Targeting super-enhancer-associated oncogenes in osteosarcoma with THZ2, a covalent CDK7 inhibitor

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Statement of Significance

Super-enhancer-associated genes contribute to the malignancy of osteosarcoma, and targeting super-enhancer-associated oncogenes with the CDK7 inhibitor THZ2 is a promising therapeutic strategy in osteosarcoma.
Translational Relevance

Despite the development of new diagnostic and advanced treatment strategies, the prognosis of patients with osteosarcoma remains poor. It is thus urgent to identify novel and effective targets and treatment regimens for osteosarcoma patients. In this study, we found that super-enhancer-associated genes contribute to the malignant potential of osteosarcoma. Knockdown of CDK7 reduced the phosphorylation of the RNA polymerase II (RNAPII) C-terminal repeat domain (CTD), which is enriched in super-enhancers and suppresses osteosarcoma cells. THZ2, a new specific CDK7 inhibitor, selectively suppressed super-enhancer-associated genes, especially oncogenes, through inhibiting the activity of CDK7 by covalent binding. This newly developed small molecular inhibitor, THZ2, exhibited a powerful anti-osteosarcoma effect in vitro and in vivo. Our findings provide an important molecular foundation for understanding the pathogenesis of osteosarcoma through super-enhancers. They also indicate that targeting super-enhancer-associated oncogenic transcriptional programs with a specific CDK7 inhibitor, THZ2, may be a promising therapeutic strategy for patients with osteosarcoma.
Abstract

**Purpose:** Malignancy of cancer cells depends on the active transcription of tumor-associated genes. Recently, unique clusters of transcriptional enhancers, termed super-enhancers, have been reported to drive the expression of genes that define cell identity. In this study we characterized specific super-enhancer-associated genes of osteosarcoma, and explored their potential therapeutic value.

**Experimental Design:** Super-enhancer regions were characterized through chromatin immunoprecipitation sequencing (ChIP-Seq). RT-qPCR was used to detect the mRNA level of CDK7 in patient specimens and confirm the regulation of sensitive oncogenes by THZ2. The phosphorylation of the initiation-associated sites of RNA polymerase II (RNAP II) C-terminal repeat domain (CTD) was measured using Western blotting. Microarray expression analysis was conducted to explore transcriptional changes after THZ2 treatment. A variety of *in vitro* and *in vivo* assays were performed to assess the effects of CDK7 knockdown and THZ2 treatment in osteosarcoma.

**Results:** Super-enhancers were associated with oncogenic transcripts and key genes encoding cell-type-specific transcription factors in osteosarcoma. Knockdown of transcription factor CDK7 reduced phosphorylation of the RNAPII CTD, and suppressed the growth and metastasis of osteosarcoma. A new specific CDK7 inhibitor, THZ2, suppressed cancer biology by inhibition of transcriptional activity. Compared with typical enhancers, osteosarcoma super-enhancer-associated oncogenes were particular vulnerable to this transcriptional disruption. THZ2 exhibited a powerful anti-osteosarcoma effect *in vitro* and *in vivo.*
Conclusions: Super-enhancer-associated genes contribute to the malignant potential of osteosarcoma, and selectively targeting super-enhancer-associated oncogenes with the specific CDK7 inhibitor THZ2 might be a promising therapeutic strategy for patients with osteosarcoma.

Introduction

Osteosarcoma is the most common primary malignant bone tumor, and the second leading cause of cancer-related death in children and adolescents (1). Rapid tumor progression and early metastasis account for medical therapy failure and death in osteosarcoma patients. Currently, the 5-year survival rate has been increased to 60-70% with the use of neoadjuvant chemotherapy, but the rate is only 20% for patients with metastatic disease (2). Therapeutic outcomes remain unsatisfactory mainly because of delayed diagnosis, distant metastasis, and chemoresistance. In addition, standard adjuvant/neoadjuvant chemotherapy has no significant anti-neoplastic effect in a portion of patients with osteosarcoma (3). Furthermore, there have been no advances in overcoming chemoresistance in the past 2 decades (4). Much of the problem is due to a lack of understanding of the mechanisms which drive the malignant properties of osteosarcoma. Thus, it is important to elucidate the pathogenesis of osteosarcoma and develop more effective treatment regimens.

Studies have shown that most cancer cells have a higher overall transcriptional output than non-malignant cells, allowing for more opportunities to engage oncogenic pathways (5). The malignant properties of tumors such as rapid proliferation, invasion,
and metastasis require continually active transcription. Enhancers, *cis*-acting regulatory elements localized distal to promoters and transcription start sites, play important roles in cell identity by controlling cell-type-specific patterns of gene expression, and there can be tens of thousands active in any one cell type (6-8). Studies have shown that the upregulated expression of tumor cell oncogenes is always associated with large clusters of transcriptional enhancers in close genomic proximity, which have been termed super-enhancers, and that transcription of oncogenes is particularly sensitive to the disruption of super-enhancers (9-12).

Super-enhancers have been identified in many cell types, and relations between super-enhancers, gene regulation, and disease have been identified (13, 14). Studies have shown that the transcription of many oncogenes, including MYC (15), STAT3 (9, 13), EGFR (10), and TAL1 (16), is related to super-enhancer activity. Some super-enhancer-associated oncogenes such as PAK4 (12) and INSM1 (11) have been identified as novel therapeutic targets. Furthermore, the expression of most super-enhancer-associated genes can be blocked by selective inhibition of their super-enhancers.

