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LncRNA BLACAT1 accelerates the proliferation and migration of osteosarcoma cells through regulating STAT3

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ABSTRACT

Background: Long non-coding RNAs (lncRNAs) have been reported in human cancers as regulators for biological processes. LncRNA bladder cancer associated transcript 1 (BLACAT1) has been found to exert oncogenic function in cervical cancer and lung cancer. However, whether it can regulate the biological processes in osteosarcoma (OS) is still unclear. This study aims to examine the potential effect of dysregulated BLACAT1 on the progression of OS.

Methods: The expression pattern of BLACAT1 in OS tissues and cell lines was detected by qRT-PCR assay. Gain or loss-of function assays were designed and conducted to determine the effect of BLACAT1 overexpression or knockdown on the OS cell proliferation, apoptosis, invasion and migration. RNA pull-down assay and western blot analysis were performed to identify the relationship between BLACAT1 and signal transducer and activator of transcription 3 (STAT3).

Results: BLACAT1 was upregulated in OS tissues and cells. Upregulation of BLACAT1 predicted unfavorable prognosis for patients with OS. Downregulation of BLACAT1 inhibited cell proliferation and invasion, whereas upregulation of BLACAT1 accelerated cell proliferation and invasion. More importantly, BLACAT1 could interact with STAT3 and regulat the phosphorylation of STAT3.

Conclusions: LncRNA BLACAT1 contributes to the proliferation and migration of OS cells by regulating STAT3.

1. Introduction

Osteosarcoma (OS), a malignant bone tumor, is ranked as the second leading cause of cancer-related death among adolescents [18]. Fast growth and early stage of metastasis are responsible for the unfavorable prognosis of osteosarcoma patients [17]. Although great progresses have been made in the diagnosis and treatment of osteosarcoma, the survival rate for osteosarcoma patients remains poor [24]. Therefore, it is urgent to investigate the underlying molecular mechanisms of OS development. Also, it is of importance to find potential diagnostic or prognostic biomarkers for OS.

As a group of transcribed RNA molecules, long non-coding RNAs (lncRNAs) are longer than 200 nucleotides [33]. Furthermore, lncRNAs participate in various biological processes such as tumor initiation, proliferation and migration [39]. Reports also revealed that lncRNAs exert multiple functions in various tumors such as lung adenocarcinoma [14], esophageal squamous cell carcinoma [40], hepatocellular carcinoma [27], renal cell carcinoma and osteosarcoma [13,30] through posttranscriptional regulation or chromatin modification. In the past decades, numerous reports have elucidated that lncRNAs play important role in osteosarcoma proliferation and migration, suggesting the involvement of lncRNAs in osteosarcoma development [3,29]. LncRNA bladder cancer associated transcript 1 (BLACAT1) was firstly identified in bladder cancer with 2616 bp in length [8]. As previously described, lncRNA BLACAT1 can regulate tumor progression by acting as an oncogene in lung cancer [4], cervical cancer [28], gastric cancer [36] and colorectal cancer [25]. However, to our knowledge, whether BLACAT1 can regulate the biological processes in osteosarcoma progression has not been studied. Thus, this study focused on exploring the role of BLACAT1 in OS progression.

In this study, we firstly examined and determined the expression pattern of BLACAT1 in OS tissues and cell lines. The prognostic potential of BLACAT1 in OS was analyzed by Kaplan-Meier method. It is well known that upregulation of lncRNAs is associated with the abnormal cell activities [9,23,26]. Therefore, we conducted gain or loss-of function assays to determine the role of BLACAT1 in regulating OS cell proliferation, invasion and migration. The oncogenic role of BLACTA1 was confirmed.

