting complexes required for transport (TSG101, Alix) and heat shock proteins (Hsp 60, Hsp70, Hsp90) (114). Apart from these proteins, exosomes also contain some specific proteins reflective of the parental cell. Epithelial tumor cells secrete exosomes carrying the epithelial cell adhesion molecule (EpCAM) (115). Melanoma-derived exosomes contain the tumor-associated antigen Mart-1 (116). Exosomes from gastric cancer, breast cancer, and pancreatic cancer express members of the human epidermal receptor family (117). This cancer specific expression of proteins has lead several groups to hypothesize that exosomes have the potential to be non-invasive cancer biomarkers.

*Exosomes in Cancer*

Cancer has traditionally been considered as a disease resulting from gene mutations, but new findings are beginning to challenge these beliefs and suggest cancer progression may also stem from non-genetic origins. It has become clear that intercellular communication plays a crucial role in cancer progression. Exchange of information is
attained through release of specific soluble (or immobilized) signaling molecules and their interaction with corresponding receptors, or through direct cell-to-cell communication that includes gap junctions, and tunneling nanotubes (118, 119). In addition to these mechanisms, a highly conserved way of intercellular communication has recently been described via exosomes.

Tumor-derived exosomes represent an important component of the tumor microenvironment, but can also take part in altering non-cancerous cells thus facilitating tumor growth, invasion, angiogenesis, metastasis, chemo-resistance, immune evasion, and escape from cell death (120). When exosomes are taken up by recipient cells, they can change the cells’ phenotype, either briefly or long term. It is still unclear whether exosomes may be able to exert long-term genomic changes, such as induction of mutations, but some oncogenes are incorporated into exosome cargo. Consequently, exosomes can act as vehicles in malignant transformations of normal cells through the transfer of membrane proteins (receptors and receptor-coupled proteins), cytosol proteins, nucleic acids (RNA and DNA) and lipids (121).

Recent investigations revealed that invasive tumors can be spread in the body not only by metastases travelling along tissues or being transported by body fluids. Tumors can also be spread by much smaller carriers in the form of exosomes containing genetic information or mutant growth factor receptors that are permanently active and provoke cell proliferation (121). Transfer of such vesicles can occur over short distances to neighboring cells or long distances by body fluids. By carrying certain enzymes such as metalloproteinases, the exosomes can adapt the microenvironment of tumor cells in favor of metastatic dissemination or implantation into certain tissues. Blocking the spreading of
exosomes could possibly slow down tumor growth or the spread of metastases (122). A thorough understanding of the biological mechanisms involved in intercellular communication by exosomes could provide a key complement to genetic factors in determination of cancer progression.

**Exosomes as Biomarkers**

Interestingly, the contents of secreted exosomes in body fluids have proven to be highly specific (123). Some recent data suggest that exosome purification may represent a more informative diagnostic tool than whole body fluids (124). Cells located towards the drainage system, such as kidney tubular cells, provide exosomal markers that are not detectable in whole urine. Urine exosomal preparations from prostate cancer patients contain mRNA of PCA3 and TMPRSS2, a product resulting from an ERG fusion chromosomal rearrangement. Similarly, in urine exosomes, diagnostic and prognostic markers of renal ischemia/reperfusion injury or antidiuretic hormone action, such as aquaporin-1 and -2, have been found. These molecules are not readily detected in urine but can be detected in purified exosomes (125).

The power of nanovesicles as biomarkers relies on the enrichment of highly selective markers, which otherwise constitute only a very small portion of the total proteome of bodily fluids. The enrichment of diagnostic markers at the exosomal source, allows for the discovery of relatively low expressed biomarkers that would normally go undetected. In healthy human-circulating exosome, approximately 66 proteins have been identified, most of which were involved in vesicular trafficking pathways (126). The exosome biomarker strategy exemplifies the concept of “less being more”, as the small
number of proteins and RNAs give very insightful information that is specifically associated with pathological conditions.

Recently, an exosome-based tumor diagnostic assay called ExoTEST™ (HansaBioMed, Estonia) was developed. ExoTEST is a sandwich ELISA developed to capture and quantify exosomes in human plasma and other body fluids, as well as in cell culture supernatants. In this method, proteins of exosomes such as CD63 and Rab-5b, and a tumor-associated marker (e.g., CAV1) are used for the detection of nanovesicles (127). In clinical studies it was observed that CD63 and CAV1 plasma exosome levels were significantly higher in melanoma patients compared with healthy donors. Consistent with these clinical findings, ExoTEST allowed sensitive detection and quantification of exosomes purified from human tumor cell culture supernatant and plasma from mice engrafted with human melanoma. Interestingly, the plasma levels of exosomes correlated with tumor size in these mice. These data suggest that diagnostic tools such as ExoTEST can be used for the determination and quantification of circulating exosomes, providing potential new sources of clinical biomarkers.

*Calcium and Exosomes*

K562 cells are a hematopoietic cell line that releases exosomes. The application of monensin (MON) generated large multivesicular bodies (MVBs) that were labeled with a fluorescent lipid. Exosome release was markedly enhanced by MON treatment, a Na$^+$/H$^+$ exchanger that induces changes in intracellular Ca$^{2+}$. To explore the possibility that the effect of MON on exosome release was caused via an increase in Ca$^{2+}$, a Ca$^{2+}$ ionophore and a chelator of intracellular Ca$^{2+}$ were used. It was shown that intracellular Ca$^{2+}$ stimulates exosome secretion. Furthermore, MON-stimulated exosome release was
completely eliminated by 1,2-bis(2-aminophenoxy)ethane-$N,N,N',N'$-tetraacetic acid acetoxymethyl ester (BAPTA-AM), implying a requirement for Ca$^{2+}$ in this process. Large MVBs generated in the presence of MON accumulated Ca$^{2+}$ as determined by labeling with Fluo3-AM, suggesting that intralumenal Ca$^{2+}$ might play a critical role in the exosome secretory process (128).

Additionally, in cells overexpressing the Rab11Q70 L mutant or Rab11 wt, an increase in the cytosolic Ca$^{2+}$ concentration induced by MON caused a significant increase in the diameter of secreted exosomes. This effect was abrogated by the membrane permeant Ca$^{2+}$ chelator BAPTA-AM. It has also been shown that Rab11 is acting in the tethering/docking of exosomes to promote homotypic fusion, but that the final fusion reaction requires the presence of Ca$^{2+}$. Finally, a rise in intracellular Ca$^{2+}$ concentration enhanced exosome secretion in Rab11 wt overexpressing cells and reversed the inhibition of the mutants. The results suggest that both Rab11 and Ca$^{2+}$ are involved in the homotypic fusion of exosomes (129).

**Goals and Hypotheses**

The goal of my work was to study the role of $\alpha$-CaMKII in the growth and metastasis of human OS. We hypothesized that CaMKII-regulated growth and metastasis of human OS could be prevented by both pharmacologic and genetic inhibition of CaMKII. To test this hypothesis we silenced or overexpressed CaMKII in different human OS cell lines and examined their tumorigenic properties *in vitro*, and their growth *in vivo* (Chapter 1). We further hypothesized that CaMKII-mediated OS tumor growth
was occurring by autocrine and paracrine mechanisms of VEGF signaling. We then showed a positive feedback loop in human OS resulting from the autocrine signaling functions of CaMKII-regulated tumor secreted-VEGF. This autocrine signaling is prevented when CaMKII and VEGF are pharmacologically inhibited (Chapter 2). Furthermore, we developed a clinically relevant metastases model by amputating the tumor containing hind limb and examined metastatic lesion formation when both CaMKII and VEGF are pharmacologically inhibited by FDA approved drugs (Chapter 3). Finally, we hypothesized that metastasis was being prevented by decreasing cancer stem cell (CSC) populations and these CSCs were maintaining pluripotency by tumor-secreted exosomes. Thus to test this hypothesis, we examined CSC populations when CaMKII was inhibited, and if dormant tumor cells phenotypically resembled CSCs when treated with tumor-secreted exosomes (Chapter 4).
ALPHA-CAMKII PLAYS A CRITICAL ROLE IN DETERMINING THE AGGRESSIVE BEHAVIOR OF HUMAN OSTEOSARCOMA

by

PAUL G. DAFT, KAIYU YUAN, JASON M. WARRAM, MICHAEL J. KLEIN, GENE P. SIEGAL, MAJD ZAYZAFOON


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ABSTRACT

Osteosarcoma is among the most frequently occurring primary bone tumors, primarily affecting adolescents and young adults. Despite improvements in osteosarcoma treatment, more specific molecular targets are needed as potential therapeutic options. One target of interest is alpha-Ca\(^{2+}\)/calmodulin-dependent protein kinase II (α-CaMKII), a ubiquitous mediator of Ca\(^{2+}\)-linked signaling, which has been shown to regulate tumor cell proliferation and differentiation. Here, we investigate the role of α-CaMKII in the growth and tumorigenicity of human osteosarcoma. We show that α-CaMKII is highly expressed in primary osteosarcoma tissue derived from 114 patients and is expressed in varying levels in different human osteosarcoma cell lines (HOS, MG-63, MNNG/HOS and 143B). To examine whether α-CaMKII regulates osteosarcoma tumorigenic properties, we genetically inhibited α-CaMKII in two osteosarcoma cell lines using two different α-CaMKII shRNAs delivered by lentiviral vectors and overexpressed α-CaMKII by retrovirus. The genetic deletion of α-CaMKII by shRNA in MG-63 and 143B cells resulted in decreased proliferation (50 and 41%), migration (22 and 25%) and invasion (95 and 90%), respectively. The overexpression of α-CaMKII in HOS cells resulted in increased proliferation (240%), migration (640%) and invasion (10,000%). Furthermore, α-CaMKII deletion in MG-63 cells significantly reduced tumor burden in vivo (65%), while α-CaMKII overexpression resulted in tumor formation in a previously non-tumor forming osteosarcoma cell line (HOS). Our results suggest that α-CaMKII plays a critical role in determining the aggressive phenotype of osteosarcoma, and its inhibition could be an attractive therapeutic target to combat this devastating adolescent disease.
INTRODUCTION

Osteosarcomas are among the most frequently diagnosed primary bone cancer in humans (1). Approximately 60% of all primary bone neoplasms occur in patients under 30 years of age, with osteosarcomas most commonly occurring in the second decade of life, between the ages of 15-19 (2). Osteosarcomas account for 5% of all pediatric tumors and roughly 20% of all primary bone tumors (2,3). Osteosarcomas most frequently develop in the highly proliferative metaphyseal region of long bones and are thought to coincide with the adolescent growth spurt (4). They are highly aggressive and frequently metastatic, with the lung being the most common site for metastasis (5). Most patients are treated with neoadjuvant multi-agent chemotherapy followed by highly invasive limb-sparing surgery or amputation. With both chemotherapy and surgery, cure rates for nonmetastatic osteosarcoma now approach 65% (6). Patients who do not respond to chemotherapy or are not disease free after surgery have a dismal prognosis with little hope for prolonged survival. With only a 30% 5 year survival rate for patients with metastatic osteosarcoma at presentation, it is necessary to develop new treatments to combat this devastating childhood disease (7).

Ca²⁺/CaM-dependent protein kinase II (CaMKII) is a ubiquitously expressed protein kinase (8). At steady state, α-CaMKII is inactive. Upon increases in intracellular Ca²⁺, α-CaMKII is activated by phosphorylation. Therefore, increases in the levels of total α-CaMKII result in correlated increases in the levels of the active form of the kinase. Furthermore, the activation of CaMKII has been shown to create a positive feedback loop by regulating the levels of intracellular Ca²⁺ through the activation of the ryanodine
receptor and several other ion channels. This complex crosstalk demonstrates the positive relationship between p-α-CaMKII and total α-CaMKII (9). Biologically active CaMKII phosphorylates a variety of substrates, regulating many aspects of cellular function in response to Ca\(^{2+}\) signaling, influencing neurotransmitter synthesis and release, transcription and translation, cytoskeletal organization and Ca\(^{2+}\) homeostasis. The CaMKII family is encoded by four genes (α, β, γ, and δ), each of which undergoes its own alternative splicing. The basic biological function of the active alpha isoform (p-α-CaMKII) is implicated in a growing number of cancers, arising in the colon, prostate, brain and breast (10-12). We have previously reported that p-α-CaMKII regulates the expression of c-fos, a member of the activator protein-1 (AP-1) family of oncogenes (13). Furthermore, we were the first to report that p-α-CaMKII regulates the growth of osteosarcoma cells by controlling cell cycle progression, ultimately contributing to the uncontrolled proliferation of this tumor (14).

In this study we investigated the role of p-α-CaMKII in the growth and tumorigenesis of human osteosarcoma in vitro and in vivo. We show that p-α-CaMKII levels are increased in primary human osteosarcoma tissues from patients and in aggressive human osteosarcoma cell lines. Furthermore, the knockdown of α-CaMKII decreases proliferation, motility and invasion, while α-CaMKII overexpression increases the tumorigenic properties of human osteosarcoma cell lines in vitro. We also show that the disruption of α-CaMKII positively controls osteosarcoma growth in vivo. These results suggest that α-CaMKII plays a critical role in the growth and aggressiveness of human osteosarcoma and compounds that inhibit α-CaMKII activation might provide novel therapies for the treatment of this malignant tumor.
MATERIALS AND METHODS

Cell Culture

Human osteosarcoma cells (HOS, MG-63, MNNG/HOS and 143B) and human mesenchymal stem cells (hMSCs) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These human-derived cell lines were authenticated by DNA short tandem repeat profiling and experiments were conducted within 6 months of resuscitation. Cells were maintained in DMEM medium containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). All cell cultures were maintained at 37°C with 5% CO₂ (14).

RNA Extraction RT-PCR

Total RNA was extracted using the TRIzol method as recommended by the manufacturer (Invitrogen). One μg of RNA was reverse-transcribed using M-MLV reverse transcriptase, and the equivalent of 10 ng was used for SYBR Green real-time quantitative RT-PCR. The expression of 18S rRNA was used for normalization of gene expression values. Primer sequences used were previously described (14).

Whole Cell Protein Extraction and Western Blot Analysis

Cells were lysed in 0.5% Nonidet P-40 lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, Immobilon-P (Millipore Co., Milford, MA, USA). Membranes were blocked with Tris-buffered saline-Blotto/Blotto B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour and
subsequently incubated overnight with antibodies directed against α-CaMKII, p-α-CaMKII, p-CREB, p-ERK, c-Fos, Lamin B1, or β-actin (Santa Cruz Biotechnology). Signals were detected using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences, Pittsburgh, PA, USA)(14).

Immunohistochemistry

Primary human osteosarcoma tissues were obtained from IMGENEX (IMH-370) (Sorrento Valley, CA, USA). Tissues were deparaffinized and rehydrated followed by antigen retrieval using 10mM sodium citrate buffer, pH 6. Samples were blocked for 1 hour in 5% goat serum (Vector Laboratories, Burlingame, CA, USA). Anti-p-α-CaMKII (Santa Cruz Biotechnology) was applied to sections and incubated overnight at 4°C. Biotin-conjugated secondary antibodies (2µg/ml) were added, followed by avidin-biotin enzyme reagents. Specimens were incubated in 3,3'-Diaminobenzidine (DAB) peroxidase substrate for 30 seconds. Tissues were counterstained with Gill’s hematoxylin for 10 seconds, dehydrated, cleared and mounted. Rabbit IgG negative controls were processed alongside the examined tissue. Photomicrographs were taken using a Nikon DS-Fi1 digital camera (14,15). The detected levels of phosphorylated α-CaMKII by IHC were scored using a semi-quantitative system as previously described (16). Two experienced pathologists independently scored the 114 tissue samples. The complete score agreement of the two pathologists was 91% of all cases, indicating that the scoring method is highly reproducible. A cut-off score was chosen at 8 (51%-75% of tumor cells staining with moderate staining intensity) (16).
Motility Assay

HOS, MG-63, MNNG/HOS and 143B cells were grown to 100% confluency in 6-well plates and scratched with the narrow end of a sterile pipette tip. Medium was changed to remove floating cells and replaced with DMEM medium containing 1% FBS. Photomicrographs were taken and the scratch width was measured immediately after initial wounding. Cells were then incubated at 37°C with 5% CO₂. After 8 hours, photomicrographs were taken at 50X magnification and the scratch width was measured. Data were expressed as percentage of the remaining width of the scratch (after 8 hours) when compared to the original width (at 0 hour) (17).

Invasion Assay

Cells (2.5x10⁴) were plated in media containing 0.1% FBS onto the Matrigel coated upper chambers of transwell invasion assay filter inserts (BD Bioscience, East Rutherford, NJ, USA). Medium containing 10% FBS was added into the lower chambers, acting as a chemoattractant. The cells were allowed to invade for 24 hours, after which the cells that invaded the Matrigel were fixed in methanol and stained with crystal violet (Cellgro, Manassas, VA, USA). Representative photomicrographs were taken at 100X magnification (17).

MTT Assay

Cell proliferation was measured by a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazoliumbromide] assay (ATCC). Cells were plated at a density of 5x10³ cells per well in 96-well plates. After treatment, MTT solution was added to the culture
medium (0.5 mmol/L) and plates were incubated for 2 hours at 37°C with 5% CO₂. Detergent solution was then added to solubilize formazan crystals. Finally, the optical density was determined at 570 nm using a Benchmark Plus microplate reader (Bio-Rad, Hercules, USA).

**Gene Silencing by shRNA**

MG-63 and 143B cells were plated at a density of 2×10^4 cells/cm² in six-well plates. Cells were incubated in Polybrene (8µg/ml) (Sigma-Aldrich) overnight at 37°C with 5% CO₂. Two specific α-CaMKII shRNAs [shCaMKIIα(1) and (2)] or one non-specific scrambled control shRNA [shCtrl] were cloned into lentiviral transduction vectors (Sigma-Aldrich) and added to the media. The media was changed 24 hours post-transfection, and the transfected cells were cultured with fresh media containing puromycin (5µg/mL) for selection (Sigma-Aldrich). Once all non-transfected cells died guaranteeing a pure culture, the transfected cells were split into 10 cm plates and maintained stably in culture. (18)

**Retrovirus Production and Infection**

We used retroviral expression vectors expressing either green fluorescent protein (GFP) (pMSCV-GFP) or α-CaMKII-GFP (pMSCV-α-CaMKII). The CMV-CaMKII-GFP was provided by Dr. Tobias Meyer (20). Retroviruses were produced by co-transfecting pMSCV vectors with pVSV-G into BOSC23 cells using Lipofectamine (Invitrogen). Twenty-four hours post transfection, the media was replaced, and retroviral supernatant was collected. For infection, 2×10^4 cells/cm² HOS cells were plated into 6-well plates. The culture media were replaced with 500 µl of retroviral supernatant containing 8 µg/ml
Polybrene (Sigma-Aldrich) and cells were incubated for 2 hours at 37 °C in 5% CO₂. Retroviral supernatant was then removed and cells were cultured in regular growth medium (19).

