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Original Article

Long non-coding RNA AK093407 promotes proliferation and inhibits apoptosis of human osteosarcoma cells via STAT3 activation

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Abstract: Osteosarcoma is a malignant tumor of the skeletal system. Long non-coding RNAs (lncRNAs) have been shown to play significant role in osteosarcoma. The present study evaluated the effects and mechanism of lncRNA AK093407 in osteosarcoma. The study included human osteosarcoma cell line, U-2OS. Cell proliferation, viability, and apoptosis were measured using Ki-67 proliferation assay, MTT assay, and Annexin V/PI staining assay, respectively. Relative mRNA and protein expressions were measured using qRT-PCR and western blot, respectively. Interaction between AK093407 and STAT3 was identified using mass spectrometry and RNA pull-down assay. Results revealed that AK093407 was highly expressed in osteosarcoma cells and tissues. Then we demonstrated that overexpression of AK093407 promoted cell proliferation and viability and inhibited apoptosis, whereas suppression of AK093407 showed opposite effects. In addition, AK093407 regulated the expression of genes and proteins (Bcl-2, TGF-β, NF-κB, and PCNA) involved in the cell proliferation, viability, and apoptosis. Furthermore, we showed that AK093407 interacted with STAT3, and promoted its phosphorylation. Lastly, we showed that STAT3 activation was essential for the effects of AK093407 on cell proliferation and apoptosis as the overexpression of AK093407 in the presence of STAT3 inhibitor did not promote cell proliferation and inhibit cell apoptosis. AK093407 is highly expressed in osteosarcoma cells and tissues, and promotes cell proliferation and viability and inhibits apoptosis of osteosarcoma cell line U-2OS via STAT3 activation.

Keywords: lncRNA, AK093407, osteosarcoma, STAT3, cell proliferation, apoptosis

Introduction

Osteosarcoma is a malignant tumor of the skeleton that is characterized by the formation of immature bone or osteoid tissue. Osteosarcoma is the most destructive primary bone tumor observed in children and adolescents [1]. Primary osteosarcoma commonly occurs in the long bones of extremities [2]. The main diagnostic characteristic of osteosarcoma is the detection of osteoid matrix produced by the neoplastic cells [3]. Studies suggested that osteosarcoma may originate from either mesenchymal stem cell-derived osteogenic progenitors or from undifferentiated mesenchymal stem cells under the influence of microenvironmental or epigenetic signals [4]. Osteosarcoma has complex etiology, characterized by highly abnormal karyotypes, vast genomic instability, and multiple genomic aberrations [5, 6]. Standard treatment of osteosarcoma includes intravenous chemotherapy administered before and after surgery. If complete surgical resection is not achieved, radiation therapy is given. This standard treatment generally achieves a 5-year survival rate in 60%-70% of patients. However, for patients with locally advanced or metastatic tumors, survival rate is below ~20% and median survival time is only 23 months [7]. Furthermore, constitutive and acquired resistance to these treatments poses a great challenge to the clinicians. Although recent research in molecular biology has provided some idea about the molecular pathogenesis of osteosarcoma [8], the exact molecular mechanisms underlying metastasis, drug resistance, and the histological heterogeneity still remains unclear. Therefore, it is necessary to develop novel tar-

gets for the diagnosis, treatment, and prognosis of osteosarcoma.

Long non-coding RNAs (lncRNAs) are a diverse class of non-protein-coding transcripts longer than 200 nucleotides [9, 10]. Recently, lncRNAs have gained attention in both academia and pharma companies, most probably due to its important roles in various biological regulatory processes. It is reported that lncRNAs are involved in a range of biological processes and regulatory mechanism. The biological processes regulated by lncRNAs include DNA damage, angiogenesis, microRNA silencing, invasion and metastasis, programmed cell death, and hormone-driven diseases [11]. In addition, lncRNAs can also regulate embryonic development, inflammation, immune cell development and tumor development [12-14]. LncRNAs are involved in several regulatory mechanisms, such as cell cycle control, gene imprinting, mRNA degradation, splicing regulation, chromatin remodeling, translocational regulation, and epigenetic regulation [11].

In cancer, many lncRNAs act as regulator of tumor suppressive and oncogenic functions [15]. Interestingly, most lncRNAs are transcribed by RNA polymerase II enzymes that are expressed in specific tissues and/or during specific development stage; indicating that lncRNAs are expressed in tissue specific manner and show aberrant expression in cancers [11]. Therefore, targeting and altering expressions of specific lncRNAs in malignancies may not have harmful side effects on normal cells. Thus, drug developers have started focusing on these novel and possibly personalized treatment options to develop nucleic acid/biological drugs.

Based on the analysis of microarray data, we found that the lncRNA AK093407 was highly expressed in osteosarcoma. In the present study, we further investigated the effects and mechanism of AK093407 in osteosarcoma. We demonstrated that AK093407 can promote cell proliferation and inhibit apoptosis of the human osteosarcoma cells U-2OS by regulation of signal transducers and activators of transcription 3 (STAT3). This study identified a novel lncRNA that functions in osteosarcoma, providing a new option for the diagnosis, prediction, and treatment of osteosarcoma.

