Systematic Kinome shRNA Screening Identifies CDK11 (PITSLRE) Kinase Expression is Critical for Osteosarcoma Cell Growth and Proliferation

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Abbreviations: CDK11, cyclin-dependent kinase 11; shRNA, short hairpin RNA; siRNA, short interfering RNA

Running title: CDK11 in Osteosarcoma

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Translational Relevance

Kinases play an essential role in cancer cell growth and survival; however, the roles of most kinases in osteosarcoma cell growth are largely uncharacterized. In the search for kinases required for osteosarcoma cell growth, we identified CDK11 (PITSLRE) as a potential target by a comprehensive human kinome-wide shRNA screening in osteosarcoma cell lines. Furthermore, knock-down of CDK11 either by lentiviral shRNA, or by synthetic siRNA independent confirmation, can inhibit cell growth or induces apoptosis in osteosarcoma cells. Immunohistochemical analysis indicated that osteosarcoma patients with high CDK11 tumor expression levels were associated with significantly shorter survival than osteosarcoma patients with low level of CDK11 expression. Systemic in vivo administration of In Vivo Ready siRNA of CDK11 reduced tumor growth in an osteosarcoma s.c. xenograft model. These observations demonstrate that CDK11 signaling is essential in osteosarcoma cell growth and survival, CDK11 may become a promising therapeutic target in the management of osteosarcoma.

Abstract

Purpose: Identification of new targeted therapies is critical to improving the survival rate of osteosarcoma patients. The goal of this study is to identify kinases based potential therapeutic target in osteosarcomas.

Experimental Design: We used a lentiviral-based shRNA kinase library to screen for kinases which play a role in osteosarcoma cell survival. The Cell Proliferation Assay was used to evaluate cell growth and survival. siRNA assays were applied to confirm the observed phenotypic changes resulting from the loss of kinase gene expression. CDK11 (PITSLRE) was identified as essential for the survival of osteosarcoma cells, and its expression was confirmed by Western blot and immunohistochemistry.
Overall patient survival was correlated with the CDC2L1 expression and its prognosis. The role of CDK11 expression in sustaining osteosarcoma growth was further evaluated in an osteosarcoma xenograft model in vivo.

**Results:** Osteosarcoma cells display high levels of CDK11 expression. CDK11 expression knocked down by either lentiviral shRNA or siRNA inhibit cell growth and induce apoptosis in osteosarcoma cells. Immunohistochemistry analysis showed that osteosarcoma patients with high CDK11 tumor expression levels were associated with significantly shorter survival than osteosarcoma patients with low level of tumor CDK11 expression. Systemic in vivo administration of In Vivo Ready siRNA of CDK11 reduced the tumor growth in an osteosarcoma s.c. xenograft model.

**Conclusions:** We demonstrate that CDK11 signaling is essential in osteosarcoma cell growth and survival, further elucidate the regulatory mechanisms controlling expression of CDK11 and ultimately develop a CDK11 inhibitor may provide therapeutic benefit against osteosarcoma.
Osteosarcoma is the most common primary malignant tumor of bone. The standard treatment for osteosarcoma incorporates surgery and chemotherapy involving several chemotherapeutic agents which include doxorubicin, cisplatin, ifosfamide, and methotrexate (1, 2). If these agents are unable to lead to favorable tumor response, further chemotherapeutic options are very limited. Despite aggressive chemotherapy, more than 30% of patients with localized osteosarcoma experience metastatic disease. Most of these patients will eventually develop multidrug resistance in late stages of osteosarcoma. The average survival period after metastases is less than one year (1-5). Therefore, there is urgent need to improve the general condition and the overall survival rate of metastatic osteosarcoma patients by identifying novel therapeutic strategies.