**Animals and Tumor Cell Inoculation**

Six-week-old male Foxn1nu mice were used in these studies, with the approval of the UAB Institutional Animal Care and Use Committee. MG-63 and HOS cells were prepared from sub-confluent cultures. Cells (1 x 10⁶ cells in 20 µl PBS) were intratibially injected using insulin syringes with 28.5 gauge needles. The knee was flexed, and the needle inserted into the tibia, boring the needle through the epiphysis and epiphyseal growth plate for delivery of the cells into the metaphysis. MG-63 osteosarcoma tumor growth was monitored by *in vivo* bioluminescence imaging at 7 and 49 days after cancer cell inoculation. Mice carrying MG-63 osteosarcoma tumors were intraperitoneally injected with D-luciferin solution (150 mg/kg) 10 minutes before bioluminescence imaging. Images were then acquired and analyzed with an IVIS 100 Imaging System (Xenogen). Regions of interest were identified and plotted as fold difference in tumor size at day 49 when compared to day 7. At the end of the study, animal were euthanized, hind limbs were excised, formalin fixed, EDTA decalcified and paraffin embedded. All tissues were sectioned and stained with hematoxylin and eosin (H&E) for histological evaluation of the tumors. Photomicrographs were taken using a Nikon DS-Fi1 digital camera (14,15).
18F-FDG positron emission tomography (PET) imaging

HOS osteosarcoma tumor growth was monitored by in vivo PET imaging at 49 days after cancer cell inoculation. Mice carrying HOS osteosarcoma tumors were anesthetized with 2.5% Isoflurane. Prior to imaging, animals were administered 200 μCi 18F-FDG intravenously through lateral tail vein followed by 200 μl saline intraperitoneally to void the bladder. Mice were imaged using a Triumph Flex PET scanner (Gamma Medica, Northridge, CA, USA). The system provided a 1.9-mm transaxial spatial resolution and 5.9% sensitivity at the center of field of view. PET images were reconstructed with maximum likelihood expectation maximization algorithm using custom scanner software (Gamma Medica, Northridge, CA, USA). Image and ROI analysis of reconstructed images were performed by a blinded reviewer using 64 bit OsiriX® imaging software (version 4.0).

Statistical Analysis

Statistical analyses were performed using the Microsoft Excel data analysis program for Student’s t-test analysis. Experiments were repeated at least three times, unless otherwise stated. Values were expressed as mean ±SE with results considered significant at p<0.05.
RESULTS

α-CaMKII in primary human osteosarcoma tissues and cell lines

To examine the levels of active α-CaMKII in human osteosarcoma tissues, immunohistochemistry staining (IHC) using an antibody targeting p-α-CaMKII was performed on clinical samples obtained from 114 primary human osteosarcoma patients, consisting of chondroblastic, osteoblastic and fibroblastic osteosarcomas and 12 normal bone samples (Figure 1). Phosphorylated α-CaMKII IHC staining was scored using a semi-quantitative system as previously described (16). A 2-sided Fisher’s exact test was performed and showed that the chondroblastic (90.4%), osteoblastic (60.1%) and fibroblastic (57.9%) subtypes of osteosarcoma have high expression of p-α-CaMKII when compared to osteoblasts and mesenchymal stromal cells in normal bones (p<0.0001). The indicated p-value is based on comparison to normal bone using exact binomial distribution (Supplementary Tables 1 and 2). These results demonstrate a significant increase in α-CaMKII activation in human osteosarcoma tissues when compared to normal bone.

In order to determine whether the increases observed for p-α-CaMKII in primary osteosarcoma tissues were also seen in human osteosarcoma cell lines, we performed IHC and western blot analysis using an antibody directed against p-α-CaMKII on several osteosarcoma cell lines (HOS, MG-63, MNNG/HOS and 143B) as well as non-transformed pre-osteoblastic human mesenchymal stem cells (hMSCs). Negative controls were processed alongside the examined tissue, but rabbit IgG was used instead of the primary antibody (data not shown). Immunohistochemical results demonstrate that the levels of p-α-CaMKII varied between osteosarcoma cell lines, with 143B cells having the
most and HOS cells having the least (Figure 2A). Additionally, western blotting analyses were performed using antibodies directed against p-α-CaMKII, α-CaMKII and β-actin. Consistent with IHC data, we show that the levels of active and total α-CaMKII in 143B cells were 800% higher than hMSCs, while levels in HOS cells were only 2 times higher (Figure 2B). Band density was measured using ImageJ software for both p-α-CaMKII and β-actin, allowing p-α-CaMKII levels to be normalized across osteosarcoma cell lines (Figure 2C).

The in vitro tumorigenic properties of osteosarcoma cell lines are positively correlated with their α-CaMKII levels.

To determine whether the variable levels of p-α-CaMKII in human osteosarcoma cell lines are correlated with aggressive phenotype, we compared the levels of in vitro proliferation, invasion and motility between these cells. Proliferation of osteosarcoma cell lines was measured by MTT assay. An MTT assay was performed every 24 hours for 4 days on HOS, MG-63, MNNG/HOS and 143B cells. Here we show that osteosarcoma cell lines expressing high levels of active α-CaMKII (MG-63, MNNG/HOS and 143B cells) proliferate more rapidly than HOS cells that express lower levels of active α-CaMKII (Figure 3A). By the fourth day of the study, 143B cells proliferated 1,200% more than HOS cells. Invasiveness of osteosarcoma cell lines was measured using a 24 hour transwell invasion assay. Invasion and motility studies were performed in serum free or 1% FBS-supplemented medium in order to suppress cell proliferation and to enable us to identify the invasive response of osteosarcoma cells independent of cell growth. Here we show that the osteosarcoma cell lines that express high levels of active α-CaMKII (MG-63, MNNG/HOS and 143B cells) are more invasive than HOS cells that
express low levels of active α-CaMKII (Figure 3B). After 24 hours, invasion of 143B cells was 11,000% more than HOS cells. Finally, motility of osteosarcoma cell lines was measured using a scratch assay. After 8 hours, the migration of HOS, MG-63, MNNG/HOS and 143B osteosarcoma cells into the cell free area was quantitated. Here we show that osteosarcoma cell lines expressing high levels of active α-CaMKII (MG-63, MNNG/HOS and 143B cells) migrate more rapidly when compared to HOS cells that express low levels of active α-CaMKII (Figure 3C). These results demonstrate that 143B cells can migrate and cover 46% of the cell free area within 8 hours, while HOS cells can only cover 5% of the cell free area during the same time period. Taken together, our data demonstrate that osteosarcoma cell lines expressing high levels of p-α-CaMKII are more proliferative, invasive and motile.

**Disruption of α-isoform in osteosarcoma cells alters their in vitro tumorigenic properties**

To determine whether differences in the tumorigenic characteristics of osteosarcoma cell lines were directly related to the levels of α-CaMKII, we generated two stable knockdowns of α-CaMKII in moderate and highly aggressive osteosarcoma cell lines (MG-63 and 143B cells, respectively), as well as an α-CaMKII overexpression model in a non-aggressive osteosarcoma cell line (HOS). 143B cells were transduced with lentiviral vectors delivering a non-specific control shRNA (shCtrl) or two different specific α-CaMKII-targeting shRNAs [shCaMKIIα(1) and shCaMKIIα(2)]. A successful knockdown of α-CaMKII gene expression (65% and 80%) was achieved by either shCaMKIIα(1) or shCaMKIIα(2), respectively, when compared with 143B cell-transduced with a non-specific control. The decreases in mRNA expression were also
confirmed by demonstrating a comparable decrease in p-α-CaMKII protein levels (Figure 4A). HOS cells were transduced with a retroviral construct overexpressing GFP-α-CaMKII (GFP-CaMKIIα) or a GFP containing retrovirus (GFP-Ctrl) as a control. An 80% decrease in α-CaMKII gene expression was observed in shCaMKIIα(2) MG-63 and 143B cells when compared to their controls, while a 400% increase in α-CaMKII gene expression was observed in HOS GFP-CaMKIIα cells when compared to GFP-Ctrl cells (Figure 4B). These data were confirmed by demonstrating that the levels of p-α-CaMKII protein were dramatically decreased in shCaMKIIα(2) cells and increased in GFP-CaMKIIα cells when compared to their respective controls (Figure 4C). Interestingly, although the levels of α-CaMKII gene expression in both 143B and HOS (GFP-CaMKIIα) are similar we constantly found that the levels of α-CaMKII protein are slightly higher (~15%) in HOS (GFP-CaMKIIα) cells (Figure 4C). To confirm that the knockdown and overexpression of α-CaMKII resulted in changes in α-CaMKII intracellular signaling, we examined the activation of three well-known downstream targets of α-CaMKII; cAMP response element-binding protein (CREB), extracellular-signal-regulated-kinase (ERK) and c-Fos by western blotting (21-23). Here we show that the knockdown of α-CaMKII in MG-63 and 143B cells dramatically decreases p-CREB, p-ERK and c-FOS when compared to cells transduced with non-specific controls, while the overexpression of α-CaMKII in HOS cells increases their activation (Figure 4C). Furthermore, the levels of p-α-CaMKII protein in knockdown and overexpressing cell lines were examined using IHC. As expected, 143B and MG-63 shCaMKIIα(2) cells show a dramatic decrease in the levels of p-α-CaMKII, while HOS GFP-CaMKIIα cells show increases in p-α-CaMKII (Figure 4D). These results validate that the
overexpression or deletion of α-CaMKII is successful in controlling α-CaMKII intracellular signaling.

To determine the specific effect of α-CaMKII knockdown and overexpression on the tumorigenic properties of osteosarcoma cell lines in vitro, we examined the proliferation, motility and invasion of shCaMKIIα(2) MG-63 and 143B cells and GFP-CaMKIIα HOS cells. Here we show that α-CaMKII knockdown decreases MG-63 and 143B cell proliferation when compared to controls, while α-CaMKII overexpression in HOS cells increases proliferation when compared to control. By day 4, shCaMKIIα(2) MG-63 and 143B cells proliferated 50 and 41% less, respectively, when compared to cells transduced with non-specific controls, while GFP-CaMKIIα HOS cells proliferated 240% more than controls (Figure 5A). Additionally, we show that shCaMKIIα(2) MG-63 and 143B cells migrate 22% and 25% less, respectively, when compared to cells transduced with non-specific controls, while GFP-CaMKIIα HOS cells migrate 640% more than GFP-Ctrl (Figure 5B). Furthermore, the knockdown of α-CaMKII in MG-63 and 143B cells decreased invasion by 95% and 90%, respectively, when compared to controls, while α-CaMKII overexpression in HOS cells increased invasion by 10,000% compared to control (Figure 5C). These results demonstrate that the deletion or overexpression of α-CaMKII in osteosarcoma cell lines leads to dramatic changes in their proliferation, motility and invasion.

**α-CaMKII positively controls the in vivo growth of human osteosarcoma**

To examine whether the observed changes of the in vitro tumorigenicity of osteosarcoma cells in response to α-CaMKII knockdown or overexpression resulted in similar changes
in osteosarcoma tumor viability in vivo, we intratibially injected shCtrl and shCaMKIIα(2) MG-63 and GFP-Ctrl and GFP-CaMKIIα HOS osteosarcoma cells into 6-week old male athymic (nude) mice. MG-63 cells were transduced with lentivirus encoding firefly luciferase allowing for in vivo monitoring of tumor growth. The in vivo tumor growth of HOS cells was monitored using 18F-FDG PET imaging. Here we show, by luminescence imaging and histology, that the deletion of α-CaMKII in MG-63 cells significantly reduced tumor burden in vivo (65%) when compared to cells transduced with non-specific control, while α-CaMKII overexpression in HOS cells resulted in a 500% increase in tumor burden when compared to control (Figures 6A and 6B). Histologically, hematoxylin and eosin staining of these tumors revealed a dramatic decrease in tumor vasculature in the α-CaMKII knockdown tumors, and an increase in tumor vasculature in α-CaMKII overexpressing tumors (Figure 6C). This was confirmed by performing immunohistochemical staining using CD31 specific antibodies targeting endothelial cells. As expected, both shCtrl MG-63 and GFP-CaMKIIα HOS osteosarcoma cells produced in vivo tumors containing numerous blood vessels. However, blood vessels were almost completely absent from the shCaMKIIα(2) MG-63 tumors, and the GFP-Ctrl HOS cells did not produce any tumors (Figure 6D).

Taken together, our data demonstrate that α-CaMKII plays a critical role in the growth and tumorigenicity of osteosarcoma cells in vitro and in vivo.
DISCUSSION

The control of intracellular Ca\(^{2+}\) levels is critical for the regulation of normal cellular functions such as proliferation, growth and gene expression (24-25). However, it is becoming increasingly evident that the in vitro growth of cancer cells is not responsive to extracellular Ca\(^{2+}\) (26-27). This phenomenon was attributed by many to the ability of cancer cells to overexpress several of Ca\(^{2+}\) downstream targets, such as CaMKII (28,29).

In the present study, we show that \(\alpha\text{-CaMKII}\) activation plays a critical role in determining the aggressive behavior of human osteosarcoma. Examination of the levels of p-\(\alpha\text{-CaMKII}\) in 114 human osteosarcoma tissues revealed that the levels of p-\(\alpha\text{-CaMKII}\) were significantly higher in osteosarcoma tissues when compared to osteoblasts in normal bone. Also, we discovered that the levels of total and active \(\alpha\text{-CaMKII}\) and tumorigenic properties of several osteosarcoma cell lines including proliferation, invasion and motility are positively correlated. Interestingly, two of the cell lines we used in this study (MNNG/HOS and 143B) are subclones originally derived from HOS cells that were carcinogen-exposed (MNNG/HOS) or ras-transformed (143B) (30-32). Neither carcinogen-exposure nor ras-transformation is known to directly impact CaMKII expression. Currently, it is unknown whether the increases in \(\alpha\text{-CaMKII}\) are a result of the more malignant phenotype of transformed HOS cells or are due to subcloning a more aggressive subpopulation of HOS cells that already have an increase in \(\alpha\text{-CaMKII}\) expression. Furthermore, it is unknown whether the demonstrated increases in the activation of \(\alpha\text{-CaMKII}\) in osteosarcoma is simply due to the increases in expression or is indirectly due to increases in an unknown upstream activator that leads to elevations in intracellular Ca\(^{2+}\). Interestingly, it has been reported that the activation of CaMKII creates
a positive feedback loop by regulating the levels of intracellular Ca\(^{2+}\) through the activation of the ryanodine receptor, and several other ion channels (9). This complex crosstalk demonstrates that increased levels of p-a-CaMKII and total a-CaMKII are positively associated.

The localization of CaMKII to different cellular compartments has been previously described. For example, in osteoblasts we have shown that α-CaMKII has distinct speckled perinuclear localization (13). Furthermore, others have shown that the localized and transient enrichment of CaMKII to dendritic sites coincided spatially and temporally with intracellular Ca\(^{2+}\) (33). Our results show that α-CaMKII in osteosarcoma cells are localized in a perinuclear pattern. It is possible that this places the kinase in proximity to Ca\(^{2+}\) stores in the ER to facilitate its activation.

Although we were the first to report that α-CaMKII is expressed in osteosarcoma cell lines and describe the role it plays in controlling cell cycle progression (14), others have also reported that the pharmacologic inhibition of CaMKII attenuates the growth and tumorigenicity of many cancer cell lines, including LN-215, LNCaP, C4-2B, CWR22Rv1 and Hep3B (34-36). To expand on our published studies, we generated osteosarcoma cell lines where α-CaMKII is either deleted (MG-63 and 143B) or overexpressed (HOS). The deletion of α-CaMKII was performed by transducing osteosarcoma cells with a lentivirus that expresses α-CaMKII shRNA, while overexpression was achieved by transducing cells with a retrovirus that overexpresses α-CaMKII. Gene delivery efficiency was ~80% for α-CaMKII deletion and 65% for the overexpression. The deletion of α-CaMKII was confirmed by the inability of MG-63 and 143B cells to activate known α-CaMKII downstream signaling molecules such as CREB, ERK and c-
Fos, while the overexpression of α-CaMKII resulted in over activation of these same molecules (37-39). The deletion of α-CaMKII significantly decreased the proliferation, motility and invasion of osteosarcoma cells, while the overexpression of α-CaMKII caused a significant increase. It remains unclear how the inhibition of α-CaMKII can mechanistically alter these phenotypic qualities of osteosarcoma cells. We have previously shown that α-CaMKII regulates the growth of osteosarcoma cells by controlling cell cycle progression in a p21-dependent mechanism (14). However, it is possible that the ability of CaMKII to directly regulate the activation of ERK and the expression of c-fos could also be responsible for controlling the proliferation of osteosarcoma cells. This is supported by several reports that describe the importance of both c-fos and ERK in cell growth (40). Furthermore, c-fos has been shown, in combination with other members of the AP-1 family of transcription factors, to regulate the expression of several matrix metalloproteinases, such as MMP-1, -9 and -13. These changes in MMP expression could be responsible for the decreased invasion observed in response to α-CaMKII deletion (41,42). Moreover, our data support other published reports describing the ability of CaMKII in cancer cells to cause remodeling of the actin cytoskeleton and increase cellular motility (43). Taken together, our results demonstrate the critical role of α-CaMKII in the in vitro tumorigenicity of osteosarcoma cells.

Similarly, we show that the deletion α-CaMKII decreases the ability of osteosarcoma cells to form tumors in vivo. Interestingly, the overexpression of α-CaMKII in HOS cells which are known to be unable to form tumors in animals and are slow proliferating cells formed relatively large tumors in the tibia of a xenograft nude mouse model. However, the ability of α-CaMKII to only regulate growth, invasion and motility cannot be
sufficient to explain the large size of the osteosarcoma tumors. Indeed, increases in angiogenesis have previously been attributed to the ability of osteosarcoma to grow into very large tumors. This was previously supported by identifying increases in several angiogenic factors such hypoxia inducible factor, vascular endothelial growth factor, basic fibroblast growth factor, Neuropilin-2 and placental growth factor in human osteosarcoma clinical tissue samples (19,44). Similarly, our data show that the deletion of α-CaMKII in osteosarcoma cells produces not only smaller tumors but also resulted less tumor vasculature as demonstrated by a decrease in the number of CD31 stained blood vessels. These findings suggest that the ability of α-CaMKII to regulate angiogenesis in vivo could be a contributing factor for the significant decrease in tumor growth. Currently, it is not known which angiogenic factor is directly regulated by α-CaMKII.

Taken together, our data support a critical role of p-α-CaMKII in regulating the pathogenesis of osteosarcoma. This could be due to the ability of p-α-CaMKII to control the activation and expression of several intracellular proteins and transcription factors, such as CREB, ERK, c-Fos and p21 that could ultimately lead to uncontrolled proliferation and growth of these cells. Furthermore, the same factors could also be altering the bone-tumor microenvironment by regulating the expression of several molecules such as MMPs and different angiogenic factors to provide a hospitable environment that facilitates the growth of osteosarcoma. Understanding the crosstalk between p-α-CaMKII and its downstream targets in osteosarcoma may thus yield novel therapeutic strategies for this devastating disease.
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Figure 1. **Primary human osteosarcoma tissues express high levels of active α-CaMKII**

Hematoxylin and eosin staining (upper panel) shows normal bone and three conventional, high-grade human osteosarcoma subtypes: chondroblastic, osteoblastic and fibroblastic. Immunohistochemical staining (lower panel) using a specific antibody directed against p-α-CaMKII (brown) in human osteosarcoma tissue and counterstained with hematoxylin (blue). Unstained osteocytes in normal bone tissue are indicated by *. Images were taken at 400X magnification.
**Figure 2.** Osteosarcoma cell lines express high levels of active α-CaMKII

**A.** Immunohistochemical staining using a specific antibody directed against p-α-CaMKII (brown) in human osteosarcoma cell lines HOS, MG-63, MNNG/HOS and 143B, with hMSCs as a control. Images were taken at 400X magnification. **B.** Immunoblots were developed using antibodies directed against p-α-CaMKII, α-CaMKII or β-actin. The autoradiograph is representative of three experiments. **C.** ImageJ software was used to quantify the levels of p-α-CaMKII normalized to β-actin. Values were obtained from three separate experiments and represent the mean ± S.E. *p<0.01.
Figure 3. The levels of α-CaMKII expression in osteosarcoma cells are positively correlated with their aggressiveness.

A. MTT assay was performed to determine the number of viable cells. Values were obtained from three separate experiments, each repeated in triplicate and represent the mean ± S.E. *p<0.01. B. Transwell invasion assay allowing cells to invade for 24 hours.
Representative photomicrographs were taken at 100X magnification from 3 independent experiments, each repeated in duplicate. Values represent the mean ± S.E. *p<0.01. 