Materials and methods

Patients

A total of 31 patients who were diagnosed as osteosarcoma in our hospital were enrolled in this study. Tumor tissues and their adjacent normal tissues were collected during surgery. The pathological diagnose for tumor tissues were confirmed according to the method described in previous study (Osteosarcoma: Pathology and classification, 1989). All the patients were informed with consent according to the requirements of our hospital.

Cell culture

The human osteosarcoma cell line U-2OS was obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37°C. Cells were subcultured at 80% to 90% confluency. Cells were subjected to no more than 20 cell passages.

Cell transfection

For suppressing AK093407, cells were transfected using pRS-siAK093407 (Shanghai GeneChem Company) and then selected with puromycin (1.5 μg/mL) for 2 weeks. For overexpressing AK093407, cells were transfected using pcDNA3-AK093407 (Invitrogen). The cells transfected with pRS-si-NC (negative control) and pcDNA3-NC, and cells without treatment were used as the control. Cell transfections were conducted using Lipofectamine 3000 reagent (Invitrogen) following the manufacturer's protocol.

Cell proliferation and viability assay

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyl-2Htetrazolium bromide (MTT) colorimetric assay according to standard methods described before [16]. Each experiment was performed three times.

Apoptosis assay

Apoptosis analysis was performed to identify and quantify the apoptotic cells using Annexin

V-FITC/PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China). Cells (1,00,000 cells/well) were seeded in a 6-well plate. The treated cells were washed twice with cold phosphate buffered saline and resuspended in buffer. The adherent and floating cells were combined and treated according to the manufacturer's instruction and measured with a flow cytometer (Beckman Coulter, USA) to differentiate apoptotic cells (Annexin-V positive and PI-negative) from necrotic cells (Annexin-V and PI-positive).

ChIP assay

Chromation IP (ChIP) assays were performed following standard protocol (Upstate Biotechnology, Inc.). Cells were treated with 1% formaldehyde for 10 min, cracked with sodium dodecyl sulfate (SDS) lysis buffer followed by ultrasonication, then incubated with proper antibodies (anti-STAT3). After washing by high salt, low salt and LiCl buffer, the elution buffer was used to harvest the chromatin fragments. Finally, the de-crosslinking was performed and enrichment was examined using real-time polymerase chain reaction (PCR).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from transfected cells using TRIzol reagent (Invitrogen) and treated with DNaseI (Promega). Reverse transcription was performed using Multiscribe RTkit (Applied Biosystems) and random hexamers or oligo (dT). The reverse transcription conditions were 10 min at 25°C, 30 min at 48°C, and a final step of 5 min at 95°C.

Western blot analysis

The proteins used for western blotting were extracted using radioimmunoassay (RIA) lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhibitors (Roche, Guangzhou, China). The proteins were quantified using BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). The western blot system was established using Bio-Rad Bis-Tris Gel system according to the manufacturer's instructions. Primary antibodies were prepared in 5% blocking buffer at a dilution of 1:1000. Primary antibody was incubated with the membrane at 4°C overnight, followed by wash and incubation with secondary antibody marked by horseradish peroxidase for 1 hour at room temperature. After rinsing, the polyvinylidene difluoride membrane-carried blots and antibodies were transferred into Bio-Rad ChemiDoc™ XRS system, and then 200 μl Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) was added to cover the membrane surface. The signals were captured and the intensity of the bands was quantified using Image Lab™ Software (Bio-Rad, Shanghai, China).

Northern blot

Total RNA was extracted from cells according to TRIZOL methods (Invitrogen) for northern blot. LncRNA and 18S fragments were cloned into PCDNA3 plasmid and northern probes were produced using Biotin RNA Labeling Mix (catalogue 11685597910, Roche). T7 RNA polymerase (catalogue 10881767001, Roche) was used for in vitro transcription. For northern blot, the samples were separated by electrophoresis using formaldehyde gel, followed by membrane transferring. The membranes were incubated with hydration buffer supplemented with proper amount of probes, and then the nucleic acid signal was detected using Chemiluminescent Nucleic Acid Detection Module (catalogue 89880, Thermo Scientific).

RNA pull-down

Biotin-labeled AK093407 was obtained using Biotin RNA Labeling Mix by T7 RNA polymerase, and then incubated with 1% formaldehyde-treated cell lysate for 4 h or overnight. Then, beads (Streptavidin, immobilized on Agarose CL-4B, for affinity chromatography, code 85881-1ML, Sigma-Aldrich) were incubated for 4 hours. After 3 times of washing with the beads, the cell lysate was added to the 5 × SDS-loading buffer, and the samples were centrifuged for 5 min at 12000 rpm. The samples were then analyzed using western blot.