Since the association between tumorigenesis and super-enhancers was reported in 2013 (9), studies have shown that super-enhancers play a role in many cancers, including breast (17, 18), colon (13, 17), lung (9, 11), brain (19), liver (20, 21), prostate (13), and pancreatic (22) cancer, as well as various types of leukemia (13, 23-25). However, the role of super-enhancers in osteosarcoma is largely unknown.

Super-enhancers are occupied by an unusually large portion of the
enhancer-associated RNA polymerase II (RNAPII), cofactors and chromatin regulators. Genes that are dependent on the high transcriptional activity of super-enhancers are exquisitely susceptible to alterations of transcription (9, 26).

CDK7 is a cyclin-dependent kinase (CDK) and a subunit of the multi-protein basal transcription factor TFIIH. It has been implicated in the regulation of cell-cycle progression and regulation of transcription, where it phosphorylates the C-terminal domain (CTD) of RNAPII (27, 28). Recent studies have shown that super-enhancer-associated genes are particularly sensitive to small perturbations in RNAPII-mediated transcription and CDK7 kinase function (29). A newly developed molecular inhibitor, THZ2, can completely inhibit the phosphorylation of the intracellular CDK7 substrate RNAPII CTD at Ser-2, -5 and -7 through irreversible covalent binding to CDK7 (10). It has been reported that THZ2 can suppress the growth of triple-negative breast cancer (30) and gastric cancer (31). However, the anticancer effect of THZ2 in other cancers is still unknown.

In this study we delineated the super-enhancer landscape in osteosarcoma cells on the basis of H3K27ac signal intensity by ChIP-Seq (13). Based on the sensitivity of super-enhancer-associated genes to transcriptional disruption and the high expression of CDK7 in osteosarcoma specimens from patients, we explored the anti-osteosarcoma effects of CDK7 inhibition by short hairpin RNA (shRNA) and THZ2. Microarray expression analyses were performed to detect transcription alterations after treatment with THZ2, and it was found that THZ2 selectively suppressed super-enhancer-associated genes which are enriched in processes
important to cancer biology. A variety of \textit{in vitro} and \textit{in vivo} cellular assay were preformed to assess the anti-tumor effects of THZ2.

\section*{Materials and Methods}

\subsection*{Human cell lines}

Human osteosarcoma cell lines SJSA-1, U2-OS, HOS, G-292, MNNG/HOS, 143B and MG-63, the osteoblast cell line hFOB1.19, and HEK-293T cells were obtained from American Type Cell Collection (ATCC). U2OS/MTX300 cells, a methotrexate-resistant derivative of the U2-OS human osteosarcoma cell line, were kindly provided by Dr. M. Serra (Instituti Ortopedici Rizzoli, Bologna, Italy). The ZOS and ZOS-M cell lines, derived from a primary osteosarcoma tumor and metastasis, respectively, have been described previously (32). All of the cells used were authenticated before experiments, and were cultured according to instructions from ATCC.

\subsection*{Compounds and reagents}

THZ2, a novel covalent inhibitor of CDK7, was a gift from Professor Zhi Shi (National Engineering Research Center of Genetic Medicine, Jinan University, Guangzhou, China, 510632). Antibodies against H2K27ac (#ab4729), β-Actin (#ab8227), Cyclin D1 (#ab134175), and total/cleaved-PARP (#ab191217) were purchased from Abcam (Cambridge, MA, USA). Antibodies against RNAPII (#A300-653A), RNAPII-CTD-SER2 (#A300-654A), and RNAPII-CTD-SER5 (#A304-408A) were purchased from Bethyl Laboratories (Montgomery, TX, USA).
Antibodies against RNAPII-CTD-SER7 (#04-1570-I) were purchased from Millipore (Billerica, MA, USA). Antibodies against CDK7 (#2916T) and P21 (#2947P) were purchased from Cell Signaling Technology (Danvers, MA, USA).

**Chromatin immunoprecipitation and sequencing data analysis**

The ChIP assays were performed according to the manufacturer’s instructions (Millipore, Danvers, MA). In brief, U2-OS and SJSA-1 cells were cultured at 37 °C in 5% CO2, and were cross-linked with 1% formaldehyde at room temperature for 10 min followed by neutralization with glycine. Cells were resuspended, lysed in lysis buffer, and sonicated on ice to shear most DNA to 200-750 bp. Magnetic beads were bound with 10 µg of the indicated antibody (anti-H3K27ac, #ab4729, Abcam). For immunoprecipitation, sonicated chromatin solutions were incubated at 4 °C overnight with magnetic beads bound with antibody to enrich for DNA fragments bound by the indicated antibody. Beads were washed 3 times with sonication buffer, and 2 times with low stringency wash buffer. DNA was eluted in elution buffer. RNA and protein were digested using RNase A (#70856, Millipore, Danvers, MA, USA) and Proteinase K, respectively, and DNA was purified with phenol chloroform extraction and ethanol precipitation.

Illumina (San Diego, CA, USA) sequencing libraries were generated, and data were processed as described by Lin et al (5). In brief, libraries were generated for ChIP samples following the NEBNext® Ultra™DNA Library Prep Kit for Illumina protocol. The reads that passed the prefiltering step were aligned with Bowtie2 v2.2.5 software to the human genome (hg19). MACS2 was used to identify enriched regions.
as enhancers with a threshold Q-value < 0.1.