C. Scratch/wound healing assay was performed on HOS, MG-63, MNNG/HOS and 143B cells. Representative photomicrographs were taken at 50X magnification from 3 independent experiments, each repeated in triplicate. Values represent the mean ± S.E. *p<0.01.
**Figure 4.** Deletion or overexpression of α-CaMKII in human osteosarcoma cell lines

MG-63 and 143B cells were transduced with lentiviruses expressing either a non-specific control (shCtrl) or two different specific α-CaMKII-targeting shRNAs [shCaMKIIα(1) and shCaMKIIα(2)]. Also, HOS cells were transduced with retroviruses expressing either GFP (GFP-Ctrl) or CaMKIIα (GFP-CaMKIIα). **A.** Real-time PCR was performed using primers specific for α-CaMKII or 18S rRNA. Immunoblots were developed using specific antibodies directed against p-α-CaMKII, α-CaMKII or β-Actin. Values were obtained from three separate experiments, each repeated in triplicate and represent the mean ±S.E. *p<0.01. **B.** Real-time PCR was performed using primers specific for α-CaMKII or 18S rRNA. Values were obtained from three separate experiments, each

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*Figure 4.* Deletion or overexpression of α-CaMKII in human osteosarcoma cell lines

MG-63 and 143B cells were transduced with lentiviruses expressing either a non-specific control (shCtrl) or two different specific α-CaMKII-targeting shRNAs [shCaMKIIα(1) and shCaMKIIα(2)]. Also, HOS cells were transduced with retroviruses expressing either GFP (GFP-Ctrl) or CaMKIIα (GFP-CaMKIIα). **A.** Real-time PCR was performed using primers specific for α-CaMKII or 18S rRNA. Immunoblots were developed using specific antibodies directed against p-α-CaMKII, α-CaMKII or β-Actin. Values were obtained from three separate experiments, each repeated in triplicate and represent the mean ±S.E. *p<0.01. **B.** Real-time PCR was performed using primers specific for α-CaMKII or 18S rRNA. Values were obtained from three separate experiments, each
repeated in triplicate and represent the mean ±S.E. *p<0.01. C. Immunoblots were developed using specific antibodies directed against p-α-CaMKII, α-CaMKII, p-CREB, p-ERK, β-Actin, c-Fos, or Lamin B. The autoradiographs are representative of three experiments. D. Immunohistochemical staining using a specific antibody directed against p-α-CaMKII (brown). Images were taken at 200X magnification and are representative of 3 independent experiments.
Figure 5

A

% change in OD570

MG-63 143B

Day 1 Day 2 Day 3 Day 4

1000 800 600 400 200 0

* shCtrl shCaMKIIα(2)

B

% change in OD570

MG-63 143B HOS

Day 1 Day 2 Day 3 Day 4

1000 800 600 400 200 0

* shCtrl shCaMKIIα(2)

C

Invasion (cells/field)

MG-63 143B HOS

0 50 100 150

* shCtrl shCaMKIIα(2)

Figure 5. Perturbation of α-CaMKII in osteosarcoma cells changes their aggressive behavior in vitro

MG-63 and 143B cells were transduced with lentiviruses expressing either a non-specific control (shCtrl) or α-CaMKII-targeting shRNAs shCaMKIIα(2). Also, HOS cells were transduced with retroviruses expressing either GFP (GFP-Ctrl) or CaMKIIα (GFP-CaMKIIα). A. MTT assay was performed to determine the number of viable cells. Values were obtained from three separate experiments, each repeated in triplicate and represent the mean ± S.E. *p<0.01. B. Scratch/wound healing assay was performed on cells cultured for 8 hours. Representative photomicrographs were taken at 50X magnification from 3 independent experiments, each repeated in triplicate. Values

65
represent the mean ± S.E. *p<0.01. C. Transwell invasion assay allowing cells to invade for 24 hours. Representative photomicrographs were taken at 100X magnification from 3 independent experiments, each repeated in duplicate. Values represent the mean ± S.E. *p<0.01.
**Figure 6.** Perturbation of α-CaMKII in osteosarcoma cells affects *in vivo* tumor formation.

MG-63 cells were transduced with lentiviruses expressing either a non-specific control (shCtrl) or α-CaMKII-targeting shRNAs shCaMKIIα(2) and HOS cells were transduced with retroviruses expressing either GFP (GFP-Ctrl) or CaMKIIα (GFP-CaMKIIα). MG-63 cells were also transduced with lentiviruses expressing firefly luciferase. **A.** Luciferase imaging (left panel) was performed at week 1 and 7 after tumor cell inoculation (n=12). PET imaging (right panel) was performed at week 7 after tumor cell inoculation (n=12). **B.** Fluorescent intensity (left graph) and 18F isotope radioactivity (right graph) were
measured and graphed. Values were obtained from seven mice in each group and represent the mean ± S.E. *p<0.01. C. Hematoxylin and eosin staining was performed on paraffin embedded tumors. Black arrows indicate blood vessels. The broken line indicates the boundary of the tumor and separates it from normal bone microenvironment. Images were taken at either 400X or 100X magnification (lower right insets). D. Immunohistochemical staining using a specific antibody directed against CD31 (brown) was performed. Images were taken at 400X or 100X magnification showing rabbit IgG control in the lower right inserts and are representative of 7 different mice.
THE GROWTH AND AGGRESSIVE BEHAVIOR OF HUMAN OSTEOSARCOMA IS REGULATED BY A CAMKII-CONTROLLED AUTOCRINE VEGF SIGNALING MECHANISM

PAUL G. DAFT, YANG YANG AND MAJD ZAYZAFOON

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ABSTRACT

Osteosarcoma (OS) is a hyperproliferative malignant tumor that requires a high vascular density to maintain its large volume. Vascular Endothelial Growth Factor (VEGF) plays a crucial role in angiogenesis and acts as a paracrine and autocrine agent affecting both endothelial and tumor cells. The alpha-Ca\(^{2+}\)/Calmodulin kinase two (α-CaMKII) protein is a critical regulator of OS growth. Here, we investigate the role of α-CaMKII-induced VEGF in the growth and tumorigenicity of OS. We show that the pharmacologic and genetic inhibition of α-CaMKII results in decreases in VEGF gene expression (50%) and protein secretion (55%), while α-CaMKII overexpression increases VEGF gene expression (250%) and protein secretion (1,200%). We show that aggressive OS cells (143B) express VEGF receptor 2 and respond to exogenous VEGF (100nm) by increasing intracellular calcium (30%). This response is ameliorated by CBO-P11 (VEGFR inhibitor), suggesting that secreted VEGF results in autocrine stimulated α-CaMKII activation. Furthermore, we show VEGF and α-CaMKII inhibition decreases the transactivation of the Hypoxia Response Element (HRE) (77%) and TPA response element (TRE) (66%). Additionally, chromatin immunoprecipitation assay shows decreased binding of HIF-1α to HRE (80%) and AP-1 to TRE (85%) on the VEGF promoter. These data suggest that α-CaMKII regulates VEGF transcription by controlling HIF-1α and AP-1 transcriptional activities. Finally, CBO-P11 (44%), KN-93 (52%) and combination therapy (74%) significantly reduced tumor burden in vivo. Our results suggest that VEGF-induced OS tumor growth is controlled by CaMKII and dual therapy.
by CaMKII and VEGF inhibitors could be a promising therapy against this devastating adolescent disease.
INTRODUCTION

Osteosarcomas (OS) are the most frequently diagnosed primary bone tumors in humans. They are hyperproliferative malignant tumors that require high vascular density to maintain their excessively large volume (1). These tumors commonly occur during the second decade of life, and account for 5% of all pediatric tumors, and 20% of all bone tumors (2). OS most frequently develop in the highly proliferative metaphyseal region of long bones, commonly coinciding with the adolescent growth spurt (3). With patient prognosis remaining stagnant since the introduction of multi-agent chemotherapy, it is necessary to identify novel molecular targets that can be used to combat this devastating childhood disease. Ca\(^{2+}\)/Calmodulin-dependent kinase II (CaMKII) is a ubiquitously expressed multifunctional serine/threonine kinase, critical for Ca\(^{2+}\) signal transduction (4). It phosphorylates a variety of substrates, that are related to many aspects of cellular function in response to Ca\(^{2+}\) signaling (5). The role of CaMKII in many human cancers has been previously described (6-11). We were the first to report that the alpha splice variant (α-CaMKII) plays a critical role in determining the aggressive phenotype of OS (12,13). However, the mechanisms of its action remain unknown.

Excessive tumor growth results in increased demand for oxygen and nutrients which are provided by an increase in a tumor’s vascular supply (14). Most primary solid tumors initially undergo an avascular state of growth in which the maximum size attainable is 1-2 mm in diameter. These small tumors eventually “switch on” angiogenesis by inhibiting anti-angiogenic molecules or by secreting pro-angiogenic molecules. The resulting
deregulation in the vascular cycle ultimately leads to the sprouting of new capillaries, which infiltrate the body of the tumor (15). The ability of tumors to induce new blood-vessel formation has been a major focus of research over the past two decades. It is now known that members of the vascular endothelial growth factor (VEGF) family are some of the major inducers of angiogenesis (16).

VEGF is known to act locally on the tumor microenvironment (paracrine), by binding to VEGF receptors 1 and 2 (VEGFR-1 and VEGFR-2) on endothelial cells leading to their subsequent recruitment, proliferation and migration (15). The paracrine mechanism of VEGF’s action dramatically increases the number and size of blood vessels, providing ample blood supply to the tumor. Furthermore, VEGF has been shown to bind to VEGFRs on VEGF-secreting tumor cells (autocrine effect) in a variety of cancers (17-21). This autocrine response leads to the activation of different signaling pathways, ultimately leading to increases in the growth and proliferation of these tumors. To date, several anti-VEGF drugs, alone or in combination with chemotherapy, have shown promising clinical anti-cancer efficacy in colorectal (22), breast (23), ovarian (24) and glioma (25), validating the potential role of VEGF pathway inhibitors as an emerging therapy for cancer.

Transcriptional regulation of the VEGF promoter is a highly controlled process in both normoxic and hypoxic conditions (26). The VEGF promoter is known to be controlled by many transcription factors, mainly hypoxia inducible factor-1 alpha (HIF-1α) and Activating Protein-1 (AP-1), which together bind to a total of four sites on the human
VEGF promoter (27). We were the first to report that the deletion of α-CaMKII in OS cells decreases AP-1 protein levels and the vascularization of intratibially grown tumors in a xenograft mouse model (12,13). These findings coupled with published reports describing the role of CaMKII in the transcription of hypoxia-inducible genes; suggest that CaMKII-induced growth of OS may be regulated via VEGF.

In this study, we investigated the roles of both the paracrine and autocrine effects of α-CaMKII-induced VEGF on human OS in vitro and in vivo. We discovered that the levels of VEGF_{165} are augmented with increased aggressiveness of human OS cell lines. Furthermore, we show that α-CaMKII positively regulates the levels of VEGF RNA and protein in OS cells. We demonstrate that the inhibition of both α-CaMKII and VEGF dramatically decreases the aggressiveness of OS in vitro and in vivo. Taken together, our findings show that α-CaMKII-induced VEGF is critical for the growth and aggressiveness of human OS and the combinatorial use of compounds that inhibit both CaMKII and VEGF might provide novel therapies for the treatment of this devastating childhood tumor.
MATERIALS AND METHODS

Cell Culture and Treatments

Human OS cells (HOS, MG-63, N-methyl-N-nitro-N-nitrosoguanidine (MNNG)/HOS and 143B) and human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These human-derived cell lines were authenticated by DNA short tandem repeat profiling and experiments were conducted within 6 months of resuscitation. OS cells were maintained in DMEM medium containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), while HUVECs were maintained in 200PRF medium (Invitrogen) supplemented with low serum growth supplement (Invitrogen). All cell cultures were maintained at 37°C with 5% CO₂. 143B cells were serum starved overnight and treated with KN-93 (10 μM) and/or CBO-P11 (1μM) (Millipore) for 24 hours (13).

Enzyme-Linked Immunosorbent Assay (ELISA)

HOS, MG-63, MNNG/HOS and 143B cells were cultured in 6-well plates at a density of 1 x 10⁵ cells per well and allowed to reach confluency. The supernatants were collected 24 hours later and analyzed for levels of secreted VEGF₁₆₅ with a sandwich ELISA (Invitrogen) according to the manufacturer’s instructions. The optical density was measured at 450 nm using a Benchmark Plus microplate reader (Bio-Rad, Hercules, USA) (28).
**Tube Formation in vitro Assay**

HUVECs were used at passages 6-8. Each well of a 24-well plate was coated with 300 µl of GELTREX reduced growth factor basement membrane (Invitrogen). The plate was then incubated at 37˚C for 30 minutes to allow for GELTREX polymerization. HUVECs (5 x 10^4/well) were then seeded on the coated plates in a total volume of 500 µl, and incubated with conditioned medium supernatant taken from each of the following cell lines: HOS, GFP-Ctrl, GFP-CaMKIIα, MNNG/HOS, MG-63, 143B, shCtrl or shCaMKIIα. Capillary-like tube formation was documented after 12 hours with photomicrographs taken at 50x magnification. The capillary tube length was quantified using ImageJ software (National Institutes of Health, USA) and is shown as percent of total tube length (29).

**RNA Extraction and real-time PCR**

Total RNA was extracted using the TRIzol method as recommended by the manufacturer (Invitrogen). One µg of RNA was reverse-transcribed using M-MLV reverse transcriptase, and the equivalent of 10 ng was used for SYBR Green real-time quantitative RT-PCR. The expression of β-Actin was used for normalization of gene expression values. The following primers were used for PCR analysis: VEGF, forward 5’-TGCAGATTATGCGGATCAAACC-‘3 and reverse 5’-TGCATTCACATTGTTGTGCTGTAG-‘3; and Actin, forward 5’-ATTGCGACAGGATGCAGAA-3’ and reverse 5’-ACATCTGCTGGAAGGTGGACAG-‘3 (13).
Whole Cell Protein Extraction and Western Blot Analysis

Cells were lysed in 0.5% Nonidet P-40 lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, Immobilon-P (Millipore Co., Milford, MA, USA). Membranes were blocked with Tris-buffered saline-Blotto/Blotto B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour and subsequently incubated overnight with antibodies directed against α-CaMKII, p-α-CaMKII, p-CREB, CREB, p-ERK, ERK, p-c-Jun, c-Fos, Lamin B1, HIF-1α, VEGFR-1, VEGFR-2 or β-actin (Santa Cruz Biotechnology and Cell Signaling Technology, Beverly, MA, USA). Signals were detected using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences, Pittsburgh, PA, USA) (13).

Immunohistochemistry

Tissues were deparaffinized and rehydrated followed by antigen retrieval using 10mM sodium citrate buffer, pH 6. Samples were blocked for 1 hour in 5% goat serum (Vector Laboratories, Burlingame, CA, USA). Antibodies directed against KI-67 (Thermo Fisher Scientific, Waltham, MA, USA) and CD-31 (Abcam, Cambridge, England) were applied to sections and incubated overnight at 4°C. Biotin-conjugated secondary antibodies (2µg/ml) were added, followed by avidin-biotin enzyme reagents. Specimens were incubated in 3,3'-Diaminobenzidine (DAB) peroxidase substrate for 30 seconds. Tissues
were counterstained with Gill’s hematoxylin for 10 seconds, dehydrated, cleared and mounted. Rabbit IgG negative controls were processed alongside the examined tissue. Photomicrographs were taken using a Nikon DS-Fi1 digital camera (13).

**Motility Assay**

143B Cells were grown to 100% confluency in 6-well plates and scratched with the narrow end of a sterile pipette tip. Medium was changed to remove floating cells and replaced with DMEM medium containing 1% FBS. Photomicrographs were taken and the scratch width was measured immediately after initial wounding. Cells were then incubated at 37°C with 5% CO₂. After 12 hours, photomicrographs were taken at 50x magnification and the scratch width was measured. Data were expressed as percentage of the remaining width of the scratch (after 12 hours) when compared to the original width (at 0 hour). Migration analysis was performed using the manual tracking suite in ImageJ (13).

**Invasion Assay**

Cells (2.5x10⁴) were plated in media containing 0.1% FBS onto the Matrigel coated upper chambers of transwell invasion assay filter inserts (BD Bioscience, East Rutherford, NJ, USA). Medium containing 10% FBS was added into the lower chambers, acting as a chemoattractant. The cells were allowed to invade for 24 hours, after which the cells that invaded the Matrigel were fixed in methanol and stained with crystal violet (Cellgro, Manassas, VA, USA). Representative photomicrographs were taken at 100X magnification. Cells were counted from 5 low-power fields per filter insert (13).
MTT Assay

Cells were plated at a density of 5x10^3 cells per well in 96-well plates. After treatment, MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] (ATCC) was added to the culture medium at a final concentration of 0.5 mmol/L and plates were incubated for 2 hours at 37°C with 5% CO₂. Detergent solution was then added to solubilize formazan crystals. Finally, the optical density was determined at 570 nm using a Benchmark Plus microplate reader (Bio-Rad, Hercules, USA) (13).

Measurement of intracellular free Ca^{2+} by Fluo-4 NW

The Fluo-4 NW Calcium Assay Kit (Invitrogen) was used according to manufacturer’s instructions. 143B cells were seeded on poly-D-lysine (PDL)-coated 8-well microscopy chamber cover slides at 1 x 10^4 cells per well and cultured overnight. Cells were then incubated at 37°C for 30 minutes in the dye loading solution dissolved in assay buffer (Component C, Invitrogen). Following incubation, the cells were washed twice with assay buffer, and equilibrated at room temperature for an additional 30 minutes. [Ca^{2+}]_i was monitored for 4 min with a 0.1-second interval between measurements and every 40th time point was recorded for analysis. Relative fluorescent intensities at 4 regions-of-interests were measured and graphed as relative fluorescence vs. time (30).

Gene Silencing by shRNA

143B cells were plated at a density of 2 x 10^4 cells/cm² in 6-well plates. Cells were incubated in Polybrene (8 mg/mL; Sigma-Aldrich) for overnight at 37°C with 5% CO₂. Two specific a-CaMKII shRNAs or one nonspecific scrambled control shRNA (shCtrl)
were cloned into lentiviral transduction vectors (Sigma-Aldrich) and added to the media. The media was changed 24 hours post-transfection, and the transfected cells were cultured with fresh media containing puromycin (5 mg/mL) for selection (Sigma-Aldrich). Once all nontransfected cells died guaranteeing a pure culture, the transfected cells were split into 10 cm plates and maintained stably in culture (13).

**Retrovirus Production and Infection**

We used retroviral expression vectors that express either pMSCV-green fluorescent protein (GFP-Ctrl) or pMSCV-GFP-α-CaMKII (GFP-CaMKIIα) (13). The pMSCV-GFP and pMSCV-GFP-α-CaMKII were provided as a gift by Dr. Tobias Meyer at Stanford University Medical Center (31). Retroviruses were produced by co-transfecting pMSCV vectors with pVSV-G into BOSC23 cells using Lipofectamine (Invitrogen). Twenty-four hours post transfection, the media was replaced, and retroviral supernatant was collected. For infection, 2×10^4 cells/cm^2 HOS cells were plated into 6-well plates. The culture media were replaced with 500 μl of retroviral supernatant containing 8 μg/ml Polybrene (Sigma-Aldrich) and cells were incubated for 2 hours at 37 °C in 5% CO₂. Retroviral supernatant was then removed and cells were cultured in regular growth medium (13).