RIP assay

For RNA immunoprecipitation (RIP), cells were treated with 1% formaldehyde and then crashed with RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP_40, 1 mM EDTA and 50 mM Tris pH 8.0) supplemented with RNase inhibitors and proteinase inhibitors for 30 min, followed by centrifugation. The super-

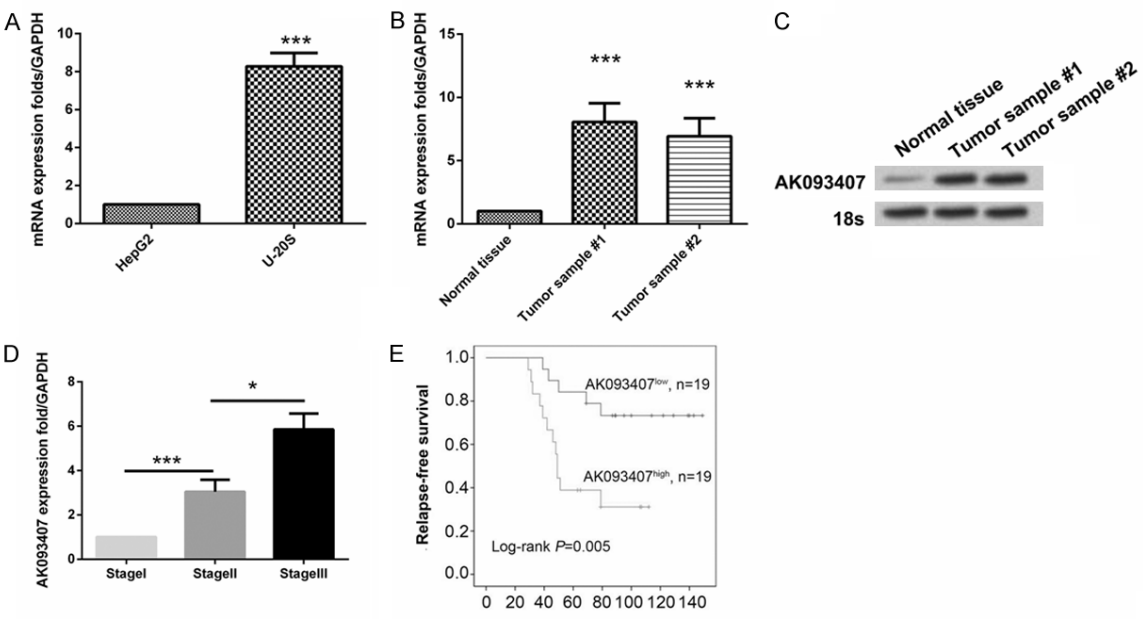

Figure 1. AK093407 is highly expressed in osteosarcoma cells and tissues. A. The relative mRNA expression of AK093407 in U-2OS cells and control cells (HepG2-liver hepatocellular cells) was measured using qRT-PCR. B, C. The relative mRNA expression and protein expression of AK093407 in normal tissue and osteosarcoma tumor samples was measured using qRT-PCR and western blot, respectively. D. The relative mRNA expression of AK093407 at cancer stages I, II, and III in tumor samples was measured using qRT-PCR. E. Kaplan-Meier survival curves of patients with osteosarcoma based on HULC expression levels. *P<0.05, ***P<0.005. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR: quantitative reverse transcription polymerase chain reaction.

natants were incubated with the indicated antibodies for 4 h, and then protein A/G beads were added. The precipitates were washed with RIPA buffer followed by de-crosslinking. Finally RNA was extracted and AK093407 enrichment was examined using real-time PCR.

Statistical analysis

All experiments were repeated three times. The results of multiple experiments are presented as mean ± SD. Statistical analyses were performed using SPSS 19.0 statistical software. The *P* values were calculated using one-way analysis of variance. *P* value of <0.05 was considered to indicate a statistically significant result.

Result

LncRNA AK093407 is highly expressed in osteosarcoma cells and tissues

The relative mRNA expression of AK093407 in U-2OS cells and control cells (HepG2-liver hepatocellular cells) was measured using qRT-PCR. The results revealed that the relative mRNA expression of AK093407 was significantly higher in U-2OS cells than in the control cells (P<0.005; **Figure 1A**). Then we determined the mRNA expression of AK093407 in normal tissue and osteosarcoma tumor samples. qRT-PCR results showed that the relative mRNA expression of AK093407 was significantly higher in the osteosarcoma tumors than in the normal tissue (P<0.005; **Figure 1B**). We observed similar results using northern blot (**Figure 1C**).

We then analyzed the expression of AK093407 at different cancer stages in tumor samples using qRT-PCR (**Figure 1D**). The results showed that the mRNA expression of AK093407 was highest in the Stage II tumor samples as compared to Stage I (P<0.005) and Stage III (P<0.05) tumor samples. Finally, we analyzed the impact of AK093407 expression on disease outcomes in osteosarcoma patients (n=38) using Kaplan-Meier survival analysis. The results showed that the relapse-free survival rate was significantly lower in the patients with high expression of AK093407 than in the patients with low expression (P<0.005; **Figure 1E**). Altogether, AK093407 was highly expressed in osteosarcoma cells and tissues.

