Cell Death and Survival

Alpha-CaMKII Plays a Critical Role in Determining the Aggressive Behavior of Human Osteosarcoma

Paul G. Daft1, Kaiyu Yuan1, Jason M. Warram2, Michael J. Klein3, Gene P. Siegal1, and Majd Zayzafoon1

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 α-CaMKII (α-CaM-KII), a ubiquitous mediator of Ca2+/calmodulin-dependent 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 (OS) cell lines [MG-63, N-methyl-N'-nitro-N-nitrosoguanidine (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 short hairpin RNA (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%), whereas 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. Mol Cancer Res; 11(4); 349–59. ©2013 AACR.

Introduction

Osteosarcomas are among the most frequently diagnosed primary bone cancers 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 to 19 years (2). Osteosarcomas account for 5% of all pediatric tumors and roughly 20% of all primary bone tumors (3, 4). Osteosarcomas most frequently develop in the highly proliferative metaphysical region of long bones and are thought to coincide with the adolescent growth spurt (5). They are highly aggressive and frequently metastatic, with the lung being the most common site for metastasis (6). Most patients are treated with neoadjuvant multiagent chemotherapy followed by highly invasive limb-sparing surgery or amputation. With both chemotherapy and surgery, cure rates for nonmetastatic osteosarcoma now approach 65% (7). 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 (8).

Ca2+/calmodulin-dependent protein kinase II (CaMK-II) is a ubiquitously expressed protein kinase (9). 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 shows the positive relationship between p-α-CaMKII and total α-CaMKII (10). Biologically active CaMKII phosphorylates a variety of substrates, regulating many aspects of cellular function in response to Ca2+.

Authors’ Affiliations: Departments of 1Pathology and 2Radiology, University of Alabama at Birmingham, Birmingham, Alabama; and 3Department of Pathology and Laboratory Medicine, Hospital for Special Surgery, New York, New York

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P.G. Daft and K. Yuan contributed equally to this work.

Corresponding Author: Majd Zayzafoon, Department of Pathology, University of Alabama at Birmingham, 813 Shelby Building, 1825 University Boulevard, Birmingham, AL 35294. Phone: 205-934-5574; Fax: 205-996-6119; E-mail: majd@uab.edu
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signaling, influencing neurotransmitter synthesis and release, transcription and translation, cytoskeletal organization, and Ca^{2+} homeostasis. The CaMKII family is encoded by 4 genes (α, β, γ, and δ), each of which undergoes its own alternative splicing. The basic biologic function of the active α-isof orm (p-α-CaMKII) is implicated in a growing number of cancers, arising in the colon, prostate, brain, and breast (11–13). 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 (14). 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 (15).

In this study, we investigated the role of p-α-CaMKII in the growth and tumorigenesis of human osteosarcoma (OS) in vitro and in vivo. We show that p-α-CaMKII levels are increased in primary OS tissues from patients and in aggressive OS cell lines. Furthermore, the knockdown of α-CaMKII decreases proliferation, motility and invasion, whereas α-CaMKII overexpression increases the tumorigenic properties of OS 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 OS, and compounds that inhibit α-CaMKII activation might provide novel therapies for the treatment of this malignant tumor.

Materials and Methods

Cell culture
OS cells [MG-63, N-methyl-N\textsuperscript{-}nitro-N-nitrosoguanidine (MNNG)/HOS, and 143B] and human mesenchymal stem cells (hMSC) were purchased from the American Type Culture Collection (ATCC). These human-derived cell lines were authenticated by DNA short tandem repeat profiling, and experiments were carried out within 6 months of resuscitation. Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS (Atlanta Biologicals), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). All cell cultures were maintained at 37°C with 5% CO\textsubscript{2} (15).

RNA extraction real-time PCR
Total RNA was extracted using the TRIzol method as recommended by the manufacturer (Invitrogen). One microgram of RNA was reverse transcribed using M-MLV reverse transcriptase, and the equivalent of 10 ng was used for SYBR Green quantitative real-time PCR (RT-PCR). The expression of 18S RNA was used for normalization of gene expression values. Primer sequences were used previously described (15).

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). Following electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, Immobilon-P (Millipore Co.). Membranes were blocked with Tris-buffered saline-Blotto/Blotto B (Santa Cruz Biotechnology) for 1 hour and subsequently incubated overnight with antibodies directed against α-CaMKII, p-α-CaMKII, p-cAMP–responsive element binding protein (p-CREB), p–extracellular signal-regulated kinase (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 (Amersham Biosciences; ref. 15).