**Chromatin Immunoprecipitation (ChIP) assay**

143B OS cells were cultured as described above. Cells were fixed with 1% formaldehyde at room temperature for 10 minutes in order to cross-link DNA protein complex. Nuclei from cross-linked cells were resuspended in Tris-EDTA buffer and sonicated (Fisher Sonic dismembrator, Model 500). The soluble chromatin was resuspended in RIPA
buffer (0.1% sodium dodecyl sulfate, 1% Triton X-100, 0.1% sodium deoxycholate, 140 mM NaCl) and immunocleared with 2 μg of salmon sperm DNA/Protein A agarose beads (Upstate Biotechnology) for 1 hr at 4°C. Immunoprecipitation was performed with antibodies directed against HIF-1α, c-Fos and normal rabbit or mouse IgG overnight at 4°C, followed by adding salmon sperm DNA/protein A agarose for 1 hr. Immunoprecipitates were sequentially washed with the following buffers: once with low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 150 mM NaCl), once with high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1) and 500 mM NaCl), once with LiCl buffer (0.25% LiCl, 1% NP-40, 1% Na-Deoxycholate, 1 mM EDTA and 10 mM Tris-HCl (pH 8.1)) and twice with Tris-EDTA buffer. Cross-linking was reversed by heating with 0.2 M NaCl at 65°C overnight. DNA was precipitated with Phenol/Chloroform and the DNA template was amplified by conventional PCR. The following primers were used for PCR analysis:

VEGF promoter -1215 to -881 (HRE-containing region), forward 5'-TTGGGCTGATAGAAGCCTTG-3' and reverse 5'-TGGCACCAAGTTTGTGGAGC-3';

VEGF promoter -1814 to -1458 (TRE containing region) forward 5'-GCTCCAGATGGCACATTGTC-3' and reverse 5'-GGAATCCTGGAGTGACCCCT-3';

VEGF promoter -780 to -590 (TRE containing region) forward 5'-GCCGACGGCCTTGGGGAGATG-3' and reverse 5'-TCCGGCGGTCTACCCCCAAAA-3'.

The product was separated by 1.5% agarose gel electrophoresis (32).
Transient transfections and luciferase reporter assays

143B OS cells were plated at a density of $2 \times 10^4$ cells/cm$^2$ in 6-well plates. Twenty-four hours after plating, cells were transfected with 1 μg of HRE or TRE luciferase plasmid (Clontech, Palo Alto, CA) using the Amaxa nucleofactor II device (Lonza, Basel, Switzerland) according to the manufacturer's instruction. Forty-eight hours post-transfection, cells were lysed and reporter activity was measured using a luciferase assay system (Promega, Madison, WI) (32).

Animal studies and tumor cell inoculation

Six-week-old male Foxn1nu mice (Harlan Laboratories, Indianapolis, IN, USA) were used in these studies, with the approval of the University of Alabama at Birmingham Institutional Animal Care and Use Committee. 143B cells were prepared from sub-confluent cultures. Cells ($1 \times 10^6$ cells in 25 µl PBS) were intratibially injected using insulin syringes with 28.5 gauge needles. The knee was flexed, and the needle inserted into the tibia, boring the needle through the epiphysis and epiphyseal growth plate for delivery of the cells into the metaphysis. Tumors were allowed to grow for 7 days. Mice were then implanted with Alzet micro-osmotic pumps (model 1002) (DURECT Corp) that allow for consistent drug delivery for 2 weeks. The reservoir of each pump was filled with 5µg/µl KN-93, 10µg/µl CBO-P11 or vehicle and was set to release 0.25 µl/1 hour (6 µl/24 hours). 143B OS tumor growth was monitored by in vivo bioluminescence imaging at 7 and 21 days after cancer cell inoculation by injecting mice with D-luciferin solution (150 mg/kg) 10 minutes before imaging. Images were then acquired and analyzed with an
IVIS 100 Imaging System (Xenogen). Regions of interest were identified and plotted as fold difference in tumor size at day 21 compared with day 7. At the end of the study, animals were euthanized, hind limbs were excised, formalin fixed, EDTA decalcified, and paraffin embedded. Tissues were sectioned and stained with hematoxylin and eosin (H&E) for histologic evaluation. Photomicrographs were taken using a Nikon DS-Fi1 digital camera (13).

**Statistical Analysis**

Statistical analyses were performed using the Microsoft Excel data analysis program for Student’s t-test analysis. Experiments were repeated at least three times, unless otherwise stated. Values were expressed as mean ±SE with results considered significant at p<0.05 (13).
RESULTS

VEGF expression and secretion are increased in the highly aggressive OS cell lines

To examine the levels of VEGF\textsubscript{165} in human OS, real-time PCR and ELISA were performed using several human OS cell lines (HOS, MG-63, MNNG/HOS and 143B). Here, we show by real-time PCR that the highly aggressive OS cell lines (MNNG/HOS and 143B) express higher levels of VEGF\textsubscript{165} when compared to less aggressive cell lines (MG-63 or HOS). VEGF gene expression is 200\% higher in the highly aggressive 143B cell line when compared to the least aggressive HOS cell line (Figure 1A). OS cells were then plated at a concentration of 1 x 10\textsuperscript{6} cells per well for 24 hours followed by the collection of conditioned media aliquots. Consistent with real-time PCR results, we discovered by ELISA that the levels of secreted VEGF\textsubscript{165} in media collected from 143B cells is 1,500\% higher than in media collected from HOS cells (Figure 1B). Finally, the ability of OS-secreted VEGF\textsubscript{165} to induce endothelial tube formation was evaluated. HUVECs were cultured on a reduced growth factor basement membrane and treated with conditioned media collected from four different OS cell lines. Here we show that media collected from the highly aggressive OS cell lines (MNNG/HOS or 143B) formed 400-650\% more endothelial tube networks when compared to conditioned media collected from less aggressive OS cells (HOS or MG-63), (Figures 1C, 1D). To specifically demonstrate that VEGF in the media is responsible for the tube formation we used two additional controls; DMEM media supplemented with 10\% FBS as well as conditioned media collected from 143B cells after removing VEGF\textsubscript{165} by immunoprecipitation using
VEGF antibody. Both treatments failed to induce tube formation suggesting that VEGF$_{165}$ secreted from the cultured 143B OS cells is the responsible factor for endothelial tube formation. Taken together, this data demonstrate that the highly aggressive human OS cell lines express and secrete higher levels of VEGF$_{165}$ that increases *in vitro* endothelial tube network formation.

**α-CaMKII promotes OS angiogenesis by up-regulating expression and secretion of VEGF**

We have previously shown that α-CaMKII levels positively correlate with the aggressive phenotype of human OS cell lines in *vitro* and with the ability of OS tumors to form blood vessels in *vivo*. In order to determine whether α-CaMKII directly regulates OS tumor microvascular density through VEGF$_{165}$, we knocked down α-CaMKII in the highly aggressive human OS cell line 143B, and overexpressed α-CaMKII in the non-aggressive OS cell line (HOS). 143B cells were transduced with lentiviral vectors expressing a control non-functioning scrambled shRNA (shCtrl) or α-CaMKII-targeting shRNA (shCaMKIIα), while HOS cells were transduced with a retroviral construct overexpressing GFP-α-CaMKII (GFP-CaMKIIα) or a GFP alone (GFP-Ctrl) as a control. Furthermore, we pharmacologically inhibited CaMKII using a pharmacologic antagonist KN-93 (10µM). We previously reported the efficiency of shCaMKIIα and GFP-CaMKIIα in modifying the levels of CaMKIIα in OS cells (13). Here we show, that α-CaMKII inhibition and knockdown decrease VEGF gene expression (50%), while α-CaMKII overexpression in HOS cells increases VEGF gene expression (250%) when compared to
controls (Figure 2A). Additionally, we show that α-CaMKII knockdown or inhibition in 143B cells result in a 55% or 52% decrease in VEGF_{165} protein secretion, respectively, when compared to controls, while GFP-CaMKIIα HOS cells secrete 1,200% more VEGF_{165} than GFP-Ctrl (Figure 2B). Furthermore, the knockdown of α-CaMKII by shRNA or its inhibition by KN-93 decrease the ability of conditioned media collected from cultured 143B cells to induce endothelial tube network formation (~80%) when compared to controls, while conditioned media collected from cultured α-CaMKII overexpressing HOS cells increases endothelial tube network formation (~700%) when compared to control (Figure 2C and 2D).

**OS cell-secreted VEGF_{165} has an autocrine effect on tumor cells**

The paracrine effect of tumor-secreted VEGF_{165} on its surrounding endothelial cells is well established (33). Interestingly, evidence continues to emerge suggesting an additional autocrine effect of VEGF_{165} on tumor cells that secrete it, which may contribute to a more severe tumor pathogenesis (21,34). An increasing number of primary tumors have been shown to express functional VEGF_{165} receptors and respond directly to increases in extracellular VEGF_{165} by increasing free intracellular Ca^{2+} [Ca^{2+}]_i (35). In order to examine if OS cells are capable of responding to extracellular VEGF_{165}, we first examined the levels of VEGFRs in OS cells. Western blot analyses were performed using antibodies directed against VEGFR-1, VEGFR-2 or β-Actin. Here we show that VEGFR-1 is highly expressed in all four of the examined human OS cell lines while VEGFR-2 is only expressed in more aggressive cell lines (MNNG/HOS and 143B), suggesting that
aggressive OS cells are potentially more capable of responding to extracellular VEGF$_{165}$ (Figure 3A). We then examined the effects of exogenous VEGF$_{165}$ (100nM) on [Ca$^{2+}$]$_i$ in 143B cells. Upon treatment with 100nM VEGF$_{165}$, a 30% increase in [Ca$^{2+}$]$_i$ was observed when compared to BSA control (Figure 3B, upper panel), while VEGF$_{165}$ inhibition by 1µM CBO-P11 (competitive VEGF receptor inhibitor) results in a 25% decrease in [Ca$^{2+}$]$_i$ when compared to control (Figure 3B, lower panel). Furthermore, we show by western blot analyses that VEGF inhibition by CBO-P11 (1µM for 24 hours) in 143B OS cells decreases the activation of α-CaMKII and its downstream signaling target, CREB (Figure 3C). This data suggests the existence of a positive signaling feedback loop in OS cells, where extracellular VEGF$_{165}$ increases the activation of α-CaMKII and its downstream targets in human OS cells that ultimately leads to further increases in its expression.

**CaMKII and VEGF$_{165}$ inhibition results in a decrease of OS proliferation, motility and invasion**

The proliferation, motility and invasion of 143B OS cells in response to VEGF$_{165}$ and/or CaMKII inhibition were then examined. Using a 5 day MTT assay, we show that the inhibition of both VEGF$_{165}$ by CBO-P11 and CaMKII by KN-93 (10µM for 24 hours) decreases the proliferation of 143B cells when compared to control. On day five of the MTT assay, the proliferation of 143B cells when compared to vehicle treated controls, was significantly less after the inhibition of CaMKII (59%), VEGF$_{165}$ (33%), or both (75%) (Figure 4A). We then examined the motility of 143B OS cells in response to
treatment with CBO-P11 and/or KN-93. Motility studies were performed in 1% FBS-supplemented medium in order to suppress cell proliferation and allow us to identify OS cell motility independent of proliferation. A scratch was made at hour 0, and the migration of 143B OS cells into the cell free area was quantitated after 12 hours. Here we show that when VEGF\textsubscript{165} or CaMKII is inhibited, 143B cells migrate 64% and 50% less, respectively, when compared to vehicle control, while a combination of both inhibitors decreases migration by 90% (Figure 4B). Finally, invasiveness of 143B OS cells was evaluated using a 24 hour transwell invasion assay. Here we show that inhibition of CaMKII or VEGF\textsubscript{165} decrease invasion by 48% and 44%, respectively, when compared to vehicle treated-control, while combined inhibition decreases invasion by 97% (Figure 4C). These results demonstrate that the inhibition of CaMKII and/or VEGF\textsubscript{165} in human 143B OS cells leads to dramatic decreases in proliferation, motility and invasion.

In order to examine whether \(\alpha\)-CaMKII-induced VEGF\textsubscript{165} is specifically responsible for the changes in OS tumorigenicity, we deleted \(\alpha\)-CaMKII by shRNA and examined the effects of rescuing the OS aggressive phenotype of 143B OS cells by treating with 100nM VEGF\textsubscript{165}. Using an MTT assay, we show that the deletion of \(\alpha\)-CaMKII by shRNA decreases the proliferation of 143B OS cells by 72% and treatment with VEGF\textsubscript{165} restored 66% of this inhibition (Figure 5A). Furthermore, we show that \(\alpha\)-CaMKII deletion decreases 143B OS cell migration by 61% and treatment with VEGF\textsubscript{165} restored 59% of this inhibition (Figure 5B). Finally, we show by an invasion assay that \(\alpha\)-CaMKII
deletion decreases the invasion of 143B OS cells by 85% and treatment with VEGF$_{165}$ restored 25% of this inhibition (Figure 5C).

**CaMKII and VEGF$_{165}$ positively regulate the levels of HIF1-α and AP-1 in OS cells**

The transcriptional regulation of VEGF is a complex and highly controlled process. The VEGF promoter is known to be controlled by many families of transcription factors, namely HIF-1 and AP-1 and their response elements (HRE and TRE respectively). Together they make a total of four binding sites on the VEGF promoter, three of which have been shown to be important in VEGF gene expression (Figure 6A). In order to examine the role of CaMKII and/or VEGF$_{165}$ in the regulation of HIF-1α and/or AP-1, we transfected 143B OS cells with 1µg HRE or TRE luciferase reporter plasmids for 24 hours. Cells were then treated with CBO-P11 and/or KN-93, for another 24 hours. At the end of the study, cells were lysed and reporter activity was measured. Here we show, that the inhibition of CaMKII by KN-93 or VEGF$_{165}$ by CBO-P11 decreases AP-1 transactivation by 33% or 41%, respectively, while dual inhibition results in a 66% decreases in AP-1 transactivation when compared to control. Furthermore, the inhibition of CaMKII or VEGF$_{165}$ decreases HRE transactivation by 66% or 45%, respectively, while inhibiting both results in 80% decrease in HRE transactivation when compared to control (Figure 6B). These results were confirmed by western blot analyses on 143B protein cell lysate after treating cells with KN-93 or CBO-P11 for 24 hours. Our data show that the inhibition of CaMKII and/or VEGF signaling dramatically decreases the activation of ERK, Jun, c-Fos and HIF-1α (Figure 6C). In order to investigate whether
the decreases in the levels of AP-1 and HIF-1 diminish their ability to bind their DNA response elements on the VEGF promoter, we performed a chromatin immunoprecipitation (ChIP) assay.

Cells treated with KN-93 and/or CBO-P11 for 24 hours were then fixed in formaldehyde and sonicated. Immunoprecipitation was performed with antibodies directed against HIF-1α, c-Fos and normal rabbit or mouse IgG. The cross-linking was then reversed and the DNA was precipitated with DNA templates amplified by conventional PCR. Here, we show that the inhibition of CaMKII and/or VEGF decreases AP-1 (TRE₁₅₂₈ and TRE₂₆₀) and HIF-1α (HRE₉₇₅) binding on the VEGF promoter of 143B OS cells (Figure 6D). Real-time PCR was performed to confirm that decreases in HIF-1α and AP-1 binding to the VEGF promoter causes decreases in VEGF gene expression. Here show significant decreases in VEGF gene expression in 143B OS cells with the inhibition of CaMKII (50%), VEGF (45%) or both (80%) (Figure 6E). Taken together, these data suggest that CaMKII and VEGF₁₆₅ are regulating VEGF gene expression by AP-1 and HIF-1α transcription factors.

Inhibition of VEGF₁₆₅ or CaMKII results in decreased OS tumor growth in animal model

We next examined whether VEGF₁₆₅ and/or CaMKII inhibition affects the growth of OS cells in vivo. We intratibially injected 143B OS cells into 6-week old male athymic (nude) mice. These cells were previously transduced with lentivirus encoding firefly luciferase allowing for in vivo monitoring of tumor growth. Tumors were allowed to
grow and establish in the bones of the animals for one week (W0). Mice were then randomly divided into four groups receiving saline as control, CBO-P11 (2 mg/kg/day), KN-93 (1 mg/kg/day), or both CBO-P11 and KN-93 for 2 weeks (W2). All treatment was delivered by subcutaneously implanted ALZET micro-osmotic pumps. Here, we show by luminescence imaging that the inhibition of VEGF$_{165}$ by CBO-P11 or CaMKII by KN-93 reduces tumor burden by 44% and 52%, respectively, while the combined treatment of both KN-93 and CBO-P11 resulted in even greater reductions in tumor size (74%) when compared to that of the saline treated control group (Figures 7A and 7B). At the end of the study (after 2 weeks of treatment), mice were euthanized and tibiae were collected. Tibiae were scanned by µ-CT and later processed for histology and IHC staining. µ-CT analyses confirmed that KN-93 and CBO-P11 treatment dramatically reduced bone destruction caused by the growth of intratibial OS tumors (Figure 7C, Micro-CT panel). H&E staining shows smaller tumors with decreased bone destruction in CBO-P11 and/or KN-93 treated mice when compared to control (Figure 7C, H&E panel). Finally, IHC staining was performed using antibodies directed against CD31, an endothelial cell specific protein, and Ki-67, a protein marker for cell proliferation (Figure 7C, lower panels). Here we show CaMKII and VEGF$_{165}$ inhibition dramatically decreases tumor microvasculature and tumor cell proliferation when compared to saline treated controls.

Taken together, our data demonstrate that α-CaMKII-induced VEGF plays a critical role in the growth and tumorigenicity of OS cells in vitro and in vivo.
DISCUSSION

Tumor growth, progression and metastasis are dependent on new blood vessel formation (neo-vascularization). VEGF is a potent angiogenic factor secreted by a variety of tumor cells, and is ubiquitously expressed at sites of angiogenesis (36,37). It has previously been shown that VEGF expression in OS is predictive of pulmonary metastasis in patients who underwent aggressive therapy (1,38). Although several studies have demonstrated poor prognosis in patients with high VEGF levels there have been few studies trying to understand the molecular mechanisms responsible for clinically aggressive behavior in OS. Here we show that the highly metastatic 143B OS cell line has increased VEGF gene expression and protein secretion when compared to parental HOS cells. Interestingly, it has recently been shown that decreases in HIF-1 by CaMKII inhibition significantly decreases VEGF expression in human macrophages (39). We show a similar effect in 143B cells, where by inhibiting CaMKII genetically by shRNA or pharmacologically by KN-93 we see a ~50% decrease in VEGF protein secretion. Additionally, when CaMKII is overexpressed in HOS cells a ~1,000% increase in VEGF protein secretion was observed. These results suggest that CaMKII may be regulating VEGF and are consistent with what was previously seen in human macrophages.

Though VEGFR signaling has mainly been described in endothelial cells, there is increasing evidence that VEGFR autocrine signaling may play a prominent role in highly metastatic cancer cell lines (40,41). Here we show that highly metastatic 143B OS cells express VEGFR-2, thereby establishing an autocrine signaling mechanism associated
with aggressive phenotypes. 143B cells express higher levels of VEGFR-2 than parental HOS cells and are sensitive to exogenous VEGF stimulation. This autocrine signaling mechanism results in increases in [Ca2+]i and leads to increased proliferation, invasion and migration. These results are consistent with previous studies that have shown increases in [Ca2+]i and CaMKII subsequent activation in vascular smooth muscle cells and hippocampal neurons upon treatment with exogenous VEGF (42,43). We show that VEGF-stimulated increases in [Ca2+]i can be inhibited by the VEGFR-2 inhibitor, CBO-P11, which ultimately results in CaMKII inhibition. These results show a novel positive feedback loop, where CaMKII-induced VEGF results in increases in [Ca2+]i and CaMKII activation.