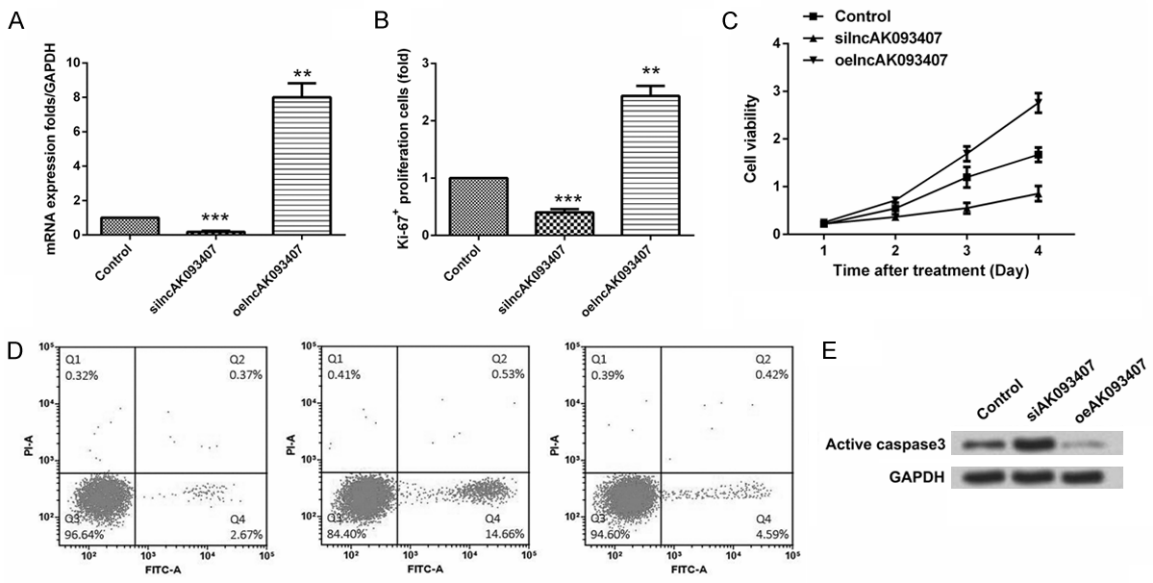

Figure 2. AK093407 promotes proliferation and inhibits apoptosis of osteosarcoma cells. For measurement of cell proliferation, viability, and apoptosis, U-2OS cells were transfected with siIncAK093407 or oeIncAK093407; un-transfected cells were used as control. A. The mRNA expression level in the transfected U-2OS cells and un-transfected cells was measured using qRT-PCR. B. Cell proliferation was measured using Ki-67 protein test; FACS detected the Ki-67 positive cells. C. MTT assay was used to measure viability of cells, transfected for 2, 3 and 4 days. D. Annexin V/PI assay was used measure apoptosis of the transfected U-2OS cells and un-transfected cells. E. Western blot measured the protein expression of pro-apoptotic protein caspase-3. **P<0.01, ***P<0.005. FACS: fluorescence-activated cell sorting; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; PI: propidium iodide; qRT-PCR: quantitative reverse transcription polymerase chain reaction.

AK093407 promotes proliferation and inhibits apoptosis of osteosarcoma cells

Cell proliferation and viability were compared between U-2OS cells that were stably transfected by siIncAK093407 or oeIncAK093407 and those not transfected. SiIncAK093407 was used to decrease the expression of AK093407 and oeIncAK093407 was used to increase the expression of AK093407, as shown in **Figure 2A**. The fluorescence-activated cell sorting (FACS) analysis was used to detect cell proliferation and demonstrated that Ki-67 positive cells were approximately 1.5-fold higher during overexpression of AK093407 (P<0.01) and ~0.5-fold lower during suppression of AK093407 (P<0.005) as compared to the control cells (**Figure 2B**). These findings indicated that AK093407 promoted proliferation of osteosarcoma cells, as the expression of Ki-67 protein is associated with cell proliferation. MTT assay was used to measure cell viability. As shown in **Figure 2C**, after U-2OS cells were cultured for 2, 3 and 4 days, overexpression of AK093407 marginally increased cell viability as compared to the control group on Day 4, while suppression of AK093407 expression showed opposite effects. These results suggested that AK093407 could promote proliferation and viability of osteosarcoma cells.

We then examined the effect of AK093407 on apoptosis in U-2OS cells using Annexin V/PI apoptosis assay and western blot. Cell apoptosis was compared between U-2OS cells transfected by siIncAK093407 or oeIncAK093407 and those not transfected. The Annexin V/PI assay detected that the overexpression of AK093407 inhibited apoptosis and suppression of AK093407 promoted apoptosis (**Figure 2D**). Western blot analysis showed that overexpression of AK093407 decreased the expression of pro-apoptotic protein (caspase-3) and suppression of AK093407 increased the expression of this protein (**Figure 2E**). These results indicated that AK093407 inhibited apoptosis in osteosarcoma cells.