The discovery of oncogenic kinases and target-specific small-molecule inhibitors has revolutionized the treatment of a select group of cancers, such as chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST). Protein kinases play important roles in regulating tumor cellular functions — proliferation/cell cycle, cell metabolism, survival/apoptosis, DNA damage repair, cell motility, and drug resistance — so it is not surprising that protein kinases are often oncogenic genes. Kinases such as c-Src, c-ABL, PI3K/ AKT, EGFR, MAP, IGF-1R, and JAK are commonly activated and highly expressed in cancer cells and are known to contribute to cancer progression (6, 7). Kinases are now firmly established as a major class of anti-cancer drug targets. Significant progress has been made in understanding kinases and their functions. There has been an explosion in the number of kinase inhibitors entering the clinic, and many more are in preclinical development. The best known kinase inhibitor is Gleevec used in the treatment of patients with CML and GIST. Gleevec inhibits the kinase activity of BCR-ABL in CML and c-Kit in GIST (6, 8). However, the question remains as to whether other highly expressed and activated kinases in tumors including osteosarcoma can be targeted in a similar manner.
In order to identify kinases based potential therapeutic target in osteosarcoma, we performed a comprehensive kinome-wide shRNA screening and found CDK11 (cyclin-dependent kinase 11, also known as CDC2L for cell division cycle 2-like or PITSLRE) is an essential kinase for osteosarcoma cell line KHOS growth and proliferation. Cyclin-dependent kinases are protein kinases that are critical for cellular processes, such as transcription (9, 10). However, to our knowledge, the oncogenic role of CDK11 has not been reported in osteosarcoma previously. Further CDK11 protein expression profiling indicated that the expression levels of CDK11 in tumor tissues is closely correlated with clinical outcome.

Materials and Methods

Cell lines and cell culture. Human osteosarcoma cell line KHOS, KHOSR2 were kindly provided by Dr. Efstathios Gonos (Institute of Biological Research & Biotechnology, Athens, Greece). Ewing sarcoma cell line TC-71 was provided by Dr Katia Scotlandi (Institute Orthopedics Rizzoli, Italy). The human osteosarcoma cell lines, U-2OS and Saos, human ovarian cancer cell line SKOV-3 and uterine sarcoma cell line MES-SA were purchased from the ATCC (Rockville, MD). Osteosarcoma cell line OSA344 was established from primary osteosarcoma tissue. Human osteoblast cells HOB-c were purchased from PromoCell GmbH (Heidelberg, Germany), osteoblast cells NHOst were purchased from Lonza Wallkersville Inc. (Walkersville, MD), and osteoblast cells hFOB were purchased from ATCC. Osteoblast cells were cultured in osteoblast growth medium (PomoCell) with 10% fetal bovine serum (FBS). All other cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100-units/ml penicillin and 100µg/ml streptomycin (Invitrogen).
**Lentiviral human kinase shRNA library screen.** The roles of protein kinases in maintaining osteosarcoma cell growth were examined using MISSION® LentiExpress™ Human Kinases shRNA library (Sigma, Saint Louis, MO). This library contains 3109 lentiviruses carrying shRNA sequences targeting 673 human kinase genes. Screening was carried out by following the manufacturer’s protocol as previously described (11, 12).

**Proliferation assay.** The initial cellular proliferation after lentiviral shRNA infection was assessed using the CellTiter 96® AQueous One Solution Cell Assay (Promega, Madison, WI) by following the manufacturer’s protocol as previously described (11).

**Synthetic CDK11 siRNA and trasfection.** Further validation of CDK11 knockdown phenotype in osteosarcoma cell lines was carried out with synthetic human CDK11 siRNA purchased from Ambion at Applied Biosystems (Foster City, CA). The siRNA sequence targeting CDK11 corresponded to coding regions (5’- AGAUCUACAUCGUGAUGAAtt-3’, antisense 5’- UUCAUCACGAUGUAGAUCUtg-3’) of the CDK11 gene. The siRNA oligonucleotides were dissolved in nuclease-free water at a concentration of 100 µM and kept at –20°C until the following transfection experiment. The nonspecific siRNA oligonucleotides (Applied Biosystems) were used as negative controls. Osteosarcoma KHOS or U-2OS cells were either plated on 96-well plates for cell proliferation assays or plated on dishes for Western blot protein isolation. Transfections were performed with Lipofectamin RNAiMax reagent (Invitrogen) according to the manufacturer’s instruction. Medium was replaced with RPMI 1640 supplemented with 10% fetal bovine serum 24 hours after transfection. Total protein was isolated with RIPA Lysis Buffer (Upstate Biotechnology) 48 hours post CDK11 siRNA transfection.