It was previously shown that Ca2+ ionophores result in increased VEGF transcription in human lung carcinoma cells (27). The human VEGF promoter contains three TRE and one HRE consensus binding regions, which might help explain the molecular mechanisms by which CaMKII is transcriptionally regulating VEGF (44). AP-1 is a protein complex that binds to the TRE consensus regions, has been shown to be tightly regulated by CaMKII in human osteoblasts (45). Here we show that both CaMKII and VEGF inhibition result in decreased HIF-1α and AP-1 binding to the VEGF promoter, which is likely responsible for the decreases observed in VEGF transcription. Furthermore, others have shown that c-fos, in combination with other members of the AP-1 family of transcription factors, regulate the expression of several matrix metalloproteinases, such as MMP-1, -9 and -13 (46). These changes in MMP expression
could be responsible for the decreased invasion observed in response to CaMKII and VEGF inhibition. Taken together, our results demonstrate the critical role of CaMKII-induced VEGF transcription in the *in vitro* aggressiveness of OS cells.

Similarly, we show that the inhibition of CaMKII and VEGF decreases OS tumor growth *in vivo*. The therapeutic benefit of anti-VEGF treatment on solid tumors is well documented. Initially, the addition of Bevacizumab, a monoclonal antibody for VEGF, to standard chemotherapy produced significant clinical benefit in patients with previously untreated and pretreated metastatic colorectal cancer, advanced non-small cell lung cancer, and metastatic breast cancer (47). Similarly, our data show that the inhibition of CaMKII and VEGF produces not only smaller tumors but also resulted less tumor vasculature as demonstrated by a decrease in the number of positively stained CD31 blood vessels. Furthermore, decreases in the osteolytic properties of OS are observed by micro-CT. These findings suggest that CaMKII and VEGF are both responsible for OS tumor angiogenesis and when used in combination therapy drastic decreases in tumor size and vasculature can be achieved.

Our present results provide novel insights into the molecular basis of the emerging interplay between CaMKII and tumor angiogenesis, and the novel VEGF/VEGFR2-mediated autocrine signaling loop. These findings help advance our understanding of the molecular mechanisms associated with poor prognosis in VEGF positive human OS patients, and provide valuable clues for future therapeutic strategies, as OS remains a major therapeutic challenge.
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**Figure 1.** VEGF expression and secretion are increased in the highly aggressive OS cell lines

**A.** Real-time PCR was performed using primers specific for VEGF or β-Actin in human OS cell lines HOS, MG-63, MNNG/HOS and 143B. Values were obtained from three separate experiments each repeated in triplicate and represent the mean ±S.E. *p<0.01. **B.** HOS, MG-63, MNNG/HOS and 143B human OS cells were seeded at 1 x 10^5 cells per well on a 6-well plate. Twenty-four hours later, aliquots of conditioned media were examined for the presence of human VEGF_{165} by ELISA. Values were obtained from three separate experiments each replicated in triplicate and represent the mean ±S.E.
*p<0.01.  **C.** HOS, MG-63, MNNG/HOS and 143B human OS cells were seeded at 1 x 10^5 cells per well on a 6-well plate. Twenty-four hours later, aliquots of supernatant were removed from dishes and added to 12-well plates seeded with 1 x 10^5 HUVEC cells. Endothelial cell tube formation was examined at 12 h. Representative photomicrographs were taken at 50x magnification from 3 independent experiments, each repeated in triplicate.  **D.** Capillary tube length was quantified using the ImageJ software. Values were obtained from three separate experiments each repeated in triplicate and represent the mean ±S.E. *p<0.01.
Figure 2. **VEGF** expression is positively regulated by CaMKII in human OS

143B cells were transduced with lentiviruses expressing either scrambled (shCtrl) or α-CaMKII-targeting shRNAs (shCaMKIIα) or treated with the CaMKII inhibitor KN-93. Also, HOS cells were transduced with retroviruses expressing either GFP (GFP-Ctrl) or CaMKIIα (GFP-CaMKIIα). A. Real-time PCR was performed using primers specific for VEGF or β-Actin. Values were obtained from three separate experiments each repeated in triplicate and represent the mean ±S.E. *p<0.01. B. α-CaMKII-inhibited 143B (shCaMKIIα or KN-93) and α-CaMKII overexpressing HOS (GFP-CaMKIIα) cells were seeded at 1 x 10^5 cells per well of a 6-well plate. Twenty-four hours later, aliquots of
supernatant were examined for human VEGF$_{165}$ by ELISA. Values were obtained from three separate experiments each repeated in triplicate and represent the mean ±S.E. *p<0.01. C. α-CaMKII inhibited 143B (shCaMKIIα or KN-93) and α-CaMKII overexpressing HOS (GFP-CaMKIIα) cells were seeded at 1 x 10$^5$ cells per well of a 6-well plate. Twenty-four hours later, aliquots of conditioned media were removed from dishes and added to 12-well plates seeded with 1 x 10$^5$ HUVEC cells. Endothelial cell tube formation was measured at 12 h. Representative photomicrographs were taken at 50x magnification from 3 independent experiments, each repeated in triplicate. D. Capillary tube length was quantified using the ImageJ software. Values were obtained from three separate experiments each repeated in triplicate and represent the mean ±S.E. *p<0.01.
Figure 3. VEGF_{165} acts on 143B human OS cells in an autocrine manner

A. VEGF binding receptors were identified by western blot. Immunoblots were developed using specific antibodies directed against VEGFR-2, VEGFR-1 or β-Actin. The autoradiograph is representative of three experiments. 

B. 143B cells were incubated with 5μM of Fluo-4-AM and then treated with 100nM VEGF (upper panel) or 1μM CBO-P11 (lower panel). BSA was used as control. Relative fluorescence intensities at four regions-of-interests were measured, with the average fluorescent intensity plotted versus time. Values were obtained from three separate experiments each repeated in triplicate and represent the mean ±S.E. *p<0.01. 

C. 143B Cells were treated with 10 μM KN-93
and/or 1 µM CBO-P11 for 24 hours. Immunoblots were developed using specific antibodies directed against p-α-CaMKII, α-CaMKII, p-CREB or CREB. The autoradiographs are representative of three independent experiments.
Figure 4. Pharmacologic inhibition of CaMKII and VEGF$_{165}$ decreases aggressiveness of 143B OS cells in vitro

A. MTT assay was performed at days 1, 2, 3, 4 and 5 to determine the number of viable cells. 5,000 143B Cells were seeded in a 96-well plate. At 24 hours after seeding, cells
were treated with 10µM KN-93 and/or 1µM CBO-P11. Values were obtained from three separate experiments, each repeated in triplicate and represent the mean ± S.E. *p<0.01.

**B.** Scratch/wound healing assay was performed on cells cultured for 12 hours. The width between the scratched areas at 0 hour was set to 100%. Representative photomicrographs were taken at 50x magnification from 3 independent experiments, each repeated in triplicate. Values represent the mean ± S.E. *p<0.01.

**C.** Transwell invasion assay allowing cells to invade for 24 hours. Representative photomicrographs were taken at 100x magnifications from 3 independent experiments, each repeated in duplicate. Values represent the mean ± S.E. *p<0.01.
Figure 5. Exogenous VEGF$_{165}$ partially rescues 143B OS cell phenotype in α-CaMKII knockdown 143B OS cells

143B cells were transduced with lentiviruses expressing either scrambled (shCtrl) or α-CaMKII-targeting shRNAs (shCaMKIIα). A. MTT assay was performed at days 1, 2, 3, 4 and 5 to determine the number of viable cells. 5x10$^3$ 143B Cells were seeded in a 96-well plate. At 24 hours after seeding, cells were treated with 100nM VEGF$_{165}$. Values were
obtained from three separate experiments, each repeated in triplicate and represent the mean ± S.E. *p<0.01. **B. Scratch/wound healing assay** was performed on cells cultured for 12 hours. The width between the scratched areas at hour 0 was set to 100%. Representative photomicrographs were taken at 50x magnification from 3 independent experiments, each repeated in triplicate. Values represent the mean ± S.E. *p<0.01. **C. Transwell invasion assay** allowing cells to invade for 24 hours. Representative photomicrographs were taken at 100x magnifications from 3 independent experiments, each repeated in duplicate. Values represent the mean ± S.E. *p<0.01.
Figure 6. CaMKII controls VEGF gene expression by regulating TRE and HRE

A. Schematic illustration of the VEGF promoter showing the binding sites for the AP-1 and HIF-1α transcription factors. B. Cells were transfected with TRE and HRE luciferase constructs. Cells were treated with 10 µM KN-93 and/or 1 µM CBO-P11 for 24 hours and harvested 24 hours later for luciferase activity measurement. Data are expressed relative to total protein, and values represent the mean ± SE of 3 separate experiments each repeated in triplicate; *p≤ 0.01. C. 143B cells were treated with 10 µM KN-93 and/or 1 µM CBO-P11 for 24 hours. Immunoblots were developed using specific...
antibodies directed against p-ERK, ERK, p-c-Jun, c-Fos, HIF-1α, Lamin B1 or β-Actin. The autoradiographs are representative of three experiments. D. Cellular DNA fragments were immunoprecipitated with antibodies against c-Fos and HIF-1α with total input material and normal rabbit IgG as controls. Immunoprecipitated DNA fragments were amplified by PCR using primers encompassing the TRE and HRE binding sites on the VEGF promoter. Three independent experiments were performed. E. Real-time PCR was performed using primers specific for VEGF$^{165}$ or β-Actin in human OS cell lines 10 μM KN-93 and/or 1 μM CBO-P11 for 24 hours. Values were obtained from three separate experiments each replicated in triplicate and represent the mean ±S.E. *p<0.01.
**Figure 7.** Inhibition of VEGF and CaMKII in OS cells dramatically decreases tumor growth in vivo.

143B cells were transduced with lentiviruses expressing firefly luciferase and allowed to grow for 1 week. ALZET micro-osmotic pumps were subcutaneously implanted into mice delivering saline (vehicle), CBO-P11 (2 mg/kg/day), KN-93 (1 mg/kg/day), or both CBO-P11 and KN-93. **A.** Luciferase imaging was performed before treatment (W0) and 2 weeks after treatment (W2) (n=8). **B.** Fluorescent intensity was measured and graphed. Values were obtained from 8 mice from each group and represent the mean ± SE. *P < 0.01. **C.** Mice were euthanized and tibiae were collected. Tibiae were scanned by μ-CT. Hematoxylin and eosin staining was performed on paraffin embedded tumors. Broken lines indicate the boundary of the tumors and separating it from normal bone.
microenvironment. Images were taken at 40x magnification. IHC staining using specific antibodies directed against CD31 and Ki-67 (brown) were performed. Images were taken at 200x magnification and are representative of 8 different mice.
TAMOXIFEN AND BEVACIZUMAB AS A NOVEL TREATMENT IN HUMAN OSTEOSARCOMA

PAUL G. DAFT, JOAN CADILLAC, JOSEPH G. PRESSEY AND MAJD ZAYZAFOON

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Abstract

Osteosarcoma (OS) is among the most frequently occurring primary bone tumors, primarily affecting adolescents and young adults. We have previously demonstrated that alpha-Ca$^{2+}$/Calmodulin kinase two ($\alpha$-CaMKII) regulates VEGF and its autocrine signaling functions in human OS. Here, we show that tamoxifen and bevacizumab inhibit CaMKII. We show that tamoxifen decreases VEGF gene expression (55%) and protein secretion (57%), while bevacizumab also decreases VEGF gene expression (40%). Furthermore we show that dual treatment with tamoxifen and bevacizumab decreased proliferation (75%), and invasion (90%) of 143B OS cells. Additionally, we show decreases in *in vivo* tumor growth with dual treatment of tamoxifen and bevacizumab (88%) when compared to control. Finally, we developed a novel preclinical xenograft mouse model to examine the recurrence and metastasis of human OS. 143B OS cells were intratibially injected into mice, and tumors were allowed to grow for 2 weeks. Hind limbs-containing tumors were then amputated, and mice were confirmed to be tumor free by bioluminescent imaging 7 days post-surgery. Mice were randomized into four treatment groups: saline, tamoxifen (500μg/kg/day), and/or bevacizumab (5μg/kg twice weekly) and monitored monthly by bioluminescent imaging for the development of metastasis. The incidence of pulmonary metastasis in saline treated mice was 100% two months after amputation. However, the incidence decreased to 66% in bevacizumab-treated mice, 11% in tamoxifen-treated mice, and 0% when both drugs were used. These
results suggest that tamoxifen and bevacizumab may be effective at both inhibiting primary tumor growth and metastasis.

INTRODUCTION

Osteosarcomas (OS) are the most commonly diagnosed primary bone tumors in humans (1). They are hyperproliferative malignant tumors that are highly vascularized and grow to excessively large volumes (2, 3). These tumors most frequently occur in adolescence between the ages of 15-19, and account for roughly 5% of all pediatric tumors, and 20% of all bone tumors (4). OS often develop in the highly proliferative metaphyseal region of long bones, which coincides with the adolescent growth spurt (5). Current OS treatment protocols consist of highly invasive wide resection surgery and systemic chemotherapy (6). Prior to multi-agent chemotherapy, when patients only received surgery, upwards of 90% of patients would develop pulmonary metastases resulting from undetectable micro-metastases (7). These high rates of tumor recurrence resulted in dismal 5-year survival rates of <20% (8). Starting in the 1970s when OS patients began receiving post- and pre-operative chemotherapy, prognoses dramatically improved to 60-70% event free 5-year survival (9). Unfortunately, patient prognosis has remained stagnant since the introduction of multi-agent chemotherapy, and patients who do not respond to chemotherapy or are not disease free after surgery have a dismal prognosis with little hope for prolonged survival (10). It is necessary to develop new treatments to more adequately prevent metastases and prolong patient survival.
Tamoxifen is a non-steroidal antioestrogen, which was originally shown to have efficacy in the treatment of advanced breast cancer (11). Tamoxifen has a low incidence of side effects and was subsequently approved as therapy for all stages of breast cancer (12). In addition to its efficacy in treating breast cancer, tamoxifen also has therapeutic activity in several other types of cancer (13, 14). Many of these tumors, including malignant gliomas, are estrogen receptor (ER) negative, suggesting an alternative role in cancer treatment independent of the estrogen receptor (15).

In addition to its anticancer effects, it has previously been shown that tamoxifen inhibits the activation of phosphodiesterase by calmodulin. Kinetic analysis subsequently demonstrated that tamoxifen is a competitive inhibitor of calmodulin (16, 17). It could be speculated that the antagonism of calmodulin by tamoxifen may be one of the mechanisms responsible for its ER-independent pharmacological actions. Additionally, tamoxifen treatment decreases CaMKII activation in breast cancer samples collected from the Breast Cancer Prevention Trial (18). We have previously shown that CaMKII plays a critical role in determining the aggressive behavior of human OS, and the inhibition of CaMKII dramatically decreases OS growth in vitro and in a pre-clinical mouse model (19). While no CaMKII specific inhibitors are FDA approved, tamoxifen appears to be a promising treatment alternative for human OS because of its previously described Calmodulin and CaMKII inhibitory effects.

Angiogenesis is crucial to tumor initiation, survival and metastasis. Vascular endothelial growth factor (VEGF) is one of the most important pro-angiogenic factors in cancer
development (20). We have previously shown that a CaMKII-regulated VEGF/VEGFR2 autocrine signaling mechanism is important for the growth of human OS. We discovered that the VEGFR-2 inhibitor, CBO-P11, decreased primary tumor growth in a xenograft mouse model. Although exciting, this data is clinically limited due to CBO-P11 being a research compound. Fortunately, bevacizumab, a humanized monoclonal antibody that blocks the binding of VEGF to its receptors and results in regression of immature tumor vasculature, normalization of remaining tumor vasculature and inhibition of further tumor angiogenesis was approved for use in the treatment of metastatic colon cancer in 2004 (21). It has since been approved for use in lung, renal, ovarian and breast cancers (22-24). Though limited, studies have shown that patients with relapsed/refractory sarcomas treated with gemcitabine, docetaxel, and bevacizumab in 3-week cycles appears to have efficacy (25). This treatment regimen is well tolerated with minimal toxicity. Two patients had a partial response and the third patient had stable disease for >6 month with this treatment. These results suggest bevacizumab may be a viable second line OS treatment.

In this study, we investigated the efficacy of treating human OS by inhibiting CaMKII with tamoxifen and VEGF with bevacizumab. We discovered that both tamoxifen and bevacizumab potently inhibit α-CaMKII in an ER-independent manner in the 143B human OS cell line. Furthermore, we show treatment with tamoxifen and bevacizumab decreases the levels of VEGF RNA and protein in 143B OS cells. We then demonstrate that treatment with these two drugs dramatically decreases the aggressiveness of OS in
vitro and in vivo. Finally, using a clinically relevant metastatic mouse model we show that tamoxifen and bevacizumab prevent detectable OS pulmonary metastases. Taken together, our findings show that the combinatorial use of tamoxifen and bevacizumab might provide a novel therapy for the treatment of this devastating childhood disease.

MATERIALS AND METHODS

Cell Culture and Treatments

143B human OS cells and human umbilical vein endothelial cells (HUVEC) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These human-derived cell lines were authenticated by DNA short tandem repeat profiling and experiments were conducted within 6 months of resuscitation. 143B cells were maintained in DMEM medium containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), while HUVECs were maintained in 200PRF medium (Invitrogen) supplemented with a low serum growth supplement (Invitrogen). All cell cultures were maintained at 37°C with 5% CO2. 143B cells were serum starved overnight and treated with tamoxifen (1 μM) and/or bevacizumab (1μM) for 24 hours.

Enzyme-Linked Immunosorbent Assay (ELISA)

143B cells were cultured in 6-well plates at a density of 1 x 105 cells per well and allowed to reach confluency. Supernatants were collected 24 hours later and analyzed for
levels of secreted VEGF165 with a sandwich ELISA (Invitrogen) according to the manufacturer’s instructions. The optical density was measured at 450 nm using a Benchmark Plus microplate reader (Bio-Rad, Hercules, USA).

Tube Formation in vitro Assay

HUVECs were used at passages 6-8. Each well of a 12-well plate was coated with 300 µl of GELTREX reduced growth factor basement membrane (Invitrogen). The plate was then incubated at 37°C for 30 minutes to allow for GELTREX polymerization. HUVECs (5 x 10^4/well) were then seeded on the coated plates in a total volume of 500 µl, and incubated with conditioned medium supernatant taken from 143B cells treated with; ICI182780, tamoxifen, bevacizumab or tamoxifen and bevacizumab. Capillary-like tube formation was documented after 12 hours with photomicrographs taken at 50X magnification. The capillary tube length was quantified using ImageJ software (National Institutes of Health, USA) and is shown as percent of total tube length.

**RNA Extraction and real-time PCR**

Total RNA was extracted using the TRIzol method as recommended by the manufacturer (Invitrogen). One µg of RNA was reverse-transcribed using M-MLV reverse transcriptase, and the equivalent of 10 ng was used for SYBR Green real-time quantitative RT-PCR. The expression of β-Actin was used for normalization of gene expression values. The following primers were used for PCR analysis: VEGF, forward 5’-TGCAGATTATGCGGATCAAACC-3’ and reverse 5’-
TGCA\text{TTCACATTGTGTGCTGTAG-}'3; and Actin, forward 5\text{'}-
ATTGCGACAGGATGCAGAA-3 and reverse 5\text{'}-
ACATCTGCTGGAAGGTGGACAG-3.

**Whole Cell Protein Extraction and Western Blot Analysis**

Cells were lysed in 0.5% Nonidet P-40 lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, Immobilon-P (Millipore Co., Milford, MA, USA). Membranes were blocked with Tris-buffered saline-Blotto/Blotto B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour and subsequently incubated overnight with antibodies directed against \( \alpha \)-CaMKII and p-\( \alpha \)-CaMKII (Santa Cruz Biotechnology). Signals were detected using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences, Pittsburgh, PA, USA).

**Invasion Assay**

Cells (2.5x104) were plated in media containing 0.1% FBS onto the Matrigel coated upper chambers of transwell invasion assay filter inserts (BD Bioscience, East Rutherford, NJ, USA). Medium containing 10% FBS was added into the lower chambers, acting as a chemoattractant. The cells were allowed to invade for 24 hours, after which the cells that invaded the Matrigel were fixed in methanol and stained with crystal violet.
(Cellgro, Manassas, VA, USA). Representative photomicrographs were taken at 100X magnification. Cells were counted from 5 fields per filter insert.