AK093407 regulates the expression of genes and proteins involved in cell proliferation and apoptosis

We then proceeded to investigate the effects of AK093407 on the expression of target genes

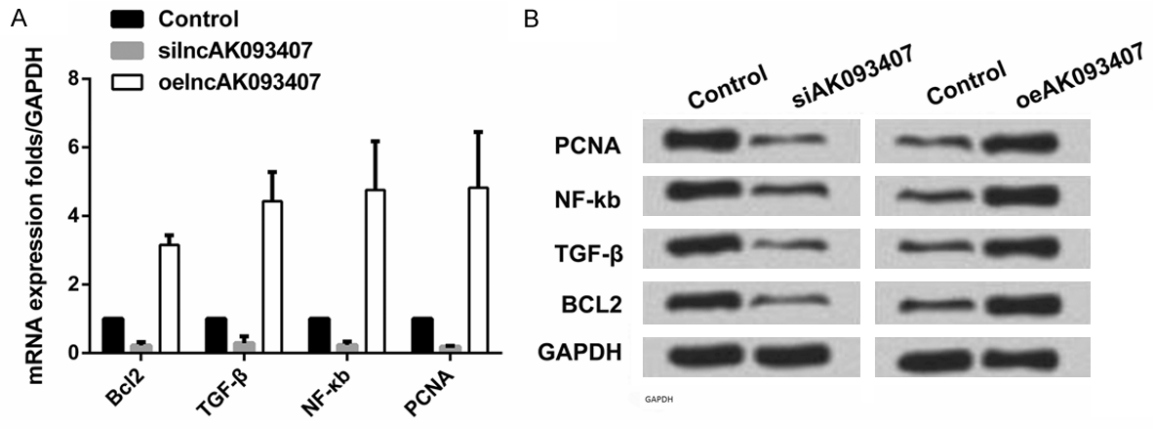

Figure 3. AK093407 regulates the expression of genes and proteins involved in cell proliferation and apoptosis. U-2OS cells were transfected with siIncAK093407 or oeIncAK093407; un-transfected cells were used as control. The relative mRNA expression level and protein level of Bcl-2, TGF-β, NFκB and PCNA in the transfected cells and un-transfected cells were measured using qRT-PCR (A) and western blot (B), respectively. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; NFκB: nuclear factor kappa B; PCNA: proliferating cell nuclear antigen; qRT-PCR: quantitative reverse transcription polymerase chain reaction; TGF-β: transforming growth factor beta.

and proteins involved in the cell proliferation, invasion, and apoptosis of osteosarcoma cells. Towards this goal, we measured the mRNA expression of Bcl-2, transforming growth factor beta (TGF-β), nuclear factor kappa B (NFκB), and proliferating cell nuclear antigen (PCNA), which are associated with cell apoptosis, proliferation and invasion, using qRT-PCR analysis. We also determined the protein expression levels of Bcl-2, TGF-β, NFκB, and PCNA using western blot analysis. The qRT-PCR results showed that overexpressed AK093407 significantly increased the mRNA expression of Bcl-2, TGF-β, NFκB and PCNA, whereas suppressed AK093407 decreased these expressions (**Figure 3A**). Western blot results revealed that overexpressed AK093407 increased the protein expression of Bcl-2, TGF-β, NFκB and PCNA, whereas suppressed AK093407 showed opposite effects (**Figure 3B**).

AK093407 interacts with STAT3 and promotes its phosphorylation

To further identify the molecular mechanism and binding partners of AK093407 in U-2OS cells, we performed mass spectrometry followed by RNA pull-down assay. Mass spectrometry showed that AK093407 interacted with STAT3; the interaction was further verified by RNA pull-down assay and western blot, confirming the interaction of AK093407 and STAT3 (**Figure 4A**). We also verified this result using RIP assay and found that AK093407 could be enriched in STAT3 precipitates ($P<0.005$), further proving that AK093407 interacts with STAT3 (**Figure 4B**). Further analysis for domain mapping by western blot showed that AK093407 interacted with STAT3 at 550-1100 bp and 1-1664 bp (**Figure 4C**); however, deletion of 550-1100 bp showed no bands (**Figure 4D**), proving that AK093407 interacts with STAT3 at 550-1100 bp only. We also studied the effect of AK093407 on STAT3 and found that overexpression of AK093407 promoted phosphorylation of STAT3 (**Figure 4E**), and this phosphorylation was maintained till 4 hours (**Figure 4F**). These findings indicated that AK093407 interacted with STAT3 and promoted and maintained its phosphorylation.