**MTT Assay.** Effects of CDK11 siRNA on cellular growth and proliferation were assessed in vitro using the MTT assay as described previously. KHOS or U-2OS were transfected with CDK11
siRNA as described above. After 72 hours of culture, 20μl of MTT (5 mg/mL in PBS, obtained from Sigma-Aldrich) were added to each well and the plates were incubated for 3 h. The resulting formazan product was dissolved with acid isopropanol and the absorbance at a wavelength of 490 nm (A490) was read on a SPECTRAmax® Microplate Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

**Apoptosis Assay.** Caspase-cleaved keratin 18 based quantification of apoptosis was evaluated using the M30-Apoptosense ELISA assay kit, as per manufacturer’s instructions (Peviva AB, Bromma, Sweden). The ELISA apoptosis detects a 21-kDa fragment of cytokeratin 18 that is only revealed after caspase cleavage of the protein. KHOS or U-2OS cell lines reverse transfected with synthetic CDK11 siRNA were seeded at 2,000 cells/ well in a 96-well plate. After 48 hours incubation, the cells were then lysed by adding 10 μl of 10% NP-40 per well, and the manufacturer's instructions for the apoptosis assay were then followed. Apoptosis was also evaluated by Western blot using whole-cell lysates immunoblotted with specific antibodies to PARP (Cell Signaling Technologies, Cambridge, MA) and its cleavage products.

**Western Blotting.** The concentration of the protein was determined by Protein Assay Reagents (Bio-Rad) with a spectrophotometer (Beckman Du-640, Beckman Instruments, Inc.). The rabbit polyclonal antibody (sc-928) to human CDK11 (PITSLRE) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal antibody to human actin was purchased from Sigma-Aldrich. Goat anti-rabbit HRP and goat anti-mouse antibodies were purchased from Bio-Rad. All other antibodies used in this study were purchased from Cell Signaling Technologies. Western blot was performed as previously reported (13).

**Immunofluorescence assay.** For immunostainings of cultured osteosarcoma cells, KHOS or U-2OS cells were grown in eight-well chambers for 24 hours and fixed in 3.7% buffered paraformaldehyde. Immunostainings were carried using antibodies against CDK11 and β-actin. After
washing the cells, they were incubated with Alexa Fluor secondary antibodies. Specifically, the slides were stained with Alexa Fluor 488 (Green) conjugated goat anti-rabbit antibody (Invitrogen) for CDK11, and Alexa Fluor 594 (Red) conjugated for goat anti-mouse antibody for β-actin.

**Human sarcoma tumor tissues.** Six of the osteosarcoma tissue samples (OST1 to OST6) were obtained from Massachusetts General Hospital sarcoma tissue bank and were used in accordance with the policies of the institutional review board (IRB) of the hospital. All diagnoses were confirmed histologically.

**Osteosarcoma tissue microarray and immunohistochemistry.** Osteosarcoma tissue microarray was purchased from Imgenex Corp (San Diego, CA) which contains 57 tumor tissues. Immunohistochemistry was performed by following the manufacturer’s instructions with HRP-DAB System Cell and Tissue Staining Kit (R&D Systems, Minneapolis, MN). In brief, primary antibody of CDK 11 (1:50 dilution) in 1% bovine serum albumin (BSA) was applied to the deparaffinized slide overnight at 4°C. After incubation with the HRP conjugated goat anti-rabbit antibody, and rinses in PBS thrice, bound antibody was detected with the substrate reagents from HRP-DAB System Cell and Tissue Staining Kit. Finally, slides were counterstained with Hematoxylin QS (Vector Laboratories) and mounted with VectaMount AQ (Vector Laboratories).

**Evaluation of immunohistochemical staining.** CDK11 positive samples were defined as those showing nuclear staining pattern of tumor tissue. CDK11 staining patterns were categorized into six groups: 0, no nuclear staining; 1+, <10% of cells nuclear stained positive; 2+, 10% to 25% positive cells; 3+, 26% to 50% positive cells; 4+, 51% to 75% positive cells; 5+, >75% positive cells. The percentage of cells showing positive nuclear staining for CDK11 was calculated by reviewing the entire spot. Categorizing of CDK11 staining was completed by two independent investigators. Discrepant scores between the two investigators were rescored to get a single final score. CDK11 images were
obtained by using a Nikon Eclipse Ti-U fluorescence microscope (Nikon Corp) with a SPORT RT digital camera (Diagnostic Instruments Inc.).