**MTT Assay**

Cells were plated at a density of 5x10^3 cells per well in 96-well plates. After treatment, MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] (ATCC) was added to the culture medium at a final concentration of 0.5 mmol/L and plates were incubated for 2 hours at 37°C with 5% CO2. Detergent solution was then added to solubilize formazan crystals. Finally, the optical density was determined at 570 nm using a Benchmark Plus microplate reader (Bio-Rad, Hercules, USA).

**Animal studies and tumor cell inoculation**

Six-week-old male Foxn1nu mice (Harlan Laboratories, Indianapolis, IN, USA) were used in these studies, with the approval of the University of Alabama at Birmingham Institutional Animal Care and Use Committee. 143B cells were prepared from sub-confluent cultures. Cells (1 x 10^6 cells in 25 µl PBS) were intratibially injected using insulin syringes with 28.5 gauge needles. The knee was flexed, and the needle inserted into the tibia, boring the needle through the epiphysis and epiphyseal growth plate for delivery of the cells into the metaphysis. Tumors were allowed to grow for 7 days. 143B OS tumor growth was monitored by in vivo bioluminescence imaging at 7 and 21 days after cancer cell inoculation by injecting mice with D-luciferin solution (150 mg/kg) 10 minutes before imaging. Images were then acquired and analyzed with an IVIS 100
Imaging System (Xenogen). Regions of interest were identified and plotted as fold difference in tumor size at day 21 compared with day 7. At the end of the study, animals were euthanized, hind limbs were excised, formalin fixed, EDTA decalcified, and paraffin embedded. Tissues were sectioned and stained with hematoxylin and eosin (H&E) for histologic evaluation. Photomicrographs were taken using a Nikon DS-Fi1 digital camera.

**Mouse tumor containing hind-limb amputation**

After inoculation of 143B OS cells through intratibial injection, tumors were allowed to establish for 14 days. After tumor establishment, the tumor containing right hind limb was amputated by joint disarticulation at the knee. Anesthesia was induced with 5% isoflurane and 2 l/min oxygen in an induction chamber and subsequently maintained with 1.5% isoflurane and 1 l/min oxygen. The tumor-inoculated hind limbs were aseptically prepared with alternating scrubs of 100% ethanol and dilute chlorhexidine acetate. A local ring block of lidocaine and bupivicaine was administered at the knee joint and infused at the site of surgery. A circumferential skin and subcutaneous fat incision was made with a number 15 scalpel blade at the level of the distal end of the tibia. The femoral artery and vein were then concurrently ligated with ligating clips and transected with scissors. Circumferential musculature was then transected proximal to the level of vessel ligation. The tibia and its remaining muscle attachments were than transected and the tumor-bearing limb removed. Musculature was closed over the distal femur with 5-0 polyglyconate in an interrupted pattern. Mice were recovered in a clean
cage on a warming pad and provided warmed SQ saline. Pre- and post-operative analgesia consisted of subcutaneous injections of 0.1 mg/kg buprenorphine (Buprenex, Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) prior to surgery and every 8–12 h for 2 days after surgery. In addition, 0.3 mg/kg/day meloxicam (Metacam, Boehringer Ingelheim Corp., Ridgefield, CT) and 31.5 mg/kg/day enrofloxacin (Baytril, Bayer HealthCare, LLC, Shawnee Mission, KS) were subcutaneously injected. Mice were examined twice daily until the surgical sites were healed (26).

**Statistical analysis**

Statistical analyses were performed using the Microsoft Excel data analysis program for Student’s t-test analysis. Experiments were repeated at least three times, unless otherwise stated. Values were expressed as mean ±SE with results considered significant at p<0.05.
RESULTS

VEGF expression and secretion are decreased in 143B OS cells treated with tamoxifen and bevacizumab

We have previously shown that α-CaMKII inhibition by KN-93 and VEGF inhibition by CBO-P11 decreases tumor growth and vascularization in vivo. In order to determine whether pharmacologically inhibiting CaMKII by tamoxifen and VEGF by bevacizumab results in similar decreases in tumor growth and vascularization, we examined the ability of these two FDA approved drugs to decrease VEGF expression and secretion. First, by western blot analysis we show that tamoxifen and bevacizumab inhibit CaMKII activation, with this inhibition being independent of the ER as demonstrated by no change in activation levels upon treatment with the ER inhibitor ICI182780 (Figure 1A). Furthermore, we show by real-time PCR that treatment with tamoxifen (58%) or bevacizumab (42%) decreases VEGF gene expression, while treatment with both drugs further decreases gene expression (75%) (Figure 1B). Consistent with real-time PCR results, we discovered by ELISA that the levels of secreted VEGF165 in media collected from 143B cells treated with tamoxifen, bevacizumab and both drugs decreases VEGF protein secretion (58%, 80% and 87% respectively) when compared to controls (Figure 1C). Finally, the ability of OS-secreted VEGF165 to induce endothelial tube formation was evaluated. HUVECs were cultured on a reduced growth factor basement membrane and treated with conditioned media collected from 143B cells treated with tamoxifen and/or bevacizumab. Here we show that media collected from tamoxifen and/or
bevacizumab significantly decreased endothelial tube networks when compared to ICI182780 and untreated control (Figures 1D). To specifically demonstrate that VEGF in the media is responsible for the tube formation we used two additional controls; DMEM media supplemented with 10% FBS as well as conditioned media collected from 143B cells after removing VEGF165 by immunoprecipitation. Both treatments failed to induce tube formation suggesting that VEGF165 secreted from the cultured 143B OS cells is the responsible factor for endothelial tube formation (data not shown). Taken together, this data demonstrate that tamoxifen and bevacizumab significantly decrease levels of VEGF165 independent of the ER.

Tamoxifen and bevacizumab treatment results in decreases in OS proliferation and invasion

The proliferation and invasion of 143B OS cells in response to tamoxifen and bevacizumab treatment were examined. Using a 5 day MTT assay, we show that tamoxifen and bevacizumab decrease the proliferation of 143B cells when compared to controls. On day five of the MTT assay, the proliferation of 143B cells when compared to vehicle treated controls, was significantly less after treatment with tamoxifen (59%), bevacizumab (33%), or both (75%) (Figure 2A). Invasiveness of 143B OS cells was then evaluated using a 24 hour transwell invasion assay. Here we show that treatment with Tamoxifen or bevacizumab decreases invasion by 75% and 20%, respectively, when compared to vehicle controls, while combinatorial treatment decreases invasion by 87% (Figure 2B). These results demonstrate that the treatment with tamoxifen and/or
bevacizumab in human 143B OS cells leads to dramatic decreases in proliferation and invasion.

**Treatment with tamoxifen and bevacizumab results in decreased OS tumor growth in an animal model**

We next examined whether treatment with tamoxifen and/or bevacizumab affects the growth of OS cells in vivo. We intratibially injected 143B OS cells into 6-week old male athymic (nude) mice. These cells were previously transduced with lentivirus encoding firefly luciferase allowing for in vivo monitoring of tumor growth. Tumors were allowed to grow and establish in the bones of the animals for one week (W0). Mice were then randomly divided into four groups receiving saline as control, bevacizumab (2 mg/kg/day), tamoxifen (1 mg/kg/day), or both bevacizumab and tamoxifen for 2 weeks (W2). Here, we show by luminescence imaging that treatment with bevacizumab or tamoxifen reduces tumor burden by 83% and 86%, respectively, while the combined treatment resulted in even greater reductions in tumor size (94%) when compared to that of the saline treated control group (Figures 3A). At the end of the study (after 2 weeks of treatment), mice were euthanized and tibiae were collected. Tibiae were scanned by µ-CT and later processed for histology and IHC staining. µ-CT analyses confirmed that tamoxifen and bevacizumab treatment dramatically reduced bone destruction caused by the growth of intratibial OS tumors (Figure 3B). H&E staining shows smaller tumors with decreased bone destruction in tamoxifen and/or bevacizumab treated mice when compared to control (Figure 3C).
Treatment with tamoxifen and bevacizumab results in decreased OS metastasis in animal model

Additionally, we developed a novel preclinical xenograft mouse model to examine the recurrence and metastasis of human OS. We intratibially injected 143B OS cells into 6-week old male athymic (nude) mice. These cells were previously transduced with lentivirus encoding firefly luciferase allowing for in vivo monitoring of tumor growth. Tumors were allowed to grow and establish in the bones of the animals for 14 days. Hind limbs-containing tumors were then amputated, and mice were confirmed to be tumor free by bioluminescent imaging 7 days post-surgery (day 21). All mice subjected to amputation underwent induction, maintenance, and recovery from general anesthesia uneventfully at the time of surgery. There were no intraoperative or post-operative surgical complications, with all surgical skin incisions healing completely within 7 days. Mice were then randomized into four treatment groups: saline, tamoxifen (500μg/kg/day), and/or bevacizumab (5μg/kg twice weekly) and monitored every 28 days by bioluminescent imaging for the development of metastasis. The incidence of pulmonary metastasis in saline treated mice was 100% 63 days after amputation (day 77). However, the incidence decreased to 66% in bevacizumab-treated mice, 11% in tamoxifen-treated mice, and 0% when both drugs were used (Figure 4). At the end of the study (day 77), mice were euthanized and tibiae were collected. H&E staining shows smaller pulmonary metastatic tumors in tamoxifen and/or bevacizumab treated mice when compared to control (Figure 4, H&E panel). These data suggest that treatment with
tamoxifen and bevacizumab could be a promising therapy in further preventing often deadly pulmonary metastases in patients.
DISCUSSION

Tamoxifen was originally described as an antagonist of the estrogen receptor in breast tissue via its active metabolite, 4-hydroxytamoxifen (27). It is the most frequently prescribed endocrine (anti-estrogen) therapy for hormone receptor-positive breast cancer in pre-menopausal women, and is also a standard in post-menopausal women along with aromatase inhibitors (28). Though its antioestrogen effects have been well documented there is increasing evidence that tamoxifen may have therapeutic potential for its ER independent actions. While the mechanisms of these actions have not been previously investigated in OS, previous studies have demonstrated tamoxifen’s Calmodulin and CaMKII inhibitory affects (16, 18). These studies, coupled with our previous work demonstrating that CaMKII plays a critical role in determining the aggressive phenotype of human OS (19), led us to examine whether tamoxifen inhibited the growth and metastasis of human OS. Here we show that tamoxifen decreases CaMKII activation in the highly metastatic 143B human OS cell line. Interestingly, CaMKII activation was not decreased when OS cells were treated with the estrogen receptor inhibitor ICI182780. These data suggest that tamoxifen is inhibiting CaMKII activation independent of its anti-ER effects.

In addition to its previously described CaMKII inhibitory effects, tamoxifen has been shown to decrease secreted VEGF in breast cancer (29). Furthermore, tamoxifen has been shown to reduce proliferation of VEGF-dependent endothelial cell lines in vitro, while orally administered tamoxifen has been shown to reduce VEGF-mediated angiogenesis in
rats (30). These findings indicate that tamoxifen may directly inhibit the effects of VEGF, which supports our previous work demonstrating that the growth of human OS is regulated by a CaMKII-controlled autocrine VEGF signaling Mechanism. In this study we show that tamoxifen and bevacizumab decrease VEGF expression and secretion in 143B human OS cells. These data suggest that our previously described VEGF/CaMKII signaling mechanism is controlled by tamoxifen and this signaling pathway could be responsible for tamoxifen’s ER-independent effects.

The approval of bevacizumab for combinatorial treatment of metastatic colorectal cancers caused a significant boom in research and drug development of anti-VEGF therapies. Bevacizumab has since had promising results on a variety of cancers and compared to other treatments it is well tolerated with limited side effects (31). Interestingly, there is little known about the clinical potential of bevacizumab treatment for OS patients. It was previously demonstrated that canine OS cells xenografted into mice have significantly delayed tumor growth when treated with low doses of bevacizumab (32). Additionally, a clinical trial was carried out on 3 patients with relapsed/refractory sarcomas treated with gemcitabine, docetaxel, and bevacizumab in 3-week cycles. The combination was well tolerated with minimal toxicity. Two patients had a partial response and the third patient had stable disease for >6 months (25). Though this data is promising it is clear that more research needs to be done investigating the possible therapeutic benefit of bevacizumab on human OS. We previously showed significant decreases in OS tumor growth with the VEGFR inhibitor CBO-P11. Here we show significant decreases in tumor growth and
metastasis when mice are treated with bevacizumab and tamoxifen. Using a xenograft animal model that mimics what is seen in conventional OS treatment we amputated the tumor containing hind-limb and monitored metastasis when mice were treated with tamoxifen and bevacizumab. Treatment with this FDA approved drugs results in significant decreases in detectable metastases. Though these results are promising, it remains unclear if these drugs are decreasing establishment in the pre-metastatic niche, or inhibiting growth of already established micro-metastases. Furthermore, more work needs to be done to determine this drug combinations efficacy for treating clinically detectable metastases, and further elucidate its potential as a second line OS therapeutic.

Our present results provide compelling evidence to further explore tamoxifen and bevacizumab as treatment for human OS. These findings may guide future therapeutic efforts in finding new second line therapies that increase prolonged survival and prevent refractory disease in human OS patients.
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**Figure 1**

A. VEGF expression and secretion are decreased in human OS treated with tamoxifen and bevacizumab

B. Real-time PCR was performed using primers specific for VEGF or β-Actin. Values were obtained from three separate experiments each repeated in triplicate and represent the mean ±S.E. *p<0.01. C. 143B cells treated with 1 µM ICI182780, 1 µM tamoxifen and/or 1 µM CBO-P11 were seeded at 1 x 10⁵ cells per well of a 6-well plate. Twenty-four hours later, aliquots of supernatant were

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**Figure 1.** VEGF expression and secretion are decreased in human OS treated with tamoxifen and bevacizumab

A. 143B cells were treated with 1 µM ICI182780, 1 µM tamoxifen and/or 1 µM CBO-P11 for 24 hours. Immunoblots were developed using specific antibodies directed against p-α-CaMKII or α-CaMKII. The autoradiographs are representative of three experiments. B. Real-time PCR was performed using primers specific for VEGF or β-Actin. Values were obtained from three separate experiments each repeated in triplicate and represent the mean ±S.E. *p<0.01. C. 143B cells treated with 1 µM ICI182780, 1 µM tamoxifen and/or 1 µM CBO-P11 were seeded at 1 x 10⁵ cells per well of a 6-well plate. Twenty-four hours later, aliquots of supernatant were
examined for human VEGF165 by ELISA. Values were obtained from three separate experiments each repeated in triplicate and represent the mean ±S.E. *p<0.01. D. 143B cells treated with 1 µM ICI182780, 1 µM tamoxifen and/or 1 µM CBO-P11 were seeded at 1 x 10^5 cells per well of a 6-well plate. Twenty-four hours later, aliquots of conditioned media were removed from dishes and added to 12-well plates seeded with 1 x 10^5 HUVEC cells. Endothelial cell tube formation was measured at 12 h. Representative photomicrographs were taken at 50x magnification from 3 independent experiments, each repeated in triplicate.
Figure 2. Tamoxifen and bevacizumab treatment results in decreases in OS proliferation and invasion

MTT assay was performed at days 1, 2, 3, 4 and 5 to determine the number of viable cells. $1 \times 10^3$ 143B Cells were seeded in a 96-well plate. At 24 hours after seeding, cells were treated with $1\mu$M ICI182780, $1\mu$M tamoxifen and/or $1\mu$M bevacizumab. Values were obtained from three separate experiments, each repeated in triplicate and represent the mean ± S.E. *p<0.01. B. Transwell invasion assay allowing cells to invade for 24 hours. Representative photomicrographs were taken at 100x magnifications from 3 independent experiments, each repeated in duplicate. Values represent the mean ± S.E. *p<0.01.
**Figure 3.** Treatment with tamoxifen and bevacizumab results in decreased OS tumor growth in an animal model.

143B cells were transduced with lentiviruses expressing firefly luciferase and allowed to grow for 1 week. **A.** Luciferase imaging was performed before treatment (W0) and 2 weeks after treatment (W2) (n=5). **B.** Fluorescent intensity was measured and graphed. Values were obtained from 5 mice from each group and represent the mean ± SE. *P < 0.01. **C.** Mice were euthanized and tibiae were collected. Tibiae were scanned by µ-CT. Hematoxylin and eosin staining was performed on paraffin embedded tumors. Broken
lines indicate the boundary of the tumors and separating it from normal bone microenvironment. Images were taken at 40x magnification and are representative of 5 different mice.
**Figure 4.** Treatment with tamoxifen and bevacizumab results in decreased OS metastasis in animal model.

143B cells were transduced with lentiviruses expressing firefly luciferase and allowed to grow for 14 days. **A.** Luciferase imaging was performed before treatment (day 14), 1 week after amputation of the tumor containing hind-limb (day 21), 4 weeks after
treatment (day 49) and 8 weeks after treatment (day 77) (n=8). Mice were euthanized and tibiae were collected. Hematoxylin and eosin staining was performed on paraffin embedded tumors. Broken lines indicate the boundary of the tumors and separating it from normal bone microenvironment. Images were taken at 40x magnification and are representative of 8 different mice.
TUMOR SECRETED EXOSOMES REGULATE OSTEOSARCOMA CANCER STEM CELLS

PAUL G. DAFT, HONGJUN WEI AND MAJD ZAYZAFOON

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ABSTRACT

In this study we discovered that the pharmacologic inhibition of α-CaMKII by tamoxifen significantly decreases the number of cancer stem cells (CSCs) and results in 68% of OS stem cells being c-kit and Stro-1 negative, which we defined as Double Negative Cells (DNCs). Furthermore, we developed a novel preclinical xenograft mouse model to examine the stemness of metastatic tumors treated with tamoxifen. We show significant decreases in the stem cell transcription factors Nanog and Sox2 in human metastatic tissue after treatment with tamoxifen. Using fluorescence-activated cell sorting, we isolated CSCs (c-Kit⁺, Stro-1⁺) and DNCs (c-Kit⁻ and Stro-1⁻) from 143B OS cells. We then discovered that CSCs secrete large amounts of nanosized vesicles (exosomes). Therefore, we hypothesized that the growth and metastasis of osteosarcoma relies on α-CaMKII controlled exosome release that regulates the development of CSCs. CSC-secreted exosomes were visualized by cryo-electron microscopy and measured to be 50–130 nM and confirmed by western blotting for their expression of flotillin-1 and CD63. Adding CSC-secreted exosomes to cultured DNCs results in a significant increase in c-Kit (400%) and Stro-1 (300%) protein levels. Finally, we intratibially injected CSCs or DNCs into 6-week old mice. We show that 100% of mice injected with CSCs develop tumors and only 25% of DNCs did so, while CSC tumors secrete 800% more exosomes in the blood than DNC tumors.
INTRODUCTION

Osteosarcoma (OS), the most common primary bone malignancy, is an aggressive and highly metastatic cancer that is often refractory to chemotherapy (1). Long term survival for patients following standard therapy, including surgery and multi-component chemotherapy, has plateaued at less than 70% (2). The propensity for OS cells to survive, proliferate and metastasize even under the pressure of multimodal, aggressive chemotherapy treatment has prompted several investigators to explore the potential role of cancer stem cells (CSCs) in this disease (3). While the experimental evidence for the existence of CSCs was first proposed for hematological malignancies, more recently CSCs have been discovered in solid tumors including breast, brain, pancreatic and now bone tumors (4-6).