STAT3 is required for the functions of AK093407 in osteosarcoma cells

We then evaluated the role of STAT3 in AK093407-induced functions in osteosarcoma cells. First, we detected whether STAT3 gets deposited at the promoter region of Bcl-2, TGF-β, and PCNA using ChIP assay, and found that STAT3 was deposited at the promoter region of Bcl-2 and TGF-β (for both, $P<0.005$), but not in PCNA (**Figure 5A**). Therefore, we then studied the effect of AK093407 expression on the deposition of STAT3 at the promoter regions of Bcl-2 and TGF-β using ChIP assay. As shown in **Figure 5B**, overexpression of AK093407 signifi-

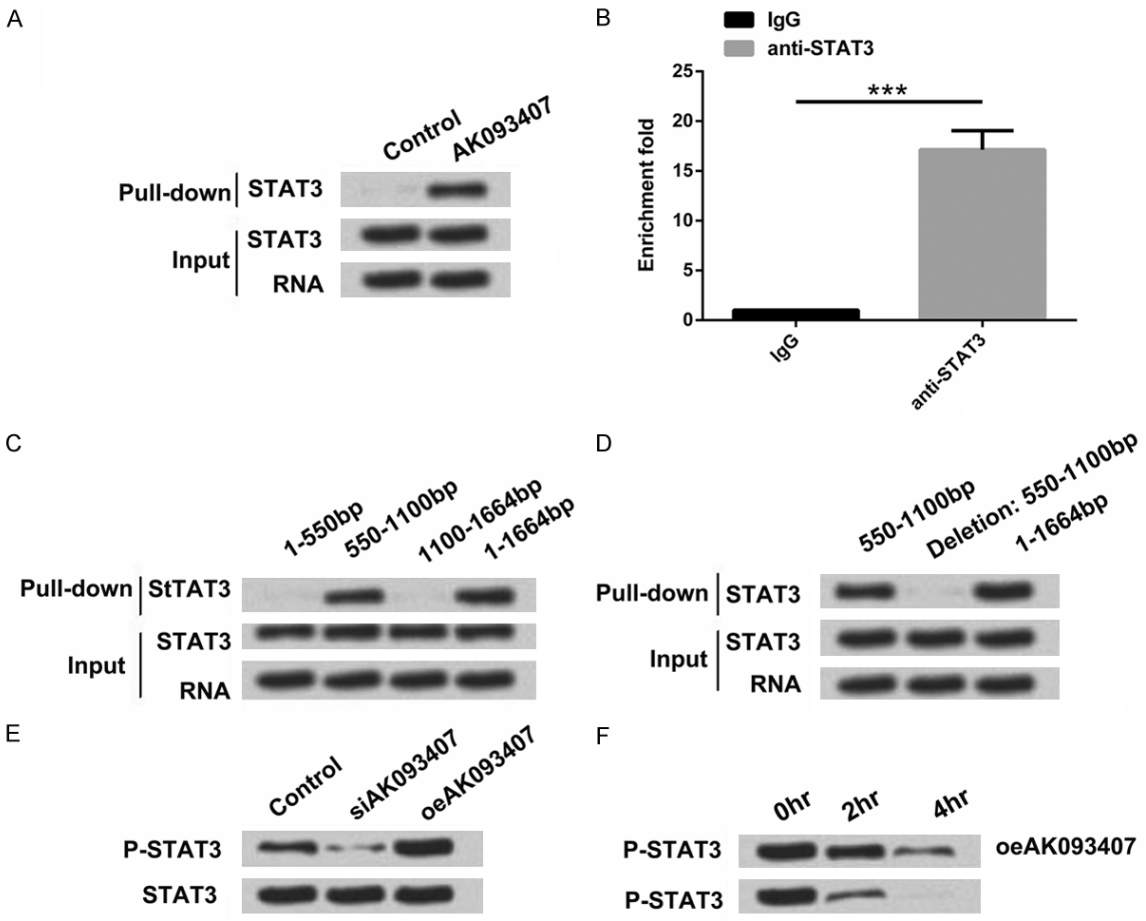

Figure 4. AK093407 interacts with STAT3 and promotes its phosphorylation. A. Mass spectrometry was performed to identify interaction between AK093407 and STAT3. The interaction between AK093407 and STAT3 was further confirmed by RNA pull-down and western blot. B. RIP was performed using anti-STAT3 and control IgG antibodies, followed by qRT-PCR to examine the enrichment of AK093407. C, D. Domain mapping of STAT3 for interaction with AK093407 using western blot. E. Effect of overexpression of AK093407 and suppression of AK093407 on phosphorylation of STAT3 was measured using western blot. F. Effect of overexpression of AK093407 on phosphorylated STAT-3 (P-STAT3) at 0, 2, and 4 hours was measured using western blot. ***P<0.005. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IgG: immunoglobulin G; qRT-PCR: quantitative reverse transcription polymerase chain reaction.

cantly increased the deposition of STAT3 at the promoter region of Bcl-2 and TGF-β (for both, P<0.05); however, suppression of AK093407 significantly decreased the deposition of STAT3 at the promoter region of Bcl-2 (P<0.005) and TGF-β (P<0.01), proving the essential role of AK093407 in STAT3 deposition at Bcl-2 and TGF-β promoters.