**Statistical analysis.** Kaplan-Meier survival analysis (GraphPad PRISM® Software; GrahPad Software, San Diego, CA) was used to analyze the correlation between the level of CDK11 expression and prognosis. The Student *t* test was used to compare the differences between groups. Results are given as mean ± SD and values with *p*<0.05 were considered statistically significant.

**CDK11 siRNA osteosarcoma tumor therapy.** Ambion® In Vivo Ready CDK11 siRNA and nonspecific siRNA were purchased from Applied Biosystems. These In Vivo Ready, validated siRNA were designed using the Silencer® Select algorithm and incorporate additional chemical modifications for superior serum stability with in vivo applications (14-17). The Crl:SHO-Prkdc<sup>SCID</sup>Hr<sup>hr</sup> nude female mice at approximately 3 to 4 weeks of age were purchased from The Charles River Laboratories (Ann Arbor, MI). To determine the effect of CDK11 siRNA on osteosarcoma cell growth in xenograft model, KHOS cells (1X10<sup>6</sup>) were inoculated subcutaneously with matrigel from BD Biosciences (San Jose, California, USA) into the right flank of the nude mouse. Two weeks after injection, the mice were randomized into 3 groups (6 mice per group). Group 1 received injection with sterile saline (0.9% NaCl), group 2 with In Vivo Ready nonspecific siRNA, group 3 with In Vivo Ready CDK11 siRNA. For intratumoral (ITu) injections, each animal was injected with 20 μl of PBS containing 10 nM of siRNA. All 3 groups were treated twice a week for two weeks. The health of the mice and evidence of tumor growth were examined daily. Tumor volumes were measured at a regular interval of for up to 4 weeks with a digital calipers. Tumor volume (mm<sup>3</sup>) was calculated as (width)<sup>2</sup> X length/2 where W is width and L is length. Data are presented as mean ± SD. Tumor tissues from the above treated animals were collected and placed in 10% formalin and embedded in paraffin for histology analysis. The silence efficiency CDK11 siRNA on CDK proteins were determined by immunohistochemical staining as
described above. Animal experiments in the present study were performed in compliance with the protocol which was approved by the Massachusetts General Hospital Subcommittee on Research Animal Care (SRAC) under the protocol number 2009N000229.

Results

**CDK11 expression is critical for osteosarcoma cell growth and survival.** To identify the potential therapeutic kinase targets in osteosarcoma cells, we performed a comprehensive kinome-wide screening of lentiviral shRNA kinase library in osteosarcoma cell line KHOS. Among the targeted kinase genes, we found that knocking down the expression of CDK11, PLK1, DYRK1B and ROCK1 led to inhibitory growth effects. Of those kinases that we found critical to osteosarcoma cell growth, the elevated expression of PLK1, DYRK1B and ROCK1 have been previously reported in various human tumors (6, 12, 18-24). However, direct evidence regarding the relationship between expression of CDK11 and cancer cell growth and survival is lacking. When Lentiviral shRNA targeting CDK11 was transduced into osteosarcoma cell lines KHOS and U-2OS, it led to significantly reduced tumor cell growth and eventual cell death as demonstrated by cellular proliferation assay (Figure 1A). In order to further characterize the function role CDK11 in osteosarcoma, we rigorously validated the results using multiple independent experiments, including multiple shRNA per CDK11 gene, and extensively tested with control nonspecific shRNA (The sequence of five shRNA target different sites of CDK11 in Supplementary Table 1). The results revealed that four out of five CDK11 shRNA inhibit osteosarcoma cell growth. We further validated these results with synthetic human CDK11 siRNA. Consistent observations of dose-dependent CDK siRNA inhibition of osteosarcoma cell growth and survival was established by MTT assay (Figure 1B). We subsequently measured the expression of CDK11 in siRNA
transfected cells. Western blot analysis suggested that down-regulated expression of CDK11 protein by CDK11 siRNA associated with the inhibition of cell growth (Figure 1B and Figure 1C).