In the cancer stem cell model of tumors, there is a small subset of cancer cells, the CSCs, which constitute a reservoir of self-sustaining cells with the ability to self-renew and maintain the tumor. These CSCs have the capacity to both divide and expand the CSC pool and to differentiate into the heterogeneous non-tumorigenic cancer cell types that in most cases appear to constitute the bulk of the tumor (5). These CSCs are resistant to therapies that have been developed to eradicate the rapidly dividing cells within the tumors, which makes current treatments unlikely to be curative and often result in relapse (7). A large area of cancer research is now aimed at developing therapeutics that targets this minority stem cell population.

CD117(c-Kit) is the receptor for stem cell factor and a known proto-oncprotein. It is also one of the markers used to isolate ovarian CSCs (8). Stro-1 is a cell surface marker
for mesenchymal stem cells (9). It has been shown that sphere cells generated from the mouse OS cell lines K7M2, 318-1, and P932 possessed characteristics of CSCs such as having increased tumorigenicity when injected subcutaneously into mice, and an ability to differentiate into multiple lineages. These mouse sphere cells also have increased expression of double positive (DP) c-Kit and Stro-1 cells. These DP cells were shown to be more resistant to doxorubicin than both double negative (DN) and parental cells. DP human HOS, and MNNG/HOS OS cells have increased tumorigenicity when subcutaneously injected into nude mice compared to DN cells derived from the same cell lines. It was also shown that lung metastases had more cells positive for the markers c-Kit and Stro-1 when compared to the primary bone tumor (9), suggesting that the OS CSCs have a greater ability to metastasize to the lung.

We have previously shown the critical role of CaMKII in OS growth and metastasis, and while it has yet to be investigated if CaMKII may play a role in maintaining CSC populations, there is strong evidence that constitutively active Wnt/β-catenin signaling may confer a stem/progenitor cell phenotype to cancer cells (10). Inhibition of this signaling pathway in colon cancer cell lines induced the expression of the cell-cycle inhibitor p21, and also induced the cells to stop proliferating and to acquire a more differentiated phenotype. Interestingly, CaMKII has previously been shown as a downstream target of the Wnt signaling pathway, and that CaMKII inhibition results in increased p21 expression in human OS (11). These data suggest that CaMKII might play a role in CSC population in human OS.
CSC have been shown to secrete large amounts of exosomes. Exosomes are small (50-120 nm) membrane bound vesicles of endocytic origin that are released into the extracellular environment by fusion of multivesicular bodies (MVB) with the plasma membrane (12). Many cells have the capacity to release exosomes, including reticulocytes (13), dendritic cells (14), B cells, T cells (15), epithelial cells (16), and tumor cells (12), but the functions of these secreted exosomes are not completely understood. Exosomes can bind to cells through receptor-ligand interactions, similar to cell-to-cell communication. Alternatively, exosomes can attach or fuse with the target-cell membrane, delivering exosomal surface proteins and perhaps cytoplasm to the recipient cell. Finally, exosomes may also be internalized by the recipient cells via endocytosis (17).

K562 cells are a hematopoietic cell line that releases exosomes. The application of monensin (MON) has been shown to generate large MVBs. Exosome release was markedly enhanced by MON treatment, a Na\(^+\)/H\(^+\) exchanger that induces changes in intracellular calcium (Ca\(^{2+}\)). Furthermore, MON-stimulated exosome release was completely eliminated by BAPTA-AM, implying a requirement for Ca\(^{2+}\) in this process. Large MVBs generated in the presence of MON accumulated Ca\(^{2+}\) as determined by labeling with Fluo3-AM, suggesting that intraluminal Ca\(^{2+}\) might play a critical role in the secretory process as well (18).

In this study we show that the pharmacologic inhibition of α-CaMKII by tamoxifen significantly decreases the number of CSCs in vitro and in vivo. Using fluorescence-activated cell sorting, we isolated CSCs (CD117\(^+\), Stro-1\(^+\)) and DNCs (CD117\(^-\) and Stro-
1) from 143B OS cells. CSC-secreted exosomes were visualized by cryo-electron microscopy and measured to be 50–130 nM and confirmed by western blotting for their expression of flotillin-1 and CD63. We then show that by adding CSC-secreted exosomes to cultured DNCs results in significant increases in c-Kit and Stro-1 protein levels. Moreover, we show that inhibiting CaMKII can decreases CSC populations and exosome secretion. This inhibition may prevent CSCs from secreting exosomes and causing surrounding tumor cell to adopt a more aggressive phenotype.
MATERIALS AND METHODS

Cell Culture

Human OS cells (143B) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These human-derived cell lines were authenticated by DNA short tandem repeat profiling and experiments were conducted within 6 months of resuscitation. Cells were maintained in DMEM medium containing 10% FBS (Atlanta Biologicals, Lawrenceville, GA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA). All cell cultures were maintained at 37°C with 5% CO₂ (19).

Whole Cell Protein Extraction and Western Blot Analysis

Cells were lysed in 0.5% Nonidet P-40 lysis buffer supplemented with protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO, USA). Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, Immobilon-P (Millipore Co., Milford, MA, USA). Membranes were blocked with Tris-buffered saline-Blotto/Blotto B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour and subsequently incubated overnight with antibodies directed against Flotiliin-1, CD-63, c-Kit, Stro-1, Nanog, Oct4, Sox2, or β-actin (Santa Cruz Biotechnology). Signals were detected using a horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence detection kit (ECL; Amersham Biosciences, Pittsburgh, PA, USA) (19).
MTT Assay

Cell proliferation was measured by a MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay (ATCC). Cells were plated at a density of $5 \times 10^3$ cells per well in 96-well plates. After treatment, MTT solution was added to the culture medium (0.5 mmol/L) and plates were incubated for 2 hours at 37°C with 5% CO$_2$. Detergent solution was then added to solubilize formazan crystals. Finally, the optical density was determined at 570 nm using a Benchmark Plus microplate reader (Bio-Rad, Hercules, USA) (19).

Animals and Tumor Cell Inoculation

Six-week-old male Foxn1nu mice were used in these studies, with the approval of the UAB Institutional Animal Care and Use Committee. 143B cells were prepared from sub-confluent cultures. Cells ($1 \times 10^6$ cells in 20 µl PBS) were intratibially injected using insulin syringes with 28.5 gauge needles. The knee was flexed, and the needle inserted into the tibia, boring the needle through the epiphysis and epiphyseal growth plate for delivery of the cells into the metaphysis. 143B OS tumor growth was monitored by in vivo bioluminescence imaging at 14 days after cancer cell inoculation. Mice were intraperitoneally injected with D-luciferin solution (150 mg/kg) 10 minutes before bioluminescence imaging. Images were then acquired and analyzed with an IVIS 100 Imaging System (Xenogen). At the end of the study, animal were euthanized, hind limbs were excised, formalin fixed, EDTA decalcified and paraffin embedded. All tissues were
sectioned and stained with hematoxylin and eosin (H&E) for histological evaluation of the tumors. Photomicrographs were taken using a Nikon DS-Fi1 digital camera (19).

**Mouse hind-limb amputation**

After inoculation of 143B OS cells through intratibial injection, tumors were allowed to establish for 14 days. After tumor establishment, the tumor containing right hind limb was amputated by joint disarticulation at the knee. Anesthesia was induced with 5% isoflurane and 2 l/min oxygen in an induction chamber and subsequently maintained with 1.5% isoflurane and 1 l/min oxygen. The tumor-inoculated hind limbs were aseptically prepared with alternating scrubs of 100% ethanol and dilute chlorhexidine acetate. A local ring block of lidocaine and bupivicaine was administered at the knee joint and infused at the site of surgery. A circumferential skin and subcutaneous fat incision was made with a number 15 scalpel blade at the level of the distal end of the tibia. The femoral artery and vein were then concurrently ligated with ligating clips and transected with scissors. Circumferential musculature was then transected proximal to the level of vessel ligation. The tibia and its remaining muscle attachments were than transected and the tumor-bearing limb removed. Musculature was closed over the distal femur with 5-0 polyglyconate in an interrupted pattern. Mice were recovered in a clean cage on a warming pad and provided warmed SQ saline. Pre- and post-operative analgesia consisted of subcutaneous injections of 0.1 mg/kg buprenorphine (Buprenex, Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) prior to surgery and every 8–12 h for 2 days after surgery. In addition, 0.3 mg/kg/day meloxicam (Metacam, Boehringer
Ingelheim Corp., Ridgefield, CT) and 31.5 mg/kg/day enrofloxacin (Baytril, Bayer HealthCare, LLC, Shawnee Mission, KS) were subcutaneously injected. Mice were examined twice daily until the surgical sites were healed (20).

**Exosome Isolation**

Cells were washed twice with PBS and grown in serum-free medium for 24 hrs. Exosomes secreted into the medium was isolated by differential ultracentrifugation. Briefly, media were centrifuged at 300 × g for 10 min to clear cells and large debris. The supernatant was then centrifuged at 2000 × g for 20 min and then at 10,000 × g for 30 min to remove residual membranous debris. The remaining supernatant was then subjected to ultracentrifugation at 100,000 × g for 70 min to pellet the exosomes. The pellets were resuspended in PBS and repelleted at 100,000 × g for 70 min to remove contaminating proteins and resuspended in PBS for further analysis. The amount of protein present in exosome pellets was determined using a Lowry protein assay, and the number and size of particles was assessed by NanoSight particle tracking (NanoSight Ltd.). Particles of size 30–120 nm were designated as exosomes (21).

**Cryo Electron Microscopy**

For electron microscopy, 3 μl of exosomes suspended in PBS were placed on a glow-discharged Formvar carbon-coated grid and negatively stained with 2% uranyl acetate solution. For cryo-electron microscopy, 3 μl of exosomes were placed on C-flat holey film, blotted, and frozen in liquid ethane. Images were taken using FEI Tecnai F20
electron microscope operated at 200 kv, and images were captured on a 4k × 4k CCD camera.

**Flourescent Activated Cell Sorting (FACS)**

Cells treated with tamoxifen were trypsinized and washed with PBS and resuspended in FACS supplemented with FC block (BD PharMingen). Cells were then incubated with Stro-1-FITC and c-Kit-PE antibodies for 30 min at room temperature. The stained samples were measured using a FACS caliber bench-top flow cytometer (Becton Dickinson). The data were analyzed using FlowJo software (Tree Star, Inc.).

**Statistical Analysis**

Statistical analyses were performed using the Microsoft Excel data analysis program for Student’s t-test analysis. Experiments were repeated at least three times, unless otherwise stated. Values were expressed as mean ±SE with results considered significant at p<0.05.
RESULTS

Tamoxifen decreases CSCs in 143B human OS cells

We have previously shown that tamoxifen inhibits α-CaMKII and dramatically decreases 143B cell pulmonary metastasis in a hind-limb amputation nude mouse model [Chapter 3]. To further elucidate the mechanism by which tamoxifen-inhibits pulmonary metastasis by CaMKII, we examined c-Kit and Stro-1 expression in 143B cells treated with tamoxifen (1µM). Fluorescence-activated cell sorting analysis (FACS) indicated that tamoxifen treatment significantly reduced the c-Kit and Stro-1 double positive (DP) OS stem cell population. We show that when 143B cells are treated with tamoxifen for 24 hours a 68% decrease in DP OS stem cells is observed (Fig. 1A). To further examine OS stem cell levels we performed western blot analysis using antibodies directed against the stem cell transcription factors Nanog, Sox2 and Oct4. We show that the levels of these stem cell-markers are significantly decreased in 143B cells treated with tamoxifen but not ICI182780, suggesting that these observed effects are ER-independent (Fig. 1B). Furthermore, when 143B OS cells are treated with tamoxifen we show significant decreases in Nanog (60%), Sox2 (75%) and Oct4 (85%) gene expression when compared to controls (Fig. 1C). These results suggest that inhibition of CaMKII by tamoxifen greatly reduces CSC populations in the 143B OS cell line.

Tamoxifen decreases metastasis and associated stemness of 143B OS cells

To examine tamoxifen’s effects on metastatic OS stem cell populations in vivo, we used a preclinical xenograft mouse model to examine the stem cell levels in metastatic lung
143B OS cells were intratibially injected into 6-week old male athymic (nude) mice. These cells were previously transduced with lentivirus encoding firefly luciferase allowing for in vivo monitoring of tumor growth. Tumors were allowed to grow and establish for 14 days. Hind limbs-containing tumors were then amputated, and mice were confirmed to be tumor free by bioluminescent imaging 7 days post-surgery (data not shown). All mice subjected to amputation underwent induction, maintenance, and recovery from general anesthesia uneventfully at the time of surgery. There were no intraoperative or post-operative surgical complications, with all surgical skin incisions healing completely within 7 days. Mice were then randomized into two treatment groups: saline or tamoxifen (500μg/kg/day and monitored monthly by bioluminescent imaging for the development of metastasis. The incidence of pulmonary metastasis in saline treated mice was 100% two months after amputation (day 77). However, the incidence decreased to 12% in tamoxifen-treated mice (Figure 2). At the end of the study (day 77), mice were euthanized and tibiae were collected. H&E staining shows smaller pulmonary metastatic tumors in tamoxifen treated mice when compared to control (Figure 2, H&E panel). To examine CSC levels in metastatic tissue, we performed immunohistochemistry (IHC) using antibodies directed against Nanog and Sox2. Negative controls were processed alongside the examined tissue, but rabbit IgG was used instead of the primary antibody (data not shown). Immunohistochemical results demonstrate that the levels of Nanog and Sox2 decreased greatly in mice treated with tamoxifen compared to control
(Figure 2, IHC panel). These data suggest that tamoxifen may be decreasing metastasis by decreasing OS stem cell populations.

**c-Kit and Stro-1 DP CSC are more tumorigenic in vivo**

One of the defining characteristics of CSC is their ability to be highly tumorigenic in vivo compared to non-CSCs. To determine whether DP Stro-1 and c-Kit 143B cells grow more aggressively in vivo than their DN counterparts, FACS analysis was used to generate stable DP and DN cell lines (Fig. 3A). These c-Kit and Stro-1 DP or DN 143B OS cell lines were intratibially injected into 6-week old male athymic (nude) mice. These cells were previously transduced with lentivirus encoding firefly luciferase allowing for in vivo monitoring of tumor growth. Here, we show by luminescence imaging 3 weeks after tumor cell inoculation that DP cells grow tumors in more mice (100%) than DN cells (25%) (Fig. 3B). At the end of the study, mice were euthanized and tibiae were collected. H&E staining shows smaller tumors in DN 143B cells when compared to DP cells (Figure 3C). These results show that increased expression of Stro-1 and c-Kit cause the 143B OS cell line to more readily grow tumors in vivo, and suggest that these cells may indeed be OS stem cells.

**Highly aggressive human OS cell lines secrete more exosomes**

Once viewed as simply a mechanism to remove waste, tumor-derived exosomes are now being viewed as mediators of tumorigenesis. It was recently shown that exosomes from highly metastatic melanomas increase the metastatic behavior of primary tumors by permanently 'educating' bone marrow progenitors through the receptor tyrosine kinase
MET (22). This seminal paper suggests that exosomes can not only promote metastasis and package proteins from cell to cell, but may be able to completely change a recipient cell’s phenotype. To begin exploring the relationship between CSCs and exosomes, we isolated exosomes from medium conditioned by stro-1°C-Kit⁺, stro-1°C-Kit⁻, tamoxifen treated, KN-93 treated and untreated 143B OS cells. We discovered that stro-1°C-Kit⁺ and untreated 143B cells secreted ~3-fold and ~2-fold higher levels of total protein in exosomes compared to controls (Fig. 4A). Furthermore, circulating exosome levels were shown to be increased in mice that developed tumors after injection with stro-1°C-Kit⁺ (Fig. 4B). The purity of exosome preparations was confirmed by electron microscopy and western blot analysis. Cryo-electron microscopy demonstrated that the vesicles isolated from OS cells were double membrane bound and within the defined size range (30–120 nm) (Fig. 4C). Additionally, western blots show high levels of the exosome markers flotillin-1 and CD63 (Fig. 4D), which are shown to be highly expressed in exosomes (23). These results show that highly aggressive OS cell lines secrete greater amounts of exosomes compared to non-aggressive cell lines. Inhibiting CaMKII results in decreased exosome secretion, which may in part be a result of decreased CSC populations.

**CSCs educate DNCs to a more aggressive phenotype**

In order to examine if CSC secreted exosomes can alter the phenotype of surrounding cell populations, we examined the changes in cultured DNC cells treated with 100µg of purified CSC-secreted exosomes. First, by PCR, we show DNCs have increased c-Kit (200%) and Stro-1 (700%) gene expression when treated with CSC-secreted exosomes
(Fig. 5A). Consistent with these results, western blot analysis shows significant increases in c-Kit and Stro-1 proteins levels in exosome treated DNCs when compared to controls (Fig. 5B). These results suggest that aggressive OS cells may have the capacity to change surrounding cell phenotype.

To determine the specific effect of Stro-1 and c-Kit increases on the tumorigenic properties of DNCs *in vitro*, we examined the proliferation, and invasion of CSCs, DNCs and DNCs treated with CSC-secreted exosomes. Here we show that exosome treatment increases DNC cell proliferation when compared to control. By day 4, exosome treated DNCs proliferated 400% more, when compared to untreated DNCs (Fig. 5C). Furthermore, exosome treatment in DNCs increased invasion by 800% when compared to control (Fig. 5D). These results demonstrate that treating cultured DNCs with exosomes secreted by a more aggressive cell line (CSCs) leads to dramatic changes in proliferation and invasion.
DISCUSSION

CSCs refer to a subset of tumor cells that has the ability to self-renew and generate the diverse group of cells that comprise the tumor (24, 25). These cells have been termed cancer stem cells to reflect their 'stem-like' properties and ability to continually sustain tumorigenesis. While the CSC hypothesis has been around for quite some time, the specific role of these CSCs in tumor biology, including metastasis, is still uncertain. While the underlying mechanism remains to be elucidated several groups have demonstrated that CSC subpopulations are essential for tumor metastasis (26). Our current work demonstrates that CaMKII inhibition by tamoxifen decreases a population of cells that may constitute the OS stem cell population. We then show decreases in metastasis and the stem-like qualities of metastatic OS tissue when CaMKII is inhibited. These results are consistent with our previous work showing decreases in metastasis of 143B cells when CaMKII is inhibited [third manuscript], and may provide an explanation for why this phenomena is observed.

Exosomes are vesicles of endocytic origin released by many cells. These vesicles can mediate communication between cells, facilitating a variety of cellular processes (27). Within the past couple years it has become increasingly evident that tumor-secreted exosomes educate selected host tissues and cells towards a pro-metastatic phenotype (28). While exciting, the majority of research has focused on development of the pre-metastatic niche, while neglecting to examine alterations in local cell populations. Last
year it was shown that triple negative breast cancer exosomes may be involved in cancer cell-to-cell communication, conferring phenotypic traits to secondary cells that reflect those of their cells of origin (29). Interestingly, it is well documented that CSC secrete high amounts of microvesicles (30). We were the first to examine if CSC-secreted exosomes are able to confer a more aggressive phenotype to surrounding cancer cells. We show that OS CSC secreted increased levels of exosomes, and by inhibiting CaMKII with tamoxifen this response can be ameliorated. We go on to show that treatment of non-aggressive tumor cells with CSC-derived exosomes results in a more metastatic phenotype by increasing invasion and proliferation. These results suggest that CSC-secreted exosomes could aid in steps of metastasis and play additional roles beyond establishing the pre-metastatic niche.

Taken together our results show that OS CSC secrete increased levels of exosomes which are able to confer an aggressive phenotype to non-aggressive human OS cells. The inhibition of CaMKII by tamoxifen not only decreases CSC populations, but also decreases exosome secretion. These data suggest that tamoxifen could be a useful tool in combating refractory disease, while also inhibiting metastasis as a frontline chemotherapeutic against this devastating childhood disease.
REFERENCES


2. Lee AF, Pawel B, Sullivan LM. Significant immunohistochemical expression of human chorionic gonadotropin in high-grade osteosarcoma is rare, but may be associated with clinically elevated serum levels. Pediatric and developmental pathology : the official journal of the Society for Pediatric Pathology and the Paediatric Pathology Society. 2014.