To characterize the functions of STAT3 in the regulation of Bcl-2 and TGF-β, we measured the effect of STAT3 overexpression on the protein expression of Bcl-2 and TGF-β using western blot. The results showed that overexpression of STAT3 using oeSTAT3 markedly increased the protein expression of Bcl-2 and TGF-β as compared to the control (**Figure 5C**). After confirming that STAT3 overexpression also increases Bcl-2 and TGF-β expression, we checked whether the inhibition of STAT3 expression affects the functions of AK093407 in osteosarcoma cells. For this purpose, we transfected U-2OS cells with oeAK093407 + vehicle or oeAK093407 + STAT3 inhibitor (Stattic, Abcam, ab120952) and measured protein expression of Bcl-2, TGF-β, and active caspase-3 using western blot, and assessed cell viability using MTT assay. The results showed that the STAT3 inhibitor decreased the protein expression of Bcl-2 and TGF-β (**Figure 5D**), decreased cell viability

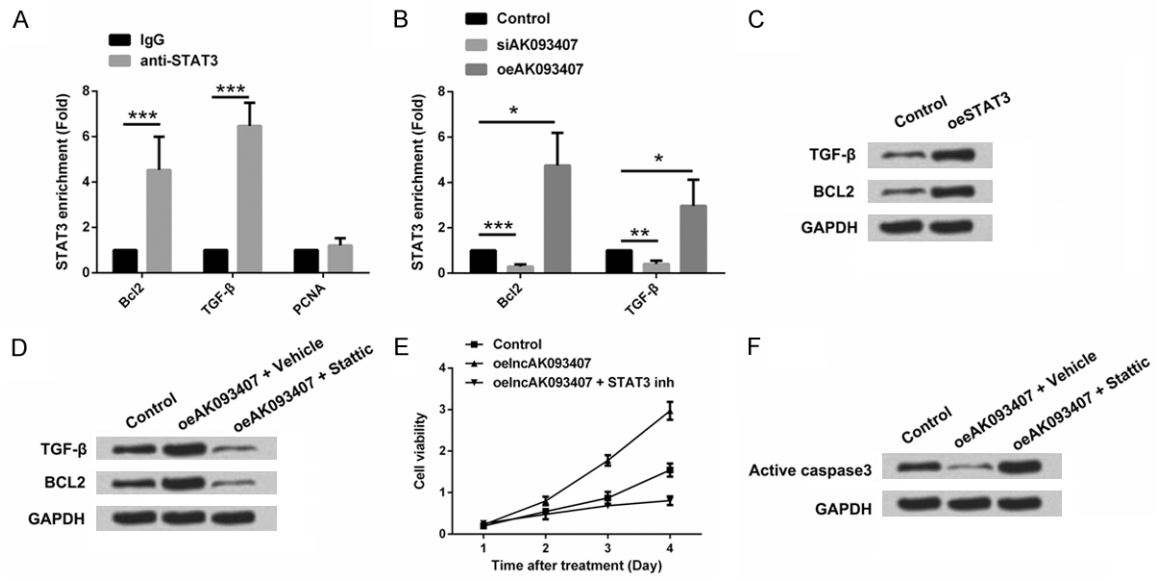

Figure 5. STAT3 is required for the functions of AK093407 in osteosarcoma cells. A. ChIP assay was performed using anti-STAT3 and control IgG antibodies and fold enrichment of the indicated regions of Bcl-2, TGF-β, and PCNA promoter was examined using qRT-PCR. B. ChIP assay was performed using U-2OS cells transfected with siIncAK093407 or oeIncAK093407 and cells without transfection (control), and enrichment of the indicated regions of Bcl-2 and TGF-β promoter was examined using qRT-PCR. C. Effect of STAT3 overexpression (by oeSTAT3) on the protein expression of Bcl-2 and TGF-β was measured using western blot. D. The protein expression of Bcl-2 and TGF-β was measured in cells transfected with oeIncAK093407 + vehicle or oeIncAK093407 + Stattic using western blot. Un-transfected cells were used as control. E. U-2OS cells were transfected with oeIncAK093407 + vehicle or oeIncAK093407 + Stattic, and then viability of these transfected cells and un-transfected control cells was measured at 1, 2, 3, and 4 days using MTT assay. F. The protein expression of pro-apoptotic protein active caspase-3 and GAPDH (control) was measured in cells transfected with oeIncAK093407 + vehicle or oeIncAK093407 + Stattic using western blot. Un-transfected cells were used as control. *P<0.05, **P<0.01, ***P<0.005. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; IgG: immunoglobulin G; PCNA: proliferating cell nuclear antigen; qRT-PCR: quantitative reverse transcription polymerase chain reaction; TGF-β: transforming growth factor beta.

(**Figure 5E**), and increased the expression of pro-apoptotic protein caspase-3 (**Figure 5F**) even in presence of AK093407 promoter. These findings suggested that suppression of STAT3 expression inhibited the effect of AK093407, and therefore STAT3 activation was essential for the functions of AK093407 in osteosarcoma cells.

Discussion

Till date, the effect of lncRNA AK093407 has not been explored for any disease conditions in any study. To our knowledge, this is the first study that explored the role of AK093407 in osteosarcoma. We investigated the effect and mechanism of AK093407 in the proliferation and apoptosis in osteosarcoma cell line U-2OS. First, we demonstrated that AK093407 was highly expressed in human osteosarcoma cell line U-2OS and in osteosarcoma tissues. AK093407 promoted the proliferation and inhibited apoptosis of osteosarcoma cells by regulating the expression of genes and proteins (Bcl-2, TGF-β, NFκB, and PCNA) involved in cell proliferation, invasion, and apoptosis. The mechanistic studies revealed that AK093407 interacts with STAT3 and promotes its phosphorylation; furthermore, STAT3 activation is essential to promote the effects of AK093407 on proliferation and apoptosis of osteosarcoma cells.