Super-enhancers were identified using the ROSE (Rank Ordering of Super-Enhancers) algorithm (https://bitbucket.org/young_computation/rose) as described previously (9, 13, 26). The union of H3K27ac peaks within 12.5 kb distance was stitched together; peaks within 2 kb from a RefSeq TSS were excluded. The stitched domains were ranked by H3K27ac signal, and super-enhancers were separated from typical enhancers by determining the point along the X-axis at which a line with a slope of 1 was tangent to the curve. Enhancers were then assigned to the transcript whose TSS was nearest the center of the enhancer.

Microarray sample preparation and analysis

Total RNA was extracted from U2-OS or SJSA-1 cells treated with DMSO (control) and different doses of THZ2, respectively, using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer’s instructions. An Affymetrix GeneChip® PrimeView™ Human Gene Expression Array was used for the microarray analysis. The hybridization data were analyzed using Affymetrix GeneChip Command Console Software (AGCC). Microarray data were normalized using the Robust Multiarray Average (RMA) method, and expression values were calculated with the Affy Suite of the Bioconductor Package (http://www.bioconductor.org), using quantile normalization of the Robust Multiarray Average method (each calculation performed at the individual probe level). Fold-changes were calculated by subtracting average log2 DMSO signal from average log2 THZ2 treatment signal. Active transcripts of each cell were defined as average log2(expression) > log2(100) in the corresponding
DMSO sample.

**Gene set enrichment analysis**

Gene Set Enrichment Analysis (GSEA) was performed using GSEA standalone desktop software (http://www.broadinstitute.org/gsea) to determine whether the set of super-enhancer-associated genes was sensitive to THZ2. All super-enhancer-associated genes of each cell line were used as gene sets, respectively, and the corresponding expression value of the sample treated with different concentrations of THZ2 were used as the expression data set.

**Cell transfection**

Lentiviral shRNAs and matching scrambled control constructs were purchased from Genecopoeia (Rockville, MD, USA). Four shRNA sequences were designed for each gene and cloned into an expression plasmid. The targeting sequences of the best shRNA (CDK7 shRNA #1: CATACAAGGCTTATTCTTA; #2: GGCTTATTCTTATTTAATCCA) were selected for further experiments. The stable cell lines were transfected with recombinant lentiviruses in the presence of 8 μg/ml polybrene according to the manufacturer's instructions, and then selected with puromycin. Puromycin (1 μg/ml) was used to treat cells for two days for selection, which eliminated all cells in an uninfected control group. Samples of protein were taken for Western blotting assays at one week after selection.

**Cell viability assay**

Osteosarcoma cells were plated in 96-well plates at a density of 2,000 cells/well. 1000 cells were plated per well for time-response assay. They were treated with
different concentrations of THZ2, or transfected with 100 nM targeting shRNA. After
the indicated times, 20 μl of MTT (5 mg/ml) was added to each well, and the plates
were incubated at 37 °C for 4 h. Then, the medium was replaced by 150 μl DMSO
and mixed thoroughly. The absorbance was determined at 490 nm using a microplate
reader. The assay was performed in triplicate.

**Clone formation assay**

Osteosarcoma cells or cells transfected with plasmids were plated in triplicate at
500 cells/well in 6-well plates with 2 ml DMEM containing 10% fetal bovine serum
(FBS). In the THZ2 experiment, plated cells were incubated without treatment, with
DMSO, or with THZ2 for 48 hours. Colonies containing > 50 cells were counted after
10 days by staining with crystal violet. Data were presented as mean ± standard
deviation (SD) from 3 independent experiments in triplicate wells.

**In vitro migration and invasion assays**

Cell migration and invasion assays were performed using Transwell cell culture
chambers with an 8-mm microporous filters that were pre-coated and pre-uncoated
with extracellular matrix coating (BD Biosciences, Palo Alto, CA, USA), respectively,
according to the manufacturer’s instructions. For the experiments, 200 μl of
osteosarcoma cell suspensions (5×10^4 cells/ml) in serum-free DMEM were seeded in
the upper chambers in 24-well plates and 500 μl of DMEM containing 10% FBS was
added to the bottom chamber. After 24 h, the cells in the upper chamber were
removed, and the cells on the lower surface of the filter were fixed, stained, and the
number in 5 random fields was counted under a light microscope. Data were
presented as mean ± SD from 3 independent experiments in triplicate chambers.

**Mouse xenograft**

Animal experiments were approved by the Institutional Review Board of the First Affiliated Hospital of Sun Yat-sen University, and were performed according to established guidelines for the Use and Care of Laboratory Animals. Female nude mice, 4 to 6 weeks old, were purchased from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China). After the mice were anesthetized with isoflurane, wildtype or plasmids transfected SJSA-1 cells were inserted to the proximal tibia through the cortex of the anterior tuberosity using a 30-gauge needle. In total, 20 µl of the cell suspension was slowly injected in the upper hole.

For study of THZ2, the mice were randomly separated into 2 groups after about 2 weeks when a tumor volume of approximately 200 mm$^3$ was reached. The mice were treated with 10% DMSO in D5W (5% dextrose in water) or 10 mg/kg THZ2 in vehicle twice daily by intraperitoneal injection. The mice were monitored every 3 days for a total of 3 weeks. As the tumors grew as almost spherical ellipsoids, the size of tumors was measured in 2 perpendicular dimensions (D1, D2). The body weight of the mice was also recorded. The tumor volume was calculated using the formula $V = \frac{4}{3} \pi \left[\frac{1}{4} (D1+D2)\right]^2$, as described previously (33). The mice were killed, and the lungs were harvested, fixed in formalin, and stained with hematoxylin and eosin (H&E). The number of the metastatic nodules in the lungs was counted.