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Figure 1. Tamoxifen decreases cancer stem cell populations

A. 143B cells were treated with Tamoxifen (1μM) for 24 h, and c-Kit and Stro-1 fluorescence-activated cell sorting (FACS) analysis was performed. B. Immunoblots were developed using antibodies directed against Nanog, Oct4, Sox2 or β-actin. The autoradiographs are representative of three experiments. C. Real-time PCR was performed using primers specific for α-CaMKII or 18S rRNA.
143B cells were transduced with lentiviruses expressing firefly luciferase and allowed to grow for 14 days. A. Luciferase imaging was performed before treatment (day 14) and 8 weeks after treatment (day 77). Mice were euthanized and tibiae were collected. Hematoxylin and eosin staining was performed on paraffin embedded tumors. Broken lines indicate the boundary of the tumors and separating it from normal bone microenvironment. Images were taken at 40x magnification. Immunohistochemical staining (right panel) using specific antibodies directed against Sox2 or Nanog (brown) in human osteosarcoma tissue and counterstained with hematoxylin (blue).
Figure 3. c-Kit and Stro-1 DP CSC are more tumorigenic in vivo

143B cells were transduced with lentiviruses expressing firefly luciferase and allowed to grow for 14 days. A. 143B cells underwent fluorescent activated cell sorting with c-Kit and Stro-1 double positive (CSC) and double negative cells (DNC) being separated. B. Luciferase imaging was performed 3 weeks after tumor cell inoculation. Fluorescent intensity was measured and graphed. Values were obtained from 4 mice from each group and represent the mean ± SE. *P < 0.01. C. Mice were euthanized and tibiae were collected. Hematoxylin and eosin staining was performed on paraffin embedded tumors. Broken lines indicate the boundary of the tumors and separating it from normal bone microenvironment. Images were taken at 40x magnification and are representative of 4 different mice.
**Figure 4.** CaMKII inhibition decreases exosome secretion from highly aggressive OS cell lines

**A.** The amount of exosome protein accumulated over 48 h in the cell medium was quantified by lowry protein assay. **B.** Exosome protein was precipitated from serum of mice with OS by ExoQuick exosome precipitation. Protein was quantified by lowry protein assay. **C.** Micrographs from cryo-electron microscopy were of the size (30–120 nm) and shape consistent with their identity as exosomes (bar = 100 nm). **D.** Immunoblots were developed using antibodies directed against Flotilin-1 or CD-63.
Figure 5. CSCs educate DNCs to a more aggressive phenotype

100 μg of exosomes isolated from CSC cell conditioned medium were added to DNC cells A. Real-time PCR was performed using primers specific for α-CaMKII or Actin. Immunoblots were developed using specific antibodies directed against c-Kit or Stro-1. Values were obtained from three separate experiments, each repeated in triplicate and represent the mean ±S.E. *p<0.01. B. Immunoblots were developed using antibodies directed against c-Kit or Stro-1. The autoradiographs are representative of three experiments. C. MTT assay was performed to determine the number of viable cells. D. Transwell invasion assay allowing cells to invade for 24 hours. Representative photomicrographs were taken at 100X magnification
SUMMARY

Ca\(^{2+}\) is a ubiquitous cellular signal. Altered expression of specific Ca\(^{2+}\) channels and pumps are common in a variety of cancers. The ability of Ca\(^{2+}\) to regulate both cell death and proliferation, combined with the potential for pharmacological modulation, offers the opportunity for a set of new drug targets in cancer.

Our lab’s previous work shows that α-CaMKII activation plays a critical role in determining the aggressive behavior of human OS. By examining the levels of p-α-CaMKII in 114 human OS tissues we show that the levels of p-α-CaMKII are significantly higher in OS tissues when compared to osteoblasts in normal bone. Although our lab was the first to report that α-CaMKII is expressed in OS cell lines and describe the role it plays in controlling cell cycle progression (49), others have also reported that the pharmacologic inhibition of CaMKII attenuates the growth and tumorigenicity of many cancer cell lines, including LN-215, LNCaP, C4-2B, CWR22Rv1 and Hep3B (130).

To more intimately examine the relationship between CaMKII and OS, we generated OS cell lines where α-CaMKII is either deleted (MG-63 and 143B) or overexpressed (HOS). The deletion of α-CaMKII significantly decreased the proliferation, motility and invasion of OS cells, while the overexpression of α-CaMKII...
caused a significant increase in these tumorigenic properties. Similarly, we show that the deletion $\alpha$-CaMKII decreases the ability of OS cells to form tumors in vivo.

Interestingly, the overexpression of $\alpha$-CaMKII in HOS cells, which are normally non-tumorigenic, formed relatively large tumors in the tibia of a xenograft nude mouse model. However, the ability of $\alpha$-CaMKII to only regulate growth, invasion and motility cannot be sufficient to explain the large size of the OS tumors. Interestingly, increases in angiogenesis have previously been attributed to the ability of OS to grow into very large tumors. This was previously supported by identifying increases in several angiogenic factors such as hypoxia inducible factor, vascular endothelial growth factor, basic fibroblast growth factor, Neuropilin-2 and placental growth factor in human OS clinical tissue samples (131, 132). Similarly, our data shows that the deletion of $\alpha$-CaMKII in OS cells produces not only smaller tumors but also resulted in less tumor vasculature as demonstrated by a decrease in the number of CD31 stained blood vessels. These initial findings suggested that the ability of $\alpha$-CaMKII to regulate angiogenesis in vivo could be a contributing factor for the significant decrease in tumor growth.

These preliminary results led us to examine CaMKII-regulated angiogenesis. Tumor growth, progression and metastasis are dependent on new blood vessel formation (neo-vascularization). VEGF is a potent angiogenic factor secreted by a variety of tumor cells, and is ubiquitously expressed at sites of angiogenesis (133). Although several studies have demonstrated poor prognosis in patients with high VEGF levels there have been few studies trying to understand the molecular mechanisms responsible for
clinically aggressive behavior in OS. We were the first to show that the highly metastatic 143B OS cell line has increased VEGF gene expression and protein secretion when compared to parental HOS cells. Interestingly, it has recently been shown that decreases in HIF-1 by CaMKII inhibition significantly decreases VEGF expression in human macrophages (84). We show a similar effect in 143B cells, where by inhibiting CaMKII genetically by shRNA or pharmacologically by KN-93 we see decreases in VEGF protein secretion. Moreover, when CaMKII is overexpressed in HOS cells we see increases in VEGF protein secretion.

Though VEGFR signaling has mainly been described in endothelial cells, there is increasing evidence that VEGFR autocrine signaling may play a prominent role in highly metastatic cancer cell lines (134). Here we show that highly metastatic 143B OS cells express VEGFR-2, thereby establishing an autocrine signaling mechanism associated with aggressive phenotypes. 143B cells express higher levels of VEGFR-2 than parental HOS cells and are sensitive to exogenous VEGF stimulation. This autocrine signaling mechanism results in increases in [Ca2+]i and leads to increased proliferation, invasion and migration. These results are consistent with previous studies that have shown increases in [Ca2+]i and CaMKII subsequent activation in vascular smooth muscle cells and hippocampal neurons upon treatment with exogenous VEGF (135). These results show a novel positive feedback loop, where CaMKII-induced VEGF results in increases in [Ca2+]i and CaMKII activation.
Similarly, we show that the inhibition of CaMKII and VEGF decreases OS tumor growth in vivo. The therapeutic benefit of anti-VEGF treatment on solid tumors is well documented. Initially, the addition of Bevacizumab, a monoclonal antibody for VEGF, to standard chemotherapy produced significant clinical benefit in patients with previously untreated and pretreated metastatic colorectal cancer, advanced non-small cell lung cancer, and metastatic breast cancer (136). Our data show that the inhibition of CaMKII and VEGF produces not only smaller tumors but also resulted in less tumor vasculature as demonstrated by a decrease in the number of positively stained CD31 blood vessels. Furthermore, decreases in the osteolytic properties of OS are observed by micro-CT. These findings suggest that CaMKII and VEGF are both responsible for OS tumor angiogenesis and when used in combination therapy drastic decreases in tumor size and vasculature can be achieved.

In an attempt to make our work as clinically relevant as possible, we expounded on these studies and showed significant decreases in tumor growth and metastasis when mice are treated with bevacizumab (VEGF inhibitor) and tamoxifen (CaMKII inhibitor). Using a xenograft animal model that mimics what is seen in conventional OS treatment we amputated the tumor containing hind-limb and monitored metastasis when mice were treated with tamoxifen and/or bevacizumab. When mice are treated with either drug, we show significant decreases in detectable metastases. Though these results were promising, it remains unclear how tamoxifen and bevacizumab are decreasing detectable metastases. They could be decreasing establishment of the pre-metastatic niche,
inhibiting growth of already established micro-metastases, or inhibiting metastasis in some other way.

These questions led us to examine CaMKII inhibition is decreasing metastasis by decreasing cancer stem cell populations. Cancer stem cells refer to a subset of tumor cells that has the ability to self-renew and generate the diverse cells that comprise the tumor (137). These cells have been termed cancer stem cells to reflect their 'stem-like' properties and ability to continually sustain tumorigenesis. While the underlying mechanism remains to be elucidated several groups have demonstrated that CSC subpopulations are essential for tumor metastasis (138). Our current work demonstrates that CaMKII inhibition by tamoxifen decreases a population of cells that may constitute the OS stem cell population. We then observe decreases in metastasis and the stem-like qualities of metastatic OS tissue when CaMKII is inhibited. These results are consistent with our previous work showing decreases in metastasis of 143B cells when CaMKII is inhibited [chapter 3], and may provide an explanation for why this phenomena occurs.

Furthermore, it is well documented that CSC secrete high amounts of microvesicles. Exosomes are vesicles of endocytic origin released by many cells. These vesicles can mediate communication between cells, facilitating a variety of cellular processes (139). Within the past couple years it has become increasingly evident that tumor-secreted exosomes educate selected host tissues and cells towards a pro-metastatic phenotype. While exciting, the majority of research has focused on development of the pre-metastatic niche, while neglecting to examine alterations in local cell populations.
Last year it was shown that triple negative breast cancer exosomes may be involved in cancer cell-to-cell communication, conferring phenotypic traits to secondary cells that reflect those of their cells of origin (140). We were the first to examine if CSC-secreted exosomes are able to confer a more aggressive phenotype to surrounding cancer cells. We show that OS CSCs secrete increased levels of exosomes and by inhibiting CaMKII with tamoxifen this response can be ameliorated. We go on to show that treatment of non-tumorigenic tumor cells with CSC-derived exosomes results in a more metastatic phenotype by increasing invasion and proliferation. These results suggest that CSC-secreted exosomes could aid in steps of metastasis and play a more crucial role than establishing the pre-metastatic niche.

Taken together our results show that OS CSC secreted increased levels of exosomes which are able to confer an aggressive phenotype to non-aggressive human OS cells. The inhibition of CaMKII by tamoxifen not only decreases CSC populations, but also decreases exosome secretion. These data suggest that tamoxifen could be a useful tool in combating refractory disease, while also inhibiting metastasis as a frontline chemotherapeutic against this devastating childhood disease.

**Future Directions**

Collectively these studies demonstrate that CaMKII inhibition results in decreases in OS growth and metastasis. Part of this inhibition can be attributed to decreases in
VEGF signaling, which contributes to a previously undescribed positive feedback loop. Additionally CaMKII inhibition results in decreased exosome secretion and chemoresistant CSC populations. These results support previous studies describing the inhibition of cancer growth by CaMKII inhibition, which had not been previously described in OS.

We have promising preliminary data that CaMKII-regulated exosome secretion may regulate CSC populations in human OS. Future studies involving intratibial injection of a variety of OS cell lines in mice, followed by injecting 5–10 μg of total exosome protein via tail vein in a total volume of 100–200 μl PBS. These exosomes will be fluorescently labeled using PKH67 membrane dye (Sigma) for exosome-tracking. These experiments will provide novel insight into the role these tumor-secreted exosomes play in primary tumor growth, as well as, examining their ability promote pulmonary metastasis and establish a pre-metastatic niche. Treating mice with and without tamoxifen will establish if the observed effects of tamoxifen on OS metastasis are due to the inhibition of exosomes.

Furthermore, dogs provide a relevant OS model that is 10 times more prevalent than the corresponding human condition and offers a unique opportunity to answer questions related to local tumor control and metastasis. Advantages of the dog model include: spontaneous development of the disease, the animal's large size, intact immune system, response to traditional chemotherapies, and owners' willingness to participate and cooperate with clinical trials for their pets. With the help of labs from the Auburn
Veterinary School we plan on examining the efficacy of tamoxifen treatment on dogs with OS. Additionally, serum will be examined for potential changes in the levels of circulating exosomes. These isolated exosomes from dogs treated with tamoxifen will undergo RNA-Seq and be compared to untreated exosomes. This will allow us to examine if tamoxifen is changing the contents of exosomes in addition to the quantity. This collaboration will bring these drugs one step closer to the clinic.
REFERENCES


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54. Hidaka H, Yokokura H. Molecular and cellular pharmacology of a calcium/calmodulin-dependent protein kinase II (CaM kinase II) inhibitor, KN-62, and


APPENDIX A

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL FORM
NOTICE OF APPROVAL

DATE: March 13, 2014

TO: MAJD ZAYZAFOON, M.D., Ph.D.
SHEL-813
(205) 934-5574

FROM: 
Robert A. Kesterson, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: The Role of CaMKII-Induced VEGF Expression in Osteosarcoma Growth & Metastasis
Sponsor: NIH
Animal Project Number: 140409650

As of April 26, 2014 the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and number of animals:

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Animal use must be renewed by April 25, 2015. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 140409650 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.
APPENDIX B

INSTITUTIONAL REVIEW BOARD PROJECT REVISION/AMMENDMENT FORM
**Project Revision/Amendment Form**

**Form version:** June 28, 2012

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1. **Today's Date**
   - 8-21-13

2. **Principal Investigator (PI)**
   - **Name (with degree):** Majd Zayzafoon, MD, PhD
   - **Department:** Pathology
   - **Office Address:** SHEL 813
   - **E-mail:** mzayzafoon@uab.edu
   - **Blazer ID:** mzayza
   - **Division (if applicable):** Molecular and Cellular
   - **Office Phone:** 4-5574
   - **Fax Number:** 5-4919

   **Contact person who should receive copies of IRB correspondence (Optional):**
   - **Name:** Traci Oden
   - **Phone:** 5-6275
   - **Office Address (if different from PI):** VH G038
   - **E-Mail:** toden@uab.edu
   - **Fax Number:** 5-7410

3. **UAB IRB Protocol Identification**
   - **Protocol Number:** X050107002
   - **Protocol Title:** Alpha-CalMKII: a New Marker and Novel Target for Treating Osteosarcoma

   **3.c. Current Status of Protocol - Check ONE box at left; provide numbers and dates where applicable**
   - [ ] Study has not yet begun
   - [x] In progress, open to accrual
   - [ ] Enrollment temporarily suspended by sponsor
   - [x] Closed to accrual, but procedures continue as defined in the protocol (therapy, intervention, follow-up visits, etc.)
   - **Date closed:**
     - Number of participants receiving interventions:
     - Number of participants in long-term follow-up only:
   - [ ] Closed to accrual, and only data analysis continues
     - **Date closed:**
     - Total number of participants entered:

4. **Types of Change**
   - Check all types of change that apply, and describe the changes in Item 5.c. or 5.d. as applicable. To help avoid delay in IRB review, please ensure that you provide the required materials and/or information for each type of change checked.

   - [ ] Protocol revision (change in the IRB-approved protocol)
   - In Item 5.c., if applicable, provide sponsor's protocol version number, amendment number, update number, etc.
   - [ ] Protocol amendment (addition to the IRB-approved protocol)
   - In Item 5.c., if applicable, provide funding application document from sponsor, as well as sponsor's protocol version number, amendment number, update number, etc.
   - [x] Add or remove personnel
   - In Item 5.c., include name, title/degree, department/division, institutional affiliation, and role(s) in research, and address whether new personnel have any conflict of interest. See "Change in Principal Investigator" in the IRB Guidebook if the principal investigator is being changed.
   - Add graduate student(s) or postdoctoral fellow(s) working toward thesis, dissertation, or publication
   - In Item 5.c., (a) identify these individuals by name; (b) provide the working title of the thesis, dissertation, or publication; and (c) indicate whether or not the student's analysis differs in any way from the purpose of the research described in the IRB-approved HSP (e.g., a secondary analysis of data obtained under this HSP)
   - [ ] Change in source of funding; change or add funding
   - In Item 5.c., describe the change or addition in detail. Include the applicable OSP proposal number(s), and provide a copy of the application as funded (or as submitted to the sponsor if pending). Note that some changes in funding may require a new IRB application.
   - [ ] Add or remove performance sites
   - In Item 5.c., identify the site and location, and describe the research-related procedures performed there. If adding site(s), attach notification of permission or IRB approval to perform research there. Also include copy of subcontract, if applicable. If this protocol includes acting as the Coordinating Center for a study, attach IRB approval from any non-UAB site added.
Add or change a genetic component or storage of samples and/or data component—this could include data submissions for Genome-Wide Association Studies (GWAS)
To assist you in revising or preparing your submission, please see the IRB Guidebook for Investigators or call the IRB office at 934-3789.

Suspend, re-open, or permanently close protocol to accrual of individuals, data, or samples (IRB approval to remain active)
In Item 5.c., indicate the action, provide applicable dates and reasons for action; attach supporting documentation.

Report being forwarded to IRB (e.g., DSMB, sponsor or other monitor)
In Item 6.c. include date and source of report, summarize findings, and indicate any recommendations.

Revise or amend consent, assent form(s)
Complete Item 5.d.

Addendum (new) consent form
Complete Item 5.d.

Add or revise recruitment materials
Complete Item 5.d.

Other (e.g., Investigator brochure)
Indicate the type of change in the space below, and provide details in Item 5.c. or 5.d. as applicable.
Include a copy of all affected documents, with revisions highlighted as applicable.

5. Description and Rationale
In Item 5.a. and 5.b., check Yes or No and see instructions for Yes responses.
In Item 5.c. and 5.d., describe—and explain the reason for—the change(s) noted in Item 4.

5.a. Are any of the participants enrolled as normal, healthy controls?
Yes [ ] No [ ]
If Yes, describe in detail how this change will affect those participants.

5.b. Does the change affect subject participation, such as procedures, risks, costs, location of services, etc.?
Yes [ ] No [ ]
If Yes, FAP-designated units complete a FAP submission and send to fap@uab.edu. Identify the FAP-designated unit in Item 5.c.
For more details on the UAB FAP, see www.uab.edu/doi.

5.c. Protocol Changes: In the space below, briefly describe—and explain the reason for—all change(s) to the protocol.

A. Paul Daft
B. Alpha-CaMKII-induced VEGF expression is critical for the growth of human osteosarcoma
C. Paul’s analysis does not differ in any way from the purpose of the research described in the IRB-approved HSP

5.d. Consent and Recruitment Changes: In the space below,
(a) describe all changes to IRB-approved forms or recruitment materials and the reasons for them;
(b) describe the reasons for the addition of any materials (e.g., addendum consent, recruitment); and
(c) indicate either how and when you will reconsent enrolled participants or why reconsenting is not necessary (not applicable for recruitment materials).

Also, indicate the number of forms changed or added. For new forms, provide 1 copy. For revised documents, provide 3 copies:
• a copy of the currently approved document (showing the IRB approval stamp, if applicable)
• a revised copy highlighting all proposed changes with “tracked” changes
• a revised copy for the IRB approval stamp.

Signature of Principal Investigator: ___________________________ Date: Aug-12-13
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*No change to IRB’s previous determination of approval criteria at 45 CFR 46.111 or 21 CFR 56.111*