**CDK11 knockdown induces apoptosis in osteosarcoma cell lines.** To investigate how CDK11 sustains tumor cell growth and survival, we investigated cellular events during the cell death caused by CDK11 knockdown in osteosarcoma cell lines KHOS and U-2OS. We investigated the potential for the induction of apoptosis using the M-30-Apoptosense ELISA and by Western blotting for the cleavage of PARP. Depletion of CDK11 by siRNA resulted in a dose-dependent cell death in KHOS and U-2OS osteosarcoma cell lines which was not observed with the non-specific siRNA transfection (Figure 2A). Consistent with these results, there was also a dose-dependent cleavage of PARP for siRNA mediated depletion of CDK11 (Figure 2B). CDK11 has been reported to play an important role in the regulation of gene transcription and pre-mRNA splicing. We hypothesized that inhibition of apoptosis induced by CDK11 may be associated with the deregulation of RNA processing associated with a subsequent decrease of antiapoptotic dependent proteins. Therefore, we examined whether the inhibition of CDK11 by siRNA could result in decreased expression of antiapoptotic proteins MCL-1, BcL-X1, survivin, Cytochrome C and Cyclin D1. Most of these antiapoptotic proteins such as MCL-1, BcL-X1 and survivin are highly expressed in osteosarcoma, and downregulation by siRNA can inhibit cell growth and induce apoptosis in osteosarcoma cells. Western blot confirmed that transfection of CDK11 siRNA significantly down-regulated the expression of several of these antiapoptotic proteins in both in KHOS and U-2OS cells (Figure 2C), whereas CDK11 knockdown did not alter actin expression. Thus, these results indicate that CDK11 can control several aspects of apoptosis signaling.

**CDK11 is highly expressed in osteosarcoma cell lines and in tumor tissues.** In order to further confirm the expression of CDK11 and determine CDK11 protein subcellular localization in osteosarcoma cell lines, immunofluorescence assay was used in KHOS and U-2OS cell lines. Previous
studies have reported that the CDK11 protein localizes both to the nucleus and cytoplasm. Our results demonstrated that the CDK11 protein is mainly localized in the nucleus of osteosarcoma cells (Figure 3A). We also extended our evaluation of the expression of CDK11 in other types of human cancer cell lines including uterine sarcoma (MES-SA), chondrosarcoma (CS-1) synovial sarcoma (SS-1), Ewing sarcoma (TC-71), and ovarian cancer (SKOV-3, 3A, 2008). These tumor cell lines exhibited a variety of expression levels of CDK11 protein as evaluated by Western blot (Figure 3B). To confirm these data in primary cancer, six freshly isolated primary osteosarcoma specimens were also examined by Western blot to exclude the possibility of CDK11 expression being an artifact induced by in vitro propagation. Different levels of CDK11 expression were once again observed in these osteosarcoma patient samples, indicating the endogenous expression of CDK11 in tumor cells. In the normal human cells, the expression of CDK11 is tightly regulated, for example, in normal human osteoblast cell lines HOB-c, NHOST and hFOB there are extremely low and almost undetectable levels of CDK11 (Figure 3C).

**CDK11 expression levels correlates with clinical prognosis in osteosarcoma patients.** To further validate the clinical relevant of CDK11 expression in osteosarcoma patients, we analyzed CDK11 protein levels by using osteosarcoma tissue microarray. The results showed the majority of tumors present on the tissue microarray had positive staining for CDK11. CDK11 nuclear staining percentage was graded into 6 groups. By comparing the clinical characteristics of low-staining (<=3) and high-staining (>=4) osteosarcomas, no correlation existed between CDK11 expression and age or tumor location (p>0.05, supplementary Table 2). Kaplan–Meier survival analysis showed that the outcome for patients in the CDK11 high-staining group was significantly worse than for those in the CDK11 low-staining group (Figure 4A). Based on 60 months survival rates, patients were grouped into survivors (survived up to 60 months post follow up) and non-survivors (deceased within 60 months of follow-up). A total of 30 (67%) samples from survivors and 15 (33%) samples from non-survivors were
collected. Comparison of CDK11 staining intensity between two group patients revealed that CDK11 staining for samples from non-survivors were significantly higher than that of survivors. The average CDK11 expression levels for survivors and non-survivors were 2.8 to 4.3, respectively (Figure 4B). The immunohistochemical staining of CDK11 protein indicated its location in the nucleus (Figure 4C) which is consistent with that of osteosarcoma cells, as measured by immunofluorescence assay (Figure 3A).