Immunohistochemistry
Primary OS tissues were obtained from IMGENEX (IMH-370; Sorrento Valley). Tissues were deparaffinized and rehydrated followed by antigen retrieval using 10 mmol/L sodium citrate buffer, pH 6. Samples were blocked for 1 hour in 5% goat serum (Vector Laboratories). Anti-p-α-CaMKII (Santa Cruz Biotechnology) was applied to sections and incubated overnight at 4°C. Biotin-conjugated secondary antibodies (2 μg/mL) were added, followed by avidin–biotin enzyme reagents. Specimens were incubated in 3,3′-diaminobenzidine peroxidase substrate for 30 seconds. Tissues were counterstained with Gill’s hematoxylin for 10 seconds, dehydrated, cleared, and mounted. Rabbit immunoglobulin G (IgG) negative controls were processed alongside the examined tissue. Photomicrographs were taken using a Nikon DS-Fi1 digital camera (15, 16). The detected levels of phosphorylated α-CaMKII by immunohistochemistry were scored using a semiquantitative system as previously described (17). Two experienced pathologists independently scored the 114 tissue samples. The complete score agreement of the 2 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; ref. 17).

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 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% CO\textsubscript{2}. After 8 hours, photomicrographs were taken at × 50 magnification and the scratch width was measured. Data were expressed as percentage of the remaining width of the scratch (after 8 hours) when compared with the original width (at 0 hour; ref. 18).

Invasion assay
Cells (2.5 × 10\textsuperscript{4}) were plated in media containing 0.1% FBS onto the Matrigel-coated upper chambers of Transwell invasion assay filter inserts (BD Bioscience). 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). Representative photomicrographs were taken at × 100 magnification (18).

**MTT Assay**

Cell proliferation was measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (ATCC). Cells were plated at a density of 5 × 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% 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).

**Gene silencing by shRNA**

MG-63 and 143B cells were plated at a density of 2 × 10^4 cells/cm^2 in 6-well plates. Cells were incubated in Polybrene (8 μg/mL; Sigma-Aldrich) overnight at 37°C with 5% CO2. Two specific α-CaMKII shRNAs (shCaMKII-t1 and t2) 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 after transfection, and the transfected cells were cultured with fresh media containing puromycin (5 μg/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 (19).

**Retrovirus production and infection**

We used retroviral expression vectors expressing either GFP (pMSCV-GFP) or α-CaMKII-GFP (pMSCV-α–CaMKII; ref. 20). The CMV-CaMKII-GFP was provided by Dr. Tobias Meyer, Stanford University (21). Retroviruses were produced by cotransfecting pMSCV vectors with pVSV-G into BOSC23 cells using Lipofectamine (Invitrogen). Twenty-four hours after 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 (20).

**Animals and tumor cell inoculation**

Six-week-old male Foxn1nu mice were used in these studies, with the approval of the University of Alabama at Birmingham (UAB; Birmingham, AL) Institutional Animal Care and Use Committee. MG-63 and HOS cells were prepared from subconfluent cultures. Cells (1 × 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. 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 200 Imaging System (Xenogen). Regions of interest were identified and plotted as fold difference in tumor size at day 49 when compared with day 7. At the end of the study, animals 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 histologic evaluation of the tumors. Photomicrographs were taken using a Nikon DS-Fi1 digital camera (15, 16).

**18F-FDG Positron emission tomography imaging**

HOS tumor growth was monitored by in vivo positron emission tomography (PET) imaging at 49 days after cancer cell inoculation. Mice carrying HOS tumors were anesthetized with 2.5% Isoflurane. Before 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). 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). Image and Region of Interest analysis of reconstructed images were conducted by a blinded reviewer using 64 bit OsiriX imaging software (version 4.0).

**Statistical analysis**

Statistical analyses were conducted using the Microsoft Excel data analysis program for Student t test analysis. Experiments were repeated at least 3 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 OS tissues, immunohistochemical (IHC) staining using an antibody targeting p-α-CaMKII was conducted on clinical samples obtained from 114 primary patients with OS, consisting of chondroblastic, osteoblastic and fibroblastic osteosarcomas, and 12 normal bone samples (Fig. 1). Phosphorylated α-CaMKII IHC staining was scored using a semiquantitative system as previously described (17). A 2-sided Fisher exact test was conducted 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 with osteoblasts and mesenchymal stromal cells in normal bones (P < 0.0001). The indicated P value is based on comparison with normal bone using exact binomial distribution (Supplementary Tables S1 and S2). These results show a significant increase in α-CaMKII activation in OS tissues when compared with normal bone.
To determine whether the increases observed for p-α-CaMKII in primary osteosarcoma tissues were also seen in OS cell lines, we conducted 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 nontransformed preosteoblastic hMSCs. Negative controls were processed alongside the examined tissue, but rabbit IgG was used instead of the primary antibody (data not shown). IHC results show that the levels of p-α-CaMKII varied between osteosarcoma cell lines, with 143B cells having the most and HOS cells having the least (Fig. 2A). In addition, Western blotting analyses were conducted 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, whereas levels in HOS cells were only 2 times higher (Fig. 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 (Fig. 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 OS 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 conducted 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 (Fig. 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 conducted in serum-free or 1% FBS-supplemented medium 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 (Fig. 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 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 2 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 nonaggressive osteosarcoma cell line (HOS). 143B cells were transduced with lentiviral vectors delivering a nonspecific control shRNA (shCtrl) or 2 different specific α-CaMKII-targeting short hairpin RNAs [shRNA; 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 cells transduced with a nonspecific control.