**Western blotting**

Western blotting was performed as previously described (34). In brief, cells were
lysed in RIPA buffer containing protease inhibitor and phosphatase inhibitor cocktails (Roche), and the cell debris were removed by centrifugation (12,000 × g, 10 min). Equal amounts of protein were boiled for 10 min, resolved by SDS-PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). Membranes were blocked in 5% non-fat dry milk with TBST for 1 h at room temperature, and then incubated with primary antibody overnight at 4 °C. After washing 3 times with TBST, the membranes were incubated with corresponding secondary antibodies. Immunoreactive proteins were then visualized using ECL detection reagents (Millipore).

**RNA extraction and quantitative real-time PCR (RT-qPCR)**

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Reverse-transcription was performed using a PrimeScript™ RT reagent kit (Takara, Kusatsu, Japan). qPCR was performed using SYBR® Premix (Takara, Kusatsu, Japan) on a Real-Time PCR System (ABIviiATM7Dx). All reactions including the no RT and no template control were carried out in a 20 μl reaction volume and performed in triplicate. The protocol included denaturing for 30 s at 95°C, 40 cycles of 2-step PCR including denaturing for 5 s at 95°C and annealing for 34 s at 60°C, with an additional 15-s detection step at 95°C, followed by a melting profile from 60°C to 95°C at a rate of 0.5°C per 10 s. Primer sequences were provided by PrimerBank (http://pga.mgh.harvard.edu/primerbank/). The relative amount of target gene mRNA was normalized to that of GAPDH.
The reverse-transcription and qPCR of the microRNA was performed using a Mir-X™ miRNA First-Strand Synthesis kit (Takara, Kusatsu, Japan). The protocol included denaturing for 10 s at 95°C, 40 cycles of 2-step PCR including denaturing for 5 s at 95°C and annealing for 20 s at 60°C, with an additional 60-s detection step at 95°C, followed by a melting profile from 55°C to 95°C at a rate of 0.5°C per 10 s. MIR21 primer sequences were designed and provided by Generay Co. Ltd. (Guangzhou, China). The RT-qPCR primer sequences used are shown in Supplementary Table S3. Results of the validation of the specificity and efficiency of all primers are provided in Supplementary Figure S5.

**Patients and clinical database**

To determine the specific expressions of the studied genes in different tissues, 35 pairs of osteosarcoma and para-carcinoma tissues from 35 patients treated at the First Affiliated Hospital of Sun Yat-sen University, Guangzhou, between March 1, 2003, and December 31, 2018 were retrospectively examined. Histological type was confirmed prior to our experiments by pathologists from the Clinical Pathology Department of the hospital. This retrospective analysis of anonymous data was approved by the Institutional Review Board of the First Affiliated Hospital of Sun Yat-sen University and conducted in accordance with Declaration of Helsinki.

**Statistical analysis**

All data were derived from at least 3 independent experiments, and the results were expressed as mean ± standard deviation. Student’s t-test or one-way ANOVA was used to analyze differences between groups. A value of P < 0.05 was considered to indicate
a statistically significant difference. Statistical analyses were performed with SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA).

**Data availability**

ChIP-seq and gene expression microarray data are deposited in GEO: GSE134605.

**Results**

**Characterization of super-enhancers in osteosarcoma cells**

Super-enhancers in osteosarcoma cells were identified through chromatin immunoprecipitation sequencing (ChIP-Seq) against the H3K27ac modification (26). We identified 308 super-enhancer-associated genes in the U2-OS cell line, and 1,526 in the SJSA-1 cell line (Fig. 1A; Supplementary Table S1). Compared with typical enhancers based on the ChIP-Seq data set, super-enhancers were specifically enriched with the H3K27ac signal in distance and density (Fig. 1B). Interestingly, we observed a number of top-ranking super-enhancer-associated genes that have been proven to be associated with osteosarcoma oncogenic transcripts in previous studies, such as MYC (35), PIM1 (36), TCF7L2 (37), and HMOX1 (38). In addition to coding RNAs, these super-enhancers have been reported to drive the expression of long non-coding RNAs such as MALAT1 (39), and microRNAs such as MIR663B (40) and MIR21 (41), which have been reported to contribute to osteosarcoma malignancy. In addition, some lineage-specific transcription factors which regulate skeletal development, such as GLI2 (42), LIF (43), MDM2 (44), RUNX2 (45), and EGFR (46), were found to be associated with super-enhancers (Fig. 1A and C).
Gene ontology (GO) analysis was performed to explore the functional enrichment of the identified super-enhancer-associated genes. We found that many genes were significantly involved in (1) regulation of RNAPII and transcriptional factor-related transcription; (2) skeletal system and cell development; and (3) tumor-related functions including cell proliferation, apoptotic processes, and cell migration (Fig. 1D; Supplementary Table S1). The super-enhancers were also associated with genes enriched in skeletal diseases, cancers and related essential signaling pathways (Supplementary Fig. S1A and B; Supplementary Table S1). These results indicated that the super-enhancer-associated genes in osteosarcoma cells were hyperactive, and may promote the development and malignancy of osteosarcoma.

**CDK7 was upregulated in osteosarcoma and plays an oncogenic role in osteosarcoma cells**

Super-enhancers are able to drive high-level expression of associated transcripts, and are vulnerable to perturbation of transcriptional activity through bound transcription factors and coactivators (26). It has been reported that super-enhancer-associated genes encode gene products involved in regulation of RNAPII-mediated transcription, and many transcription factors are important for cancer states and might represent candidate oncogenes (11). CDK7 is a component of the general transcription factor IIH, which regulates RNAPII initiation and elongation (47). Compared with normal tissue, the expression of CDK7 was significantly higher in tumor tissues from 35 patients with osteosarcoma (Fig. 2A). We retrieved the expression data of CDK7 from the GEO database (GSE36001), and found that the
expression of CDK7 in osteosarcoma tissues is higher than in normal bone (Fig. 2B).

To explore the role of CDK7 in osteosarcoma, we depleted CDK7 expression in U2-OS and SJSA-1 cells by shRNA-mediated knockdown. Decreased phosphorylation of initiation-associated serine 5 (S5) and serine 7 (S7), and of elongation-associated serine 2 (S2) of the RNAII CTD, which are regulated by CDK7, were observed in both cell lines (Fig. 2C). The MTT, colony formation, and Transwell assays demonstrated a significant decrease in osteosarcoma cell growth, migration, and invasion, respectively, after knockdown of CDK7 (Fig. 2D-F).

To further confirm the anti-tumor potential of downregulating CDK7, we establish an osteosarcoma orthotropic animal model by injecting stable knockdown SJSA-1 cells or control in nude mice. The results indicated that tumor volume and weight were dramatically decreased in the CDK7 knockdown groups compared with the control group (Fig. 2G and H; Supplementary Fig. S2A). In addition, osteosarcoma xenograft metastasis to the lungs was also decreased after CDK7 knockdown (Supplementary Fig. S2B).

These observations indicated that suppressing the phosphorylation of RNAPII CTD by targeting CDK7 has promising anti-osteosarcoma effects.

*Selective suppression of super-enhancer associated genes by the specific CDK7 inhibitor, THZ2*

Based on the biological process enrichments of super-enhancer-associated genes and the experimental results thus far, we hypothesized that suppressing CDK7 activity could inhibit the malignant properties of osteosarcoma through targeting the
super-enhancer-associated genes involved in osteosarcoma pathogenesis. In order to explore the correlations between CDK7 and super-enhancer-associated genes, and find an innovative and effective drug against osteosarcoma, we used THZ2, a new CDK7 inhibitor (10, 12), to suppress the CDK7 activity of osteosarcoma cells. A dose-dependent decrease in phosphorylation at S2, S5, and S7 with increasing THZ2 concentration was observed in U2-OS and SJSA-1 cell lines (Fig. 3A). Gene expression profiling was then performed to investigate THZ2-induced transcription alterations, and identify the subsets of sensitive genes in these 2 osteosarcoma cell lines. Cells were treated with 25, 100, and 400 nM THZ2 for 6 hours (Fig. 3B; Supplementary Table S2). We observed there were a small number of sensitive genes that began to be down-regulated at a concentration of 100 nM in both cell lines (Fig. 3C). GO analysis showed many subsets of the sensitive genes were involved in transcriptional regulation, cell cycle regulation, apoptotic processes, and other critical cellular functions with respect to osteosarcoma (Fig. 3D). The sensitive genes identified are also involved in skeletal diseases, musculoskeletal diseases, cancers and a number pathways in various cancers, similar to that of the super-enhancer-associated genes we identified (Supplementary Fig. S3A and B).

We sought to explore whether super-enhancer-associated genes, including oncogenes, are disproportionately suppressed by CDK7 inhibition. We observed that an abundance of super-enhancer-associated transcripts were preferentially down-regulated upon THZ2 treatment as compared with that of typical-enhancers (Fig. 3E). GSEA of all active transcripts after treatment with 100 nM THZ2 showed that
these THZ2-sensitive genes were significantly enriched in the gene sets associated with super-enhancers (Fig. 3F). Notably, many super-enhancer-associated genes which have been reported to be involved in the malignant potential of osteosarcoma, such as MYC (35), RUNX2 (45), MDM2 (44), SRSF3 (48), and TCF7L2 (49), were among the top 10% sensitive to THZ2 treatment (Fig. 3G). The downregulation of these critical osteosarcoma-related genes was confirmed on the RNA level by quantitative PCR (Fig. 3H).

These results suggested that super-enhancer-associated genes, especially osteosarcoma-related genes, were particularly vulnerable to inhibition of CDK7, and THZ2 might be a potential anti-osteosarcoma agent through selective targeting super-enhancer-associated oncogenes.

**THZ2 suppresses proliferation and metastatic potential in osteosarcoma cells**

To investigate the effect of THZ2 on osteosarcoma cells, dose response experiments showed that all 10 osteosarcoma cell lines were highly sensitive to THZ2, with IC50 values in the range of 57.6 to 719.4 nM, which is much lower than that of a human osteoblast cell line (hFOB1.19) of 2210.4 nM (Fig. 4A). At concentrations as low as 25, 50 or 100nM, THZ2 potently reduced cell viability of U2-OS and SJSA-1 in a time-dependent manner, but not hFOB 1.19 (Fig. 4B). To further corroborate the anti-proliferative effect of THZ2, a colony-formation assay was performed which showed that THZ2 decreased colony formation from transplanted osteosarcoma cells (Fig. 4C).