**CDK11 siRNA inhibit tumor growth.** The significant association of CDK11 expression with clinical outcome led us to further verify the essential role of CDK11 in sustaining osteosarcoma growth in vivo. KHOS osteosarcoma cells (1X10^6) were injected s.c into the flank of nude mice. By 2 weeks, visible tumors had developed at injection sites (mean tumor volume=52 mm^3). CDK11 siRNA was then intratumorally injected, twice a week for two weeks. As shown in Figure 5A, CDK11 In Vivo Ready siRNA significantly suppressed tumor growth as compared with vehicle (saline) and nonspecific siRNA treatment. The immunohistochemical staining indicated a significant decrease of CDK11 expression in tumor treated with CDK11 In Vivo Ready siRNA (Figure 5B). No gross adverse effects, i.e the loss of body weight were observed during the experimental period.

**Discussion**

To identify essential kinases which are responsible for osteosarcoma growth, we first performed a kinome-wide shRNA screen. CDK11, PLK1, DYRK1B and ROCK1 were the primary hits with loss of expression of these kinases significantly reduced cell growth and survival. We and others had previously found that targeting PLK1, DYRK1B and ROCK1 kinases inhibits osteosarcoma cell growth and survival by using similar shRNA or siRNA kinase library screenings (11, 12, 18, 25-27). However, the relationship between the expression of CDK11 and osteosarcoma growth and survival was lacking. The important functional role of CDK11 in osteosarcoma cells was further validated by using CDK11 gene
specific synthetic siRNA knockdown endogenous CDK11. Both initial shRNA screenings and follow up siRNA validation assay results highlighted the importance of CDK11 in osteosarcoma.

CDK11 is a serine/threonine-protein kinase and encoded by the CDK11 gene on chromosome 1p36.3 (9, 10). The function of CDK11 has not been described in osteosarcoma. There is only one CDK11 gene in mouse, whereas in human there are two CDK11 genes that encode two almost identical protein kinases. There are three CDK11 protein isoforms, p110, p58 and p46 (28). CDK11 p58 protein is specifically translated from an internal ribosome entry site and expressed only in the G2/M phase of the cell cycle. These different CDK11 isoforms appear to play multiple roles in transcription, RNA processing, regulating cell cycle progression, cytokinesis and apoptosis (10, 28). CDK11 knockout mice display an earlier phenotype and death during the blastocyst stage of embryonic development (29). The CDK11-null cells exhibit proliferative defects, mitotic arrest, and apoptosis, thus suggesting that CDK11 kinase is critical for embryonic development and cellular viability (29). By kinome-wide siRNA screen for Hedgehog (Hh) regulators, CDK11 has been shown to directly participate in the Hh pathway. CDK11 is necessary and sufficient for activation of the Hh pathway, functioning downstream of Smo and upstream of the Glioma-associated (Gli) transcription factors (19). CDK11 is also a modulator of autophagy in human cells (30). Although the function of CDK11 has been examined in different model systems, its potential role in tumors has not been fully investigated due to a lack of data regarding CDK11 expression and tumor cell growth. CDK11 was initially proposed as a tumor suppressor candidate gene since the CDK11 chromosomal location region 1p36.3 is frequently deleted or translocated in a number of different human tumors, including neuroblastoma, breast cancer and melanoma (31-33). However, a study of neuroblastoma by FISH analysis excludes the CDK11 genes as a tumor suppressor gene (34). On the contrary, CDK11 knockdown by RNAi in HeLa cells induces abnormal spindle assembly, mitotic arrest by checkpoint activation, and cell death (20, 35). An unbiased
high-throughput RNAi screening demonstrated that CDK11 is a positive modulator of the Wnt/β-cat pathway in colon cancer (36). Consistent with these studies, a more recent kinome-wide siRNA study in human multiple myeloma identified CDK11 as one out of fifteen survival kinases (including PLK1, AKT1 and GRK6) that was repeatedly vulnerable in myeloma cells (37). Interestingly, a more recent RNAi lethality screening of the druggable genome also found CDK11 is the survival gene in multiple myeloma (21). Our results in osteosarcoma are compatible with HeLa and myeloma cells in which CDK11 inhibition leads to decreased cell growth and induces apoptosis.