Effective control of cell proliferation, cell cycle and apoptosis is critical for the prevention of oncogenesis and for successful cancer therapy [17]. It is reported that lncRNAs play an important role in cell proliferation, invasion and apoptosis of cancer cells [15]. Therefore, identification of cancer-associated lncRNAs and study of their functions and clinical significance may provide new options in cancer research. Recent studies have reported that lncRNAs play a crucial role in osteosarcoma by regulating cell

development, differentiation, proliferation, invasion, and apoptosis [18-21]. Yun-Bo et al showed that lncRNA TUG1 is up-regulated and promoted cell proliferation in osteosarcoma [18]. Li et al demonstrated that overexpression of lncRNA UCA1 promoted osteosarcoma cell proliferation and inhibited apoptosis [20]. Sun et al showed that increased expression of HULC promoted osteosarcoma cell proliferation, migration, and invasion [21]. In consistent with these studies, we demonstrated that overexpression of lncRNA AK093407 promoted cell proliferation and viability and inhibited apoptosis of osteosarcoma cells.

We then investigated the effects of AK093407 on the expression of target genes and proteins (Bcl-2, TGF-β, NF-κB, and PCNA) involved in cell proliferation, invasion, and apoptosis of osteosarcoma cells. The BCL-2 family play significant role in cell survival and oncogenesis. The Bcl-2 subfamily promoted cell survival and inhibited apoptosis [22]. TGF-β signaling pathway regulated cell growth, apoptosis, invasion, angiogenesis, extracellular matrix production, and immune response [23]. Generally, the expression of TGF-β mRNA and protein is increased in human cancers; therefore, TGF-β is commonly targeted for cancer therapy research [24]. The NFκB family of transcription factors has an essential role in cancer initiation and progression. Increased expression of NFκB in cancer cells lead to up-regulation of anti-apoptotic genes, thereby providing a survival mechanism to cancer cells and causing drug resistance [25]. PCNA is a molecular marker for cell proliferation. Increased PCNA expression has been shown to be related to a shorter disease-free period and overall survival time in breast cancer patients [26, 27]. In our study, we found that overexpression of AK093407 increased the expression of Bcl-2, TGF-β, NF-κB, and PCNA, indicating that AK093407 exerts its effects on osteosarcoma cells via regulating the expression of these proteins.

Finally, we explored the underlying mechanism through different analyses with an important transcription factor, STAT3. Transcription factors are essential for gene transcription and cell fate determination [28, 29]. STAT3 is a transcription factor that mediates the expression of various genes in response to cell stimuli. Thus, STAT3 plays an important role in cell growth and apoptosis. STAT3 is activated through phosphorylation in response to various cytokines and growth factors [30]. It has been reported that STAT3 is activated in many types of cancer. In addition, evidences suggest that aberrant expression of STAT3 promotes initiation and progression of cancer by either inducing cell proliferation, angiogenesis, invasion, and metastasis or inhibiting apoptosis [31].

In our study, we demonstrated that AK093407 interacts with STAT3 and promotes its phosphorylation. Further analysis revealed that STAT3 gets deposited at the promoter region of Bcl-2 and TGF-β, and overexpression of AK093407 further increases this deposition. Then we showed that overexpression of STAT3 increases the expression of Bcl-2 and TGF-β and inhibition of STAT3 decreased these expressions. These findings confirmed that STAT3 also regulates the proteins involved in cell proliferation and apoptosis of osteosarcoma cells. Then we checked whether inhibition of STAT3 expression affects the functions of AK093407 in osteosarcoma cells, and found that the inhibition of STAT3 decreased the protein expression of Bcl-2 and TGF-β, decreased cell viability, and increased the expression of pro-apoptotic protein caspase-3 even in presence of AK093407 promoter. This finding suggests that if STAT3 was suppressed, AK093407 cannot promote cell viability and inhibited apoptosis of osteosarcoma cells. Therefore, STAT3 was essential for the effects of AK093407 on cell viability and apoptosis. STAT3 expression is also associated with other lncRNAs. Wu et al showed that lncTCF7 promotes hepatocellular carcinoma aggressiveness via IL-6/STAT3 transactivation [32]. Wang et al showed that lnc-DC controls human dendritic cell differentiation via STAT3 activation [33].

In summary, the lncRNA AK093407 was highly expressed in human osteosarcoma cell line U-2OS and in osteosarcoma tissues and overexpression of AK093407 promotes cell proliferation and viability and inhibits apoptosis of osteosarcoma cells by regulating the expression of Bcl-2, TGF-β, NFκB, and PCNA. Importantly, activation of the transcription factor STAT3 is essential for the effects of AK093407 on cell proliferation and apoptosis in osteosarcoma cells.

Disclosure of conflict of interest

None.

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