Metastasis and invasion are also key processes during tumor development and
progression. Experiments using the osteosarcoma cell lines U2-OS, SJSA-1, 143B, and MG-63, showed that THZ2 reduced the migratory and invasive properties of osteosarcoma cells (Fig. 4D). These results demonstrated that THZ2 has powerful inhibitory effects on the growth, migration and invasion of osteosarcoma in vitro.

**THZ2 inhibits cell-cycle progression and induce apoptosis in osteosarcoma cells**

To examine whether THZ2 affects cell-cycle distribution, human osteosarcoma cell lines were treated with THZ2 (100 nM for 24 h), followed by flow cytometry analysis. G2-M phase arrest was observed in U2-OS, SJSA-1, 143B, and MG-63 cells (Fig. 5A). The percentage of U2OS, SJSA-1, 143B, and MG-63 cells in G2-M increased by 10.2%, 20.6%, 8.1%, and 20.1%, respectively, and the percentage of cells in G0-G1 decreased by 12.7%, 9.1%, 6.9%, and 12.0%, respectively (Fig. 5A).

Immunoblotting was performed to determine the effects of THZ2 on the expression of CyclinD1, which is related to G0-G1 to S-phase transition, and expression of P21, which is a potent inhibitor of cell-cycle progression. The results showed that treatment of U2-OS cells with THZ2 resulted in a reduction of Cyclin D1 expression and an increase of P21 expression in a dose-dependent and time-dependent manner (Fig. 5C and D). Annexin V/PI staining showed the percentage of apoptotic cells increased markedly in all tested cell lines after THZ2 treatment (Fig. 5B). The number of apoptotic cells in the U2-OS, SJSA-1, 143B and MG-63 cell lines increased by 18.1%, 24.3%, 12.2% and 3.7% with a THZ2 concentration of 100 nM (Fig. 5B).

As a marker of apoptosis, total and cleaved PARP was detected by Western blotting and the results showed that protein expression was increased after exposure to THZ2.
Taken together, these results indicated that THZ2 can affect cell-cycle progression and induce apoptosis.

**THZ2 exhibits a powerful antitumor effect against osteosarcoma xenografts in nude mice**

On the basis of *in vitro* data, we explored the anti-osteosarcoma activity of THZ2 using an osteosarcoma orthotopic animal model. Mice bearing SJSA-1 cells were randomly separated into 2 groups (vehicle and THZ2), and received either vehicle or THZ2 (10 mg/kg) twice daily. The tumor growth curves showed a significant anti-tumor effect of THZ2, as compared with the control (Fig. 6A).

Tumor-bearing mice were killed on day 21. Examination revealed tumor swelling in the lower legs. Radiographic features characteristic of human primary osteosarcoma, including central osteolysis with specular new bone formation and extension into surrounding soft tissues, were more apparent in the vehicle group compared with the THZ2 treatment group (Fig. 6B and C). A lower tumor weight was observed in the THZ2 group (Fig. 6D).

Immunohistochemistry (IHC) and TUNEL assay examination of tumor samples showed that THZ2 dramatically inhibited cell proliferation and promoted cell apoptosis (Fig. 6E). In addition, the number of observable lung metastatic nodules was lower in the THZ2 treatment group (Fig. 6F and G). Importantly, no significant difference in mouse body weight was observed between the vehicle group and the THZ2 group (Fig. 6H), and no obvious pathological changes were observed in the heart, kidney, liver, lung, and spleen in the THZ2 group as determined by H&E.
staining (Supplementary Fig. S4). Collectively, these results revealed that THZ2 possesses potent anti-tumor properties against human osteosarcoma cells in vivo.

Discussion

Despite advances in the diagnosis and treatment of osteosarcoma, the prognosis remains poor (50, 51). The effectiveness of standard chemotherapy is hampered by the development of chemoresistance in a large portion of patients (4). A limited understanding of the pathogenesis of osteosarcoma has impeded improvement in the outcomes of patients in the last 4 decades.

Super-enhancers, large clusters of enhancers that are enriched in size and binding of the mediator complex, have been characterized in various cancer cells and have been reported to promote the expression of genes that define cell identity (9, 14, 52). Recent studies have suggested that a number of different cancer cell types generate super-enhancers at oncogenes, and the acquisition of specific super-enhancers near oncogenes contributes to tumorigenesis (11, 13, 26). However, there have been no studies examining super-enhancers in osteosarcoma. In the present study, we characterized the super-enhancer landscape of osteosarcoma for the first time and identified the associated transcripts. We found that super-enhancer-associated transcripts are important for the regulation of RNAII-mediated transcriptional activity, as well as cellular processes representing ‘cancer hallmarks’ (53) and lineage-specific processes, such as skeletal system development. A number of osteosarcoma oncogenes, including both coding and non-coding RNAs, such as GLI2 (54), TCF7L2 (37), MYC (35), MDM2 (55), MALAT1 (39) and MIR21 (41) are associated with
super-enhancers. Based on our findings, we speculate that super-enhancer-associated genes play an important role in the malignancy and cell identity of osteosarcoma.