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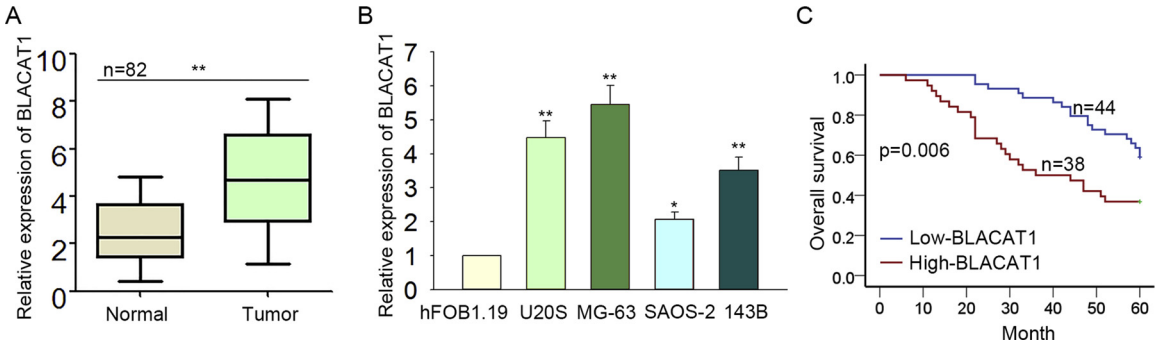

Fig. 1. Upregulation of BLACAT1 is associated with poor prognosis of OS patients.
(A) The relative expression of BLACAT1 in 82 pairs of OS tissues and matched normal tissues was detected by qRT-PCR. The results were normalized to the expression of GAPDH. (B) The relative expression of BLACAT1 was measured in four OS cell lines (MG-63, SAOS-2, U20S, 143B) and one human normal osteoblastic cell line (hFOB1.19) by qRT-PCR assays. (C) Overall survival rate of OS patients with high or low level of BLACAT1 was analyzed by using Kaplan-Meier method. $^{*}P < 0.05$, $^{**}P < 0.01$.

Table 1
Correlation between the expression of BLACAT1 and clinicopathological features of osteosarcoma patients. (n = 82).

Variable	BLACAT1 Expression		P-value
	Low	High	
Age			
< 20	16	15	0.0772
≥20	28	23	
Gender			
Male	21	14	0.320
Female	23	24	
TNM Stage			
I–II	29	12	0.002**
III–IV	15	26	
Tumor Size			
< 5cm	26	12	0.013*
≥5cm	18	26	
Lymph Node Metastasis			
No	29	13	0.004**
Yes	15	25	

Low/high decided by the expression mean of BLACAT1. Pearson χ^2 test.
* P < 0.05 was considered statistically significant.
** P < 0.01 was considered statistically significant.

More importantly, previous studies have revealed that lncRNAs can interact with signal transducer and activator of transcription 3 (STAT3) to regulate tumor progression [32,35]. In the present study, pull-down and western blot analysis were designed and conducted to validate the relation between BLACAT1 and STAT3. Collectively, this study revealed the involvement of BLACAT1 in OS progression.

2. Material and methods

2.1. Tissues samples

82 pairs of OS tissues and adjacent normal tissues were obtained and collected from OS patients who were diagnosed at Hefei Binhu Hospital. This study was approved by the Research Medical Ethics Committee of the Hefei Binhu Hospital. Informed consent was signed by each osteosarcoma patient.

2.2. Cell culture

Four human osteosarcoma cell lines (MG-63, SAOS-2, U20S, 143B) and one human normal osteoblastic cell line (hFOB1.19) were purchased from the American Type Culture Collection (Manassas, VA, USA). MG-63 cell was cultured in DMEM medium (Gibco Co., New York, NY, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). SAOS-2 cell was grown in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO, USA) fixed with 15% FBS (Invitrogen). U20S and 143B cell lines were maintained in RPMI-1640 (Gibco) medium containing 10% FBS (Invitrogen). hFOB 1.19 cell line was cultured in DMEM/F-12 medium (Gibco) fixed with 10% FBS (Invitrogen). Cells were all maintained in a moist atmosphere at 37 °C with 5% CO_2.

2.3. Cell transfection

For the knockdown of BLACAT1, the specific shRNA targeting BLACAT1 (sh-BLACAT1) and corresponding control shRNA (sh-NC) were synthesized by Gene Pharma (Shanghai, China). To overexpress BLACAT1, the pc-DNA3.1 vector containing the whole length of BLACAT1 and the empty vector (NC) were synthesized by Gene Pharma. The shRNA sequence for BLACAT1: 5′-AGGCUGGUUUCUGC CCUCAUCCUUU-3′. The negative control shRNA sequence: 5′-UUUCU CCGAACGUGUCACGUTT-3′. According to the manufactures' instructions, the Lipofectamine 2000 (Invitrogen-Life Technologies, Carlsbad, CA, USA) was used for transfection. After 48 h transfection, cells were collected for subsequent experiments.

2.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from osteosarcoma tissues and cells by Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The quality and concentration of RNA were detected by using NanoDrop 2000 (Quawell, San Jose, CA, USA) at 260 and 280 nm. Reverse transcription (RT) of first-strand cDNAs was performed by using PrimeScript RT Master Mix (Perfect Real Time; Takara Bio, Inc., Tokyo, Japan). PCR reactions were conducted in an ABI PRISM 7900 Real-Time system (Applied Biosystems, Foster City, CA, USA) with the SYBR Premix ExTaq kit (Takara Bio, Inc.). The primer sequences for PCR are presented as below: BLACAT1: 5′-GTCTCTGCC CTTTTGAGCCT-3′ (forward) and 5′-GTGGCTGCAGTGTCATACCT-3′ (reverse); GAPDH: 5′-GGGAAACTGTGGCGTGAT-3′ (forward) and 5′-GAGTGGGTGTCGCTGTTGA-3′ (reverse). The results were measured by using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal reference.

2.5. CCK-8

Cells were cultured in a 96-well plate and were maintained at 37 °C incubator overnight. 100 µl CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added into each well at different time stage (0, 24, 48, 72 h). After 4 h incubation, the absorbance at 450 nm was detected by microplate reader (Bio Tek Instruments, Winooski, VT, USA). CCK-8 assay was thrice conducted independently.

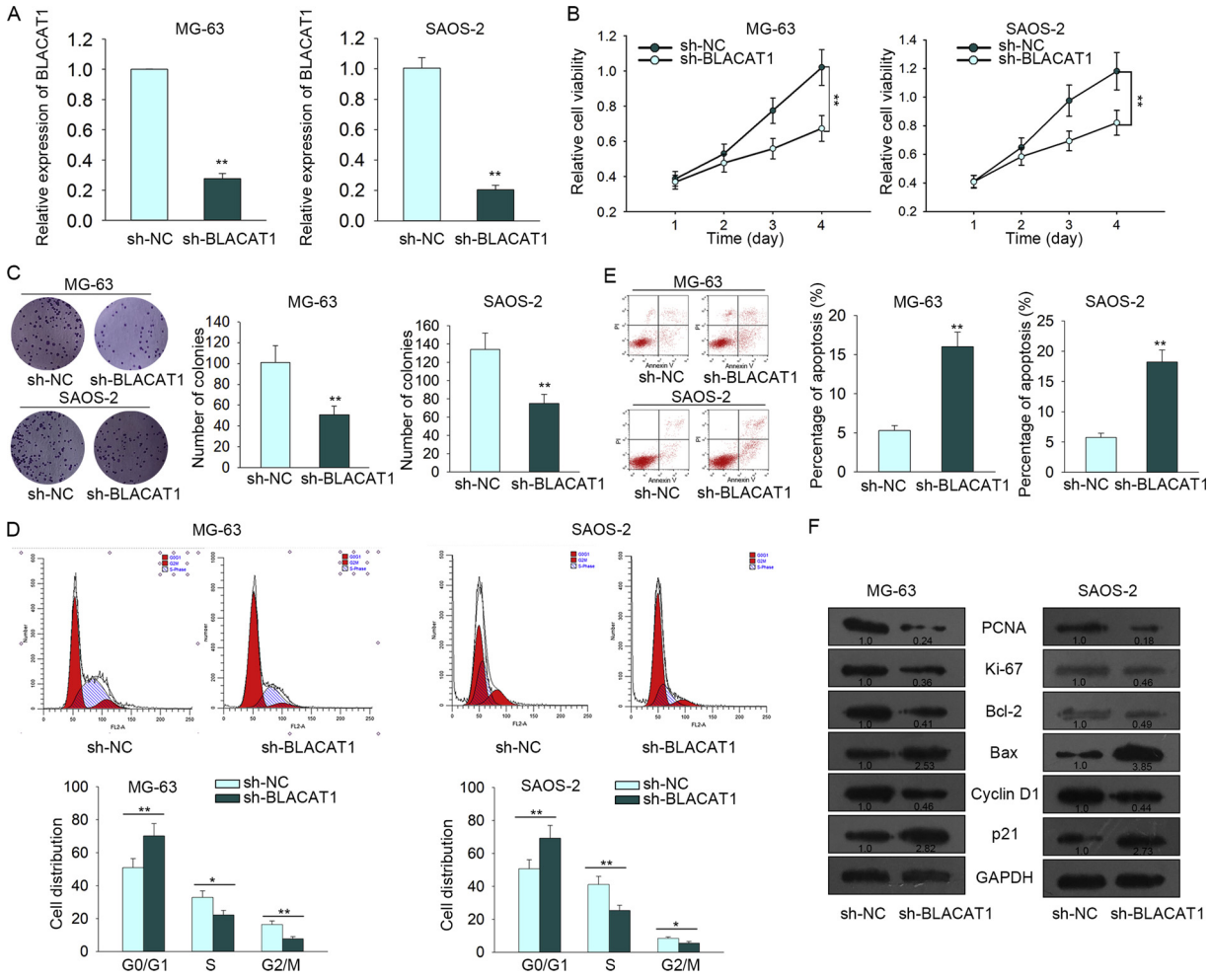

Fig. 2. Silencing of BLACAT1 inhibited OS cell proliferation, led to cell cycle arrest and promoted cell apoptosis.
(A) The knockdown efficiency for sh-BLACAT1 was assessed with qRT-PCR assay at 48 h' post-transfection. (B–C) The effect of BLACAT1 knockdown on proliferative abilities of MG-63 and SAOS-2 cells was evaluated by CCK-8 and colony formation assays. (D–E) The cell cycle distribution and cell apoptosis were observed in BLACAT1-downregulated OS cells by performing flow cytometry analyses. (F) The levels of proteins involved in proliferation, cell cycle progress and apoptosis were tested in OS cells transfected with sh-BLACAT1 or sh-NC. $^{*}P < 0.05$, $^{**}P < 0.01$.

2.6. Colony formation assay

After transfection, SAOS-2 and MG-63 cells were plated in six-well plates (500 cells/well) and cultured in RPMI-1640 medium with 10% FBS for 48 h at 37 °C. 2 weeks later, the colonies were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, and then stained with 0.1% crystal violet. At last, colonies were imaged and counted by a light microscope (Olympus Corp., Tokyo, Japan). The final result was acquired from more than two independent experiments.

2.7. Cell cycle analysis

Cells were seeded in a six-well plates for 48 h. Then, cells were harvested and fixed with 70% ethanol for 10 min on ice, followed by washing and re-suspending in pre-cold PBS and cultured at 37 °C for 30 min with 10 mg/ml RNase and 1 mg/ml propidium iodide (PI) (Sigma-Aldrich, St Louis, MO, USA). The cell cycle stage was measured by using the Cell Cycle Analysis Kit (Beyotime, Shanghai, China) following the manufacturer's instructions. Each experiment was carried out in triplicate.

2.8. Cell apoptosis analysis

After transfection, cells were seeded in six-well plates. After 48 h, cells were harvested and re-suspended by using binding buffer. Based on the manufacturers' instruction, The FITC Annexin V/PI Apoptosis Detection Kit I (Ribobio, Guangzhou, China) was used to detect cell apoptosis. Cells were incubated with 5 μl of Annexin V-FITC at normal temperature for 15 min, followed by the incubation with 10 μl of propidium iodide (PI, 10 mg/ml) in a dark room for 5 min. Finally, a FACScan flow cytometer was used for apoptosis analysis. The result of cell apoptosis analysis was obtained from three different replications.

2.9. Wound healing assay

To assess cell migration, indicated OS cells were seeded into 6-well plates and allowed to grow to 90–95% confluence. Wounds were introduced into monolayer cells by using a 200 μl sterile pipette tip. PBS was used to wash wounded monolayer cells three times to remove cell debris. The speed of wound closure was observed and photographed at different time intervals (0–24 h). Wound healing assay was performed independently for more than two times.

2.10. Transwell invasion assay

Cell invasion assay was carried out by using Control Cell Culture Inserts (BD Biosciences, San Jose, CA, USA) with 24-well plates. Transfected cells (1×10^4) were suspended in 100 μl of serum-free

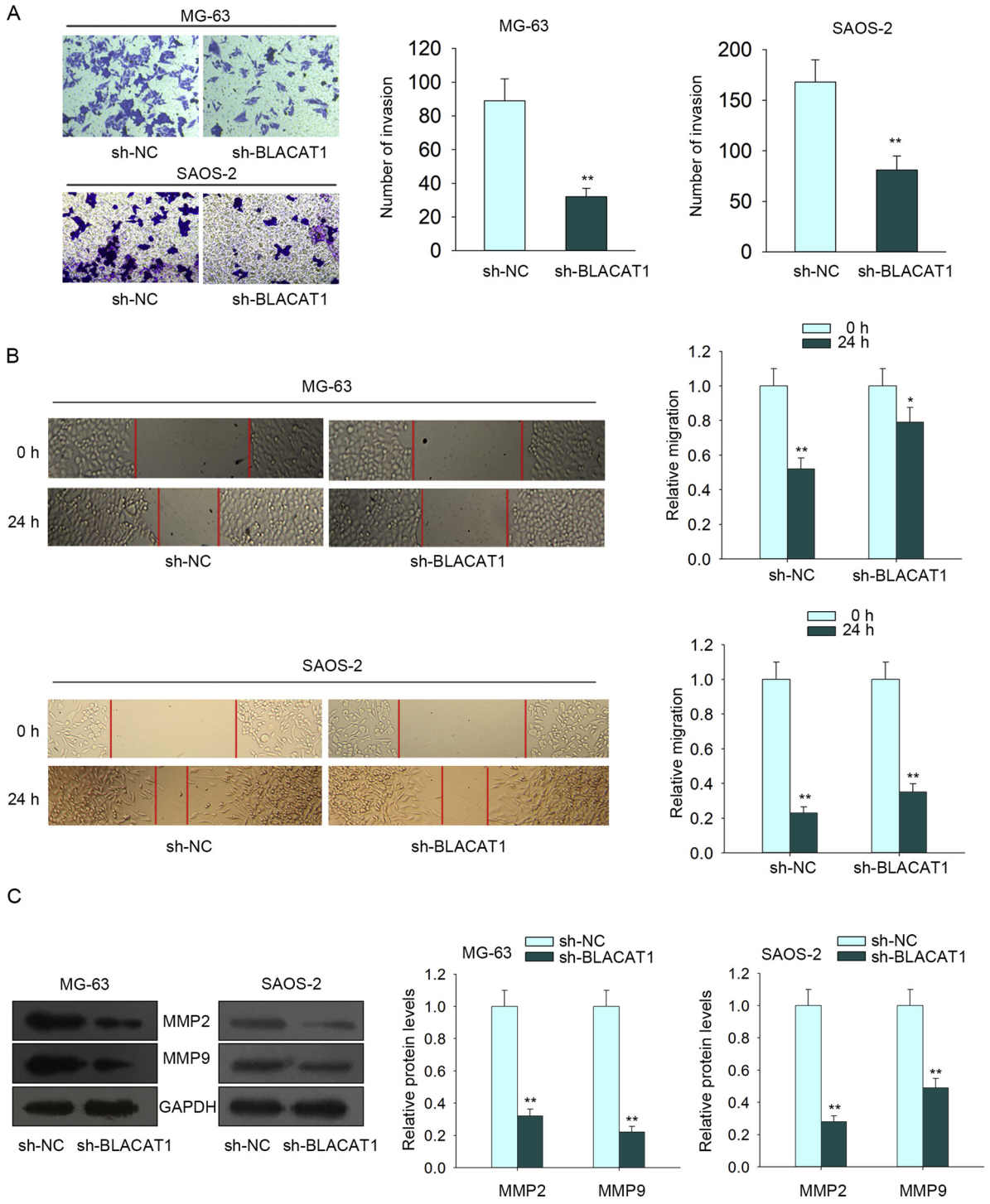

Fig. 3. Knockdown of BLACAT1 suppressed OS cell migration and invasion.
(A) The invasive number of BLACAT1-downregulated OS cells was detected with transwell invasion assays. (B) The effect of BLACAT1 knockdown on cell migration was measured using wound-healing assay. (C) The levels of migration-related proteins were examined in cells transfected with sh-BLACAT1 or sh-NC. $^{*}P < 0.05$, $^{**}P < 0.01$.

medium and added into the upper chamber pre-coated with Matrigel (BD Biosciences). Whereas, the lower chamber was suffused with 600 μl of medium containing 20% FBS. After 48 h of incubation, the cells on the lower chamber, namely the invasive cells, were fixed in 4% paraformaldehyde at 4 °C for an hour and stained with 1% crystal violet at room temperature for 12 min. Cells remaining on the upper chamber were removed using the cotton swabs. The number of invasive cells was determined randomly from five visual fields per well by a light microscope (Olympus Corp, Tokyo, Japan). Each experiment procedure was carried out in triplicate.

2.11. Western blot assay

Cells were placed into six-well plates and transfected with plasmids. Total protein was isolated from osteosarcoma tissues and cells using RIPA lysis buffer (Beyotime, Shanghai, China). The protein

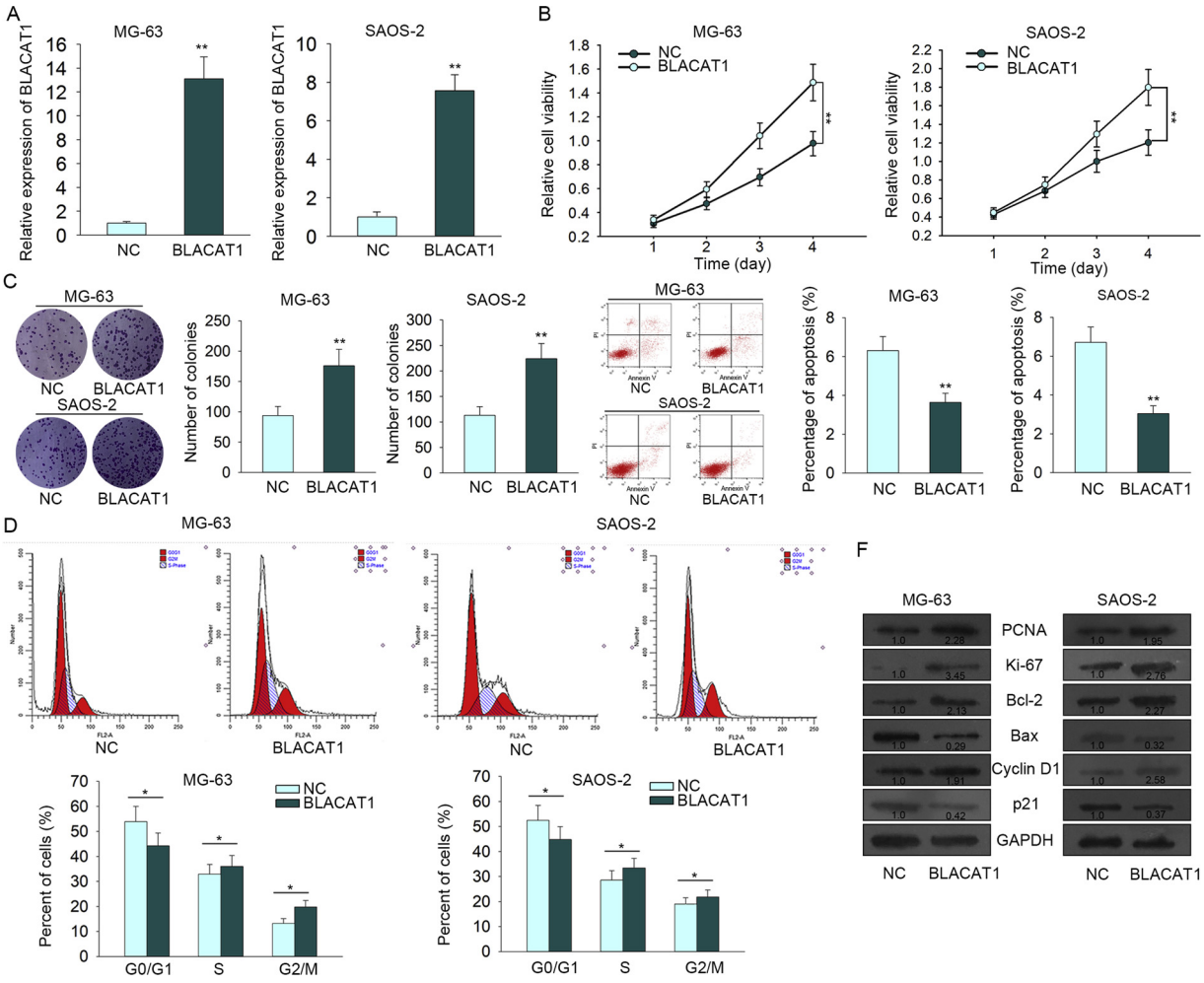

Fig. 4. Overexpression of BLACAT1 promoted cell proliferation, cell cycle progress and inhibited cell apoptosis.
(A) Overexpression efficiency for pcDNA-BLACAT1 (BLACAT1) was evaluated by qRT-PCR. (B–C) The proliferative abilities of BLACAT1-overexpressed OS cells were assessed with CCK-8 and colony formation assays. (D–E) Flow cytometry analyses were applied to evaluate the cell cycle distribution and cell apoptosis in OS cells transfected with BLACAT1 expression vector. (F) Western blot analysis was used to examine the levels of proteins involved in proliferation, cell cycle progress and apoptosis in OS cells transfected with BLACAT1 expression vector or NC. $^{*}P < 0.05$, $^{**}P < 0.01$.

concentration was measured by BCA Protein Assay Reagent Kit (Beyotime) in line with the user guide. Proteins were separated by 10% SDS-PAGE and transferred onto 0.45 μm PVDF membranes (Millipore, Billerica, MA, USA) on ice all night. After blockade in 5% skim milk at room temperature for 2 h, the membranes were cultured with the primary antibodies, including anti- STAT3 (ab68153, 1:1000, Abcam, Cambridge, MA, USA) and anti-p-STAT3 (ab30647, 1:1000, Abcam) overnight at 4℃. After thrice washing with TBST, HPR-conjugated secondary antibodies (1:2000, Abcam) were cultured with the membranes at room temperature for two hours. At last, the protein bands were exposed to X-ray films and visualized using ECL Western blotting kit (Bio-Rad lab, Hercules, CA, USA) following the standard method. GAPDH was used as an internal reference. Experimental procedures were conducted at least three times.

2.12. RNA pull-down assay

For the RNA pull-down assay, the plasmids containing BLACAT1 or the short fragments of BLACAT1 were constructed and synthesized, followed by linearization in 3′end using the restriction enzyme EcoRI. The linearized plasmids were used as templates and biotinylated in vitro using T7 RNA polymerase. After purification on G-50 Sephadex RNA Columns (Roche Diagnostics, Indianapolis, IN, USA), the size of biotinylated RNA was assessed by agarose gel electrophoresis. Thereafter, biotinylated RNA was cultured at various temperatures to generate the folded appropriate RNA secondary structure. The prepared streptavidin-agarose beads were utilized to collect nuclear lysates from cells. The complex containing folded biotinylated RNAs and nuclei lysates was cultured in turn with tRNA and streptavidin-agarose beads. After rinsing, pellets were boiled in loading buffer. At last, the protein level of STAT3 was analyzed by western blot assay. The total protein (input) was seen as the control. RNA pull-down assay was conducted in triplicate.

2.13. Statistical analysis

Statistical analysis was performed with the SPSS software package (version 17.0, SPSS Inc., Armonk, NY, USA) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Student's *t*-test and Chi-square test were carried out to test the significance of statistical analysis. Survival analysis was performed by using the Kaplan-Meier method and the log-rank test. All results were expressed as mean ± standard deviation (SD). P value less than 0.05 indicates statistical significance.