The decreases in mRNA expression were also confirmed by showing a comparable decrease in p-α-CaMKII protein levels (Fig. 4A). HOS cells were transduced with a retroviral vector encoding either a nonspecific control shRNA (shCtrl) or a specific α-CaMKII-targeting short hairpin RNA [shRNA; shCaMKIIα(1)] to ensure that the differences observed were not due to the retroviral vector alone.
Figure 4. Deletion or overexpression of α-CaMKII in OS cell lines. MG-63 and 143B cells were transduced with lentiviruses expressing either a nonspecific control (shCtrl) or 2 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, RT-PCR was conducted 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 3 separate experiments, each repeated in triplicate and represent the mean ± SE. *P < 0.01. B, RT-PCR was conducted using primers specific for α-CaMKII or 18S rRNA. Values were obtained from 3 separate experiments, each repeated in triplicate and represent the mean ± SE. *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 3 experiments. D, IHC staining using a specific antibody directed against p-α-CaMKII (brown). Images were taken at ×200 magnification and are representative of 3 independent experiments.

construct overexpressing GFP-α-CaMKII (GFP-CaMKII(α)) 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 with their controls, whereas a 400% increase in α-CaMKII gene expression was observed in HOS GFP-CaMKIIα cells when compared with GFP-Ctrl cells (Fig. 4B). These data were confirmed by showing that the levels of p-α-CaMKII protein were dramatically decreased in shCaMKIIα(2) cells and increased in GFP-CaMKIIα cells when compared with their respective controls (Fig. 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 (Fig. 4C). To confirm that the knockdown and overexpression of α-CaMKII resulted in changes in α-CaMKII intracellular signaling, we examined the activation of 3 well-known downstream targets of α-CaMKII; CREB, ERK, and c-Fos by Western blotting (22–24). 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 with cells transduced with nonspecific controls, whereas the overexpression of α-CaMKII in HOS cells increases their activation (Fig. 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, whereas HOS GFP-CaMKIIα cells show increases in p-α-CaMKII (Fig. 4D). These results validate that the overexpression or deletion of α-CaMKII...
is successful in controlling \( \alpha \)-CaMKII intracellular signaling.

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

\( \alpha \)-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 \( \alpha \)-CaMKII knockdown or overexpression resulted in similar changes in osteosarcoma tumor viability in vivo, we intratibially

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**Figure 5.** Perturbation of \( \alpha \)-CaMKII in osteosarcoma cells changes their aggressive behavior in vitro. MG-63 and 143B cells were transduced with lentiviruses expressing either a nonspecific control (shCtrl) or \( \alpha \)-CaMKII-targeting shRNAs shCaMKII(\( \alpha \))(2). Also, HOS cells were transduced with retroviruses expressing either GFP (GFP-Ctrl) or CaMKII(\( \alpha \)) (GFP-CaMKII(\( \alpha \))). A, MTT assay was conducted to determine the number of viable cells. Values were obtained from 3 separate experiments, each repeated in triplicate and represent the mean \( \pm \) SE. \( * \) \( P < 0.01 \). B, scratch/wound healing assay was conducted on cells cultured for 8 hours. Representative photomicrographs were taken at \( \times \) 50 magnification from 3 independent experiments, each repeated in triplicate. Values represent the mean \( \pm \) SE. \( * \) \( P < 0.01 \). C, transwell invasion assay allowing cells to invade for 24 hours. Representative photomicrographs were taken at \( \times \) 100 magnification from 3 independent experiments, each repeated in duplicate. Values represent the mean \( \pm \) SE. \( * \) \( P < 0.01 \).
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 with cells transduced with nonspecific control, whereas α-CaMKII overexpression in HOS cells resulted in a 500% increase in tumor burden when compared with control (Figs. 6A and B). Histologically, H&E 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 (Fig. 6C). This was confirmed by conducting IHC staining using CD-31–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 (Fig. 6D).