Super-enhancers are occupied by a large portion of the RNAPII, cofactors and chromatin regulators, which can explain how they contribute to high-level transcription of associated genes (13). Recent studies have found that super-enhancers are sensitive to perturbations of transcriptional activity through certain bound transcription factors and coactivators, as well as associated genes (9, 26). CDK7 is a cyclin-dependent kinase and a subunit of the multi-protein basal transcription factor TFIIH which regulates transcription initiation and elongation by phosphorylating the CTD of the largest subunit (RPB1) of RNAPII (56). Based on the high expression of CDK7 in human osteosarcoma tissues, and suppression of the malignant properties of osteosarcoma cells after CDK7 knockdown, we reasoned that targeting CDK7 might be a potential treatment strategy for osteosarcoma through suppressing super-enhancer-associated oncogenes.

THZ1 is a selective CDK7 inhibitor that covalently binds to CDK7 and suppresses its kinase activity, and has a very high level of selectivity because of modification of a unique cysteine residue (29). Although recent studies have showed that THZ1 has the potential of promising anti-cancer activity in various malignancies, its translational significance and application are limited to the short half-life (30). THZ2 is an newly developed analog of THZ1 with a 5-fold increase of half-life, and has been reported to suppress the growth of triple-negative breast cancer and gastric cancer (30, 31). However, the effect of THZ2 in other cancers, including osteosarcoma, is still
unknown. In order to identify a potential drug against osteosarcoma and confirm the relations between the activity of CDK7 and the expression of super-enhancer-associated genes in osteosarcoma, we used THZ2 in our investigations.

Microarray expression analysis was used to detected alterations of total transcripts in osteosarcoma cells treated with various concentrations THZ2. We observed that a cluster of genes was particularly sensitive to THZ2 treatment, and was also enriched in cancer-related biological processes and pathways, such as apoptosis, cell migration, PI3K-Akt signaling, and MAPK signaling, etc. Notably, they were similarly to the processes in which super-enhancer-associated genes are involved. Based on these interesting results, we explored the relation between the THZ2-sensitive genes and super-enhancers we identified, and found that THZ2 could selectively suppress super-enhancer-associated genes. Notably, a large number of well-defined oncogenes associated with identified super-enhancers were found to be present in the subset most sensitive to THZ2. These results indicated that oncogenes associated with super-enhancers in osteosarcoma are particularly vulnerable to THZ2.

Moreover, we identified some seldom reported transcripts in osteosarcoma associated with super-enhancers that were also sensitive to THZ2, such as CEP55 and LACTB (Supplementary Table S1 and S2). The identification and analysis of super-enhancer-associated genes in our study provides an important molecular foundation to understand the pathogenesis of osteosarcoma, and a catalogue of genes which may be valuable for further research.
To explore the anti-osteosarcoma potential of THZ2, we examined changes of malignant features \textit{in vitro} and \textit{in vivo} after THZ2 treatment. Our results showed that THZ2 suppressed the proliferation of osteosarcoma cells in time- and dose-dependent manners, and exhibited less cytotoxicity in the human osteoblast cell line hFOB1.19. Osteosarcoma has a strong tendency to metastasize, and pulmonary metastasis is the main cause of medical failure and death in osteosarcoma patients. The effect of THZ2 on the metastatic potential of osteosarcoma cells was examined using the Transwell assay. The results showed that THZ2 markedly suppressed the migration and invasion ability of osteosarcoma cells. In addition, we also found that THZ2 induced G2-M cell-cycle arrest and apoptotic cell death in osteosarcoma cells. Furthermore, an orthotopic murine model was established to assess the anti-tumor potential of THZ2 \textit{in vivo}. THZ2 potently suppressed tumor growth in nude mice, and fewer lung metastatic nodules were found in mice treated with THZ2 as compared to those not treated. Notably, no severe side effects or pathological changes in important organs were observed in mice treated with THZ2. Taken together, our results indicate that THZ2 has a powerful anti-tumor activity against osteosarcoma \textit{in vitro} and \textit{in vivo}, and may have clinical value for the treatment of patients with osteosarcoma.

In summary, our results showed significant correlations between super-enhancers and the malignant potential of osteosarcoma, and indicated that targeting super-enhancer-associated genes with a CDK7 inhibitors such as THZ2 may be a promising treatment for osteosarcoma patients. Moreover, our data provides an important molecular foundation towards understanding the pathogenesis of osteosarcoma.
osteosarcoma modified by epigenetic patterns.

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**Reference**


14. Niederriter AR, Varshney A, Parker SC, Martin DM. Super Enhancers in Cancers,


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Figure 1. Super-enhancers in osteosarcoma cells are associated with oncogenic and lineage-specific genes.

(A) Enhancers ranked by increasing H3K27Ac ChIP-seq signal (length×density, input normalized) in their stitched regions. (B) The mean density of the H3K27ac signal in typical enhancers and super-enhancers of the osteosarcoma cell lines. (C) ChIP-seq binding profiles at representative super-enhancer-associated gene loci in both cell lines. (D) Selected gene ontology (GO) functional categories of the osteosarcoma super-enhancer-associated genes.

Figure 2. CDK7 inhibition suppressed growth of osteosarcoma in vitro and in vivo

(A) Comparative quantification of CDK7 mRNA in paired primary osteosarcoma tissues and no-tumor tissues from 35 patients. Data represent mean ± SD. (B) CDK7 mRNA expression of normal bone and osteosarcoma tissues from data sets GSE36001 downloaded from Gene Expression Omnibus (GEO). Data represent mean ± SD. (C) Immunoblotting analyses of RNAPII, RNAPII CTD phosphorylation (S2, S5 and S7), and CDK7 in U2-OS and SJSA-1 cells stably transfected with shRNA targeting CDK7. (D) MTT assay and (E) colony formation assay were used to evaluate the cell growth of U2-OS and SJSA-1. The suppression of CDK7 dramatically inhibit the growth of osteosarcoma cells. Data represent mean ± SD. (F) The suppression of CDK7 significantly reduced the migration/invasion ability of the osteosarcoma cells by Transwell assay. Data represent mean ± SD. (G) Tumor growth curves of mice in which CDK7 knockdown and control-SJSA-1 cells implanted in the proximal tibia.
Data represent mean ± SD. (H) Photographs of resected tumors from control and knockdown groups excised on day 35. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 3. Super-enhancer-associated genes in osteosarcoma cells are particularly sensitive to THZ2 treatment.

(A) Immunoblotting analyses of RNAPII, RNAPII CTD phosphorylation (S2, S5 and S7), and CDK7 in U2-OS and SJSA-1 cells treated either with THZ2 or DMSO at the indicated concentrations for 6 hours. (B) Heatmap showing the change of global active transcripts in U2-OS and SJSA-1 cells following treatment with 25, 100, and 400 nM THZ2 for 6 hours. (C) Scatter plot displaying the log2-fold change of all active genes altered by 100 nM THZ2. (D) Enriched gene ontology (GO) functional categories of transcripts were reduced over 2-fold in U2-OS and SJSA-1 cells following treatment with 100nM THZ2 for 6 hours. (E) Box plots showing log2-fold changes of transcripts associated with the total pool of all enhancers (ALL), typical-enhancers (TE), and super-enhancers (SE) upon treatment with 100 nM THZ2 for 6 hours. Data represent mean ± SD. (F) Gene Set Enrichment analysis (GSEA) showing the super-enhancer-associated transcript signature enriched in 100 nM THZ2-treated cells versus DMSO-treated cells. (G) Venn diagram showing overlap between super-enhancer-associated genes identified with ChIP-seq, and ranking among the top 10% of THZ2-sensitive active transcripts in 2 osteosarcoma cell lines respectively. The genes had been reported involved in malignant properties of osteosarcoma were listed. (H) Quantitative PCR to detect expression of indicated gene
transcripts in DMSO-treated and 100 nM THZ2-treated U2-OS and SJSA-1 cells respectively. Data represent mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 4. THZ2 impedes the proliferation and metastasis of osteosarcoma in vivo.

(A) Dose-response curves of 10 osteosarcoma cell lines and 1 osteoblast cell line (hFOB1.19) after treatment with THZ2 for 48 hours. Cell viability was assessed with the MTT assay. IC50 data are presented as mean with 95% confidence interval. (B) Time-response curves of U2-OS, SJSA-1, and hFOB 1.19 upon treatment with THZ2 at concentrations as low as 25, 50 and 100 nM. Data represent mean ± SD. (C) THZ2 impaired colony formation of osteosarcoma cells. The colony formation ability of osteosarcoma cells (U2-OS, SJSA-1, 143B, and MG-63) was examined after treatment with various concentrations THZ2 for 10 days. The quantification of cell growth in the right panel is presented as mean ± standard deviation (SD). (D) THZ2 inhibits the migration and invasion ability of osteosarcoma cells. The indicated cells were treated with THZ2 (100 nM) or vehicle. Data represent mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 5. The effect of THZ2 on cell-cycle progression and apoptosis in osteosarcoma cells.

(A) Flow cytometry were used to detected and analyze cell-cycle distribution of osteosarcoma cells treated with vehicle or 100 nM THZ2 for 24 hours. The length of each cell-cycle phase was calculated. Data represent mean ± SD. (B) Annexin V/PI
staining assay were used to detect the apoptosis rate of osteosarcoma cells treated with vehicle or 100 nM THZ2 for 24 hours. The proportion of apoptotic cells was calculated. Data represent mean ± SD. (C-D) Western blot analyses were performed using the indicated antibodies, including Total/cleaved PARP, P21, and CyclinD1. THZ2 affects the protein levels of the apoptosis marker cleaved PARP and cell cycle-related genes P21 and CyclinD1 in dose/time-dependent manner. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure 6. THZ2 suppress the growth and lung metastatic potential in orthotopic mice model.

(A) Tumor growth curves of mice treated with either vehicle or THZ2 for 21 days (twice daily, 10 mg/kg, i.p.). Data represent mean ± standard deviation SD. (B) The mice bearing induced tibial osteosarcoma upon vehicle (upper) or THZ2 (lower) treatment are shown in the RGB images and the X-ray images of the whole body. (C) Photographs and (D) weights of resected tumors from both vehicle and THZ2 treatment groups. Data represent mean ± SD. (E) H&E and immunohistochemical (IHC) staining of Ki67, and TdT-mediated DUTP nick end labeling (TUNEL) assay in tumor tissue sections. Each image was taken at a magnification of 400×. (F) Representative images of lung sections after H&E staining. Each image was taken at a magnification of 40× and 100×. (G) Statistical results for the number of visible lung metastatic nodules. Data represent mean ± SD. (H) The body weight of mice over the treatment time. Data represent the mean ± SD. ns=not significant. *p < 0.05, **p <
0.01, ***p < 0.001.
Figure 1
Figure 2
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Figure 4
Figure 5
Figure 6
Targeting super-enhancer-associated oncogenes in osteosarcoma with THZ2, a covalent CDK7 inhibitor

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