It has been reported that treatment of the Fas-activated T cells with a serine protease inhibitor prevented apoptotic death and led to the accumulation of CDK11 p110 isoform, but not the CDK11 p58 isoform (22). To establish the mechanisms of CDK11 knockdown induced cell growth inhibition and apoptosis in osteosarcoma cells, we examined antiapoptotic protein expression. Several antiapoptotic proteins were reduced by CDK11 knockdown, suggesting that CDK11 can control several aspects of apoptosis signaling.

Many studies have found survival-promoting kinase genes to be highly expressed in human cancer, especially in high-grade tumors (10, 38). For CDK11, we show that osteosarcoma cell lines and tumors express high level of CDK11 protein in comparison to normal osteoblasts. Most importantly, the levels of CDK11 expression are significantly associated with clinical outcome in osteosarcoma. Overexpression of CDK11 was correlated with poor prognosis. Furthermore, silencing of CDK11 reduced tumor volume in an osteosarcoma xenograft mouse model. These in vivo studies have confirmed the anticancer effects of CDK11 inhibition in vitro and provide a rationale for pharmacological investigation of CDK11 as a novel therapy target.

Taken together, this study identified that CDK11 as essential for osteosarcoma cell growth and survival. Experiments are underway to understand the mechanisms behind CDK11 signaling in human
cancer. In turn, these findings may lead to targeting CDK11 through gene therapy or kinase specific inhibitors in the treatment of osteosarcoma.

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References


Figure Legends

Fig. 1. Effects of CDK11 inhibition by shRNA and siRNA in osteosarcoma cell lines. (A) Results of lentiviral shRNA targeting CDK11 in KHOS and U-2OS cell lines. Proliferation was determined by the CellTiter 96® Aqueous One Solution Cell Assay. The data represent a single well of the 96-well plate of MISSION® LentiExpress™ Human kinases shRNA library as described. (B) Results of synthetic siRNA against CDK11 in KHOS and U-2OS cell lines. Proliferation was assessed by MTT as described in the Materials and Methods. The data represent the mean ± SE of two experiments carried out in triplicate. (C) Dose-dependent CDK siRNA down-regulated expression of CDK11. KHOS or U-2OS cells were transfected with CDK siRNA in a dose-dependent manner. For Western blot analysis, 25 μg of total cellular proteins was used for immunoblotting with specific antibody to CDK11. The results were detected by a chemiluminescence detection system as described in Materials and Methods.

Fig. 2. Synthetic siRNA targeting CDK11 induces apoptosis in osteosarcoma cells. (A) KHOS or U-2OS cells were transfected with CDK siRNA as dose-dependent manner. The cells were lysed with 10%
NP40 after 48 hours transfection and the apoptosis was determined by M30-Apoptosense ELISA assay as described in Materials and Methods. (B) Confirmation of CDK11 siRNA induced apoptosis of PARP cleavage by Western blot. Total cellular proteins were subjected to immunoblotting with specific antibody to PARP as described in Materials and Methods. (C) CDK11 siRNA decreases CDK11 expression and down-regulated antiapoptotic proteins expression. For Western blot analysis, 25 μg of total cellular proteins was subjected to immunoblotting with specific antibody to CDK11, MCL-1, BcL-XL, survivin, cytochrome C, cyclin D1 and β-actin. The results were detected by a chemiluminescence detection system as described in Materials and Methods.

**Fig. 3.** Expression of CDK11 in osteosarcoma cell lines and osteosarcoma tissues. (A) Expression of CDK11 in KHOS or U-2OS cells was assessed by immunofluorescence with antibodies to CDK11 and β-actin. Cells were visualized under a fluorescence microscope after incubated with secondary fluorescent conjugated antibodies Alexa Fluor 488 goat anti-rabbit IgG (green) and Alexa Fluor 594 goat anti-mouse IgG (red) as described in the Materials and Methods. (B) Levels of CDK11 expression were determined by Western blot in osteosarcoma (U-2OS, KHOS), and other types of human cancer cell lines, including uterine sarcoma (MES-SA), chondrosarcoma (CS-1), synovial sarcoma (SS-1), Ewing sarcoma (TC-71) and ovarian cancer (SKOV-3, 3A, 2008). (C) Levels of CDK11 expression were determined by Western blot in osteosarcoma tissues (OST1 to OST6) and in normal osteoblast cell lines (HOB-c, NHOst and hFOB). OST1–OST6 represents tissues samples from six patients.

**Fig. 4.** Association of CDK11 expression with clinical outcome in osteosarcoma. (A) Kaplan–Meier survival curve of osteosarcoma patients are subgrouped as either CDK11 low staining (CDK11 staining <=3). CDK11 high staining CDK11 staining>=4). (B) Distribution of CDK11 staining
scores among the survivors and nonsurvivors. (C) Representative images of different immunohistochemistry staining intensities of CDK11 are shown in osteosarcoma tissues. For CDK11 immunohistochemical staining, the percentage of cells showing positive nuclear staining for CDK11 was calculated by reviewing the entire spot. On the basis of the percentage of cells with positive nuclear staining, the staining patterns were categorized into six groups: 0, no nuclear staining; 1+, <10% of cells stained positive; 2+, 10% to 25% positive cells; 3+, 26% to 50% positive cells; 4+, 51% to 75% positive cells; 5+, >75% positive cells.

**Fig. 5.** Inhibition of tumor growth by *In Vivo* Ready CDK11 siRNA in a xenograft mouse model. (A) CDK11 *In Vivo* Ready siRNA, vehicle (saline) control and nonspecific siRNA were injected into the tumor region. Day 1 corresponds to 2 weeks after inoculation of KHOS cells when tumor volume was 50 to 60 mm$^3$. Tumor diameters were measured at a regular interval of 4 days for up to 4 weeks with a digital caliper, and the tumor volume was calculated. (B) Histological analyze effect of CDK11 siRNA on CDK11 staining in osteosarcoma tumor tissues show downregulation of CDK11 compared with vehicle or nonspecific siRNA.
Fig. 1

A

Cell viability (%)

KHOS

U-2OS

B

Cell viability (Absorbance)

KHOS

U-2OS

C

CDK11 110kD

Actin 42kD

KHOS

U-2OS

1: Cells only
2: Cells + nonspecific siRNA
3: Cells + CDK11 siRNA 20nM
4: Cells + CDK11 siRNA 40nM
5: Cells + CDK11 siRNA 80nM

CDK11 shRNA (TRCN0000006206)
CDK11 shRNA (TRCN00000062067)
CDK11 shRNA (TRCN0000006208)
CDK11 shRNA (TRCN00000062069)
CDK11 shRNA (TRCN000000620610)
pLKO.1 vector shRNA
Non target shRNA
Media control
Fig. 2

A

CK18 (relative levels)

KHOS

U-2OS

B

PARP

1 2 3 4 5

KHOS

U-2OS

C

CDK11

MCL-1

Bcl-XL

Survivin

Cyto C

Cyclin D1

Actin

1 2 3 4 5

KHOS

U-2OS

1: Cells only
2: Cells + nonspecific siRNA
3: Cells + CDK11 siRNA 20nM
4: Cells + CDK11 siRNA 40nM
5: Cells + CDK11 siRNA 80nM

118kd PARP

85kd cleavage

Cells only

Cells + nonspecific siRNA

Cells + CDK11 siRNA 20nM

Cells + CDK11 siRNA 40nM

Cells + CDK11 siRNA 80nM
Fig. 3

A

KHOS

U-2OS

CDK11

Actin

Merge

B

CDK11 110 kDa

Actin 42 kDa

C

CDK11 110 kDa

Actin 42 kDa
Fig. 4

A

Survival proportions

Percent survival

0  50  100  150  200

Months

P=0.0006

B

CDK11 staining

Survivor  Non-survivor

Score 2+

Score 5+

P=0.0016

C

HE (40X)

CDK11 (40X)
Fig. 5

A

![Graph showing tumor volume over time](image)

- Black line: Vehicle controls
- Blue line: Nonspecific siRNA
- Red line: In vivo ready CDK11 siRNA

B

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Systematic Kinome shRNA Screening Identifies CDK11 (PITSLRE) Kinase Expression is Critical for Osteosarcoma Cell Growth and Proliferation

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