Taken together, our data show 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 Ca2+ levels is critical for the regulation of normal cellular functions such as proliferation, growth, and gene expression (25, 26). However, it is becoming increasingly evident that the in vitro growth of cancer cells is not responsive to extracellular Ca2+ (27, 28). This

![Figure 6](Image) Perturbation of α-CaMKII in osteosarcoma cells affects in vivo tumor formation. MG-63 cells were transduced with lentivirus expressing either a nonspecific 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) was conducted at week 1 and 7 after tumor cell inoculation (n = 12). PET imaging (right) was conducted 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 7 mice in each group and represent the mean ± SE. *P < 0.01. C, H&E staining was conducted 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 ×400 or ×100 magnification (bottom right insets). D, IHC staining using a specific antibody directed against CD-31 (brown) was conducted. Images were taken at × 400 or × 100 magnification showing rabbit IgG control in the bottom right inserts and are representative of 7 different mice.
phenomenon was attributed by many to the ability of cancer cells to overexpress several of calcium downstream targets, such as CaMKII (29, 30). In the present study, we show that α-CaMKII activation plays a critical role in determining the aggressive behavior of human osteosarcoma. Examination of the levels of p-α-CaMKII in 114 OS tissues revealed that the levels of p-α-CaMKII were significantly higher in osteosarcoma tissues when compared with osteoblasts in normal bone. Also, we discovered that the levels of total and active α-CaMKII and tumorigenic properties of several osteosarcoma cell lines including proliferation, invasion, and motility are positively correlated. Interestingly, 2 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; refs. 31–33). Neither carcinogen exposure nor ras transformation is known to directly impact CaMKII expression. Currently, it is unknown whether the increases in α-CaMKII are a result of the more malignant phenotype of transformed HOS cells or are due to subcloning of a more aggressive subpopulation of HOS cells that already have an increase in α-CaMKII expression. Furthermore, it is unknown whether the shown increases in the activation of α-CaMKII in osteosarcoma are simply due to the increases in expression or are indirectly due to increases in an unknown upstream activator that leads to elevations in intracellular Ca2+. Interestingly, it has been reported that the activation of CaMKII creates a positive feedback loop by regulating the levels of intracellular Ca2+ through the activation of the ryanodine receptor, and several other ion channels (10). This complex crosstalk shows that increased levels of p-α-CaMKII and total α-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 (14). Furthermore, others have shown that the localized and transient enrichment of CaMKII to dendritic sites coincided spatially and temporally with intracellular calcium (34). 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 Ca2+ stores in the endoplasmic reticulum 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 (15), 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 (35–37). To expand on our published studies, we generated osteosarcoma cell lines in which α-CaMKII is either deleted (MG-63 and 143B) or overexpressed (HOS). The deletion of α-CaMKII was conducted by transducing osteosarcoma cells with a lentivirus that expresses α-CaMKII shRNA, whereas overexpression was achieved by transducing cells with a retrovirus that overexpresses α-CaMKII. Gene delivery efficiency was approximately 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, whereas the overexpression of α-CaMKII resulted in overactivation of these same molecules (38–40). The deletion of α-CaMKII significantly decreased the proliferation, motility, and invasion of osteosarcoma cells, whereas 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 (15). 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 (41). 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 (MMP), 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 (42, 43). 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 (44). Taken together, our results show 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, VEGF, basic fibroblast growth factor, Neuropilin-2, and placental growth factor in OS clinical tissue samples (20, 45). Similarly, our data show that the deletion of α-CaMKII in osteosarcoma cells produces not only smaller tumors but also resulted in less tumor vasculature as shown by a decrease in the number of CD-31–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 expression and activation 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-CaM-KII and its downstream targets in osteosarcoma may thus yield novel therapeutic strategies for this devastating disease.

Disclosure of Potential Conflicts of Interest

M.J. Klein is employed by Weitz and Luxenberg. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: P.G. Daft, K. Yuan, M. Zayzafoon
Development of methodology: P.G. Daft, K. Yuan, M. Zayzafoon
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.G. Daft, K. Yuan, J.M. Warram, M.J. Klein, M. Zayzafoon
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.G. Daft, K. Yuan, M.J. Warram, M.J. Klein, G.P. Siegal, M. Zayzafoon
Writing, review, and/or revision of the manuscript: P.G. Daft, K. Yuan, J.M. Warram, M.J. Klein, G.P. Siegal, M. Zayzafoon
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.G. Daft, K. Yuan, G.P. Siegal, M. Zayzafoon
Study supervision: P.G. Daft, M. Zayzafoon

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Paul G. Daft, Kaiyu Yuan, Jason M. Warram, et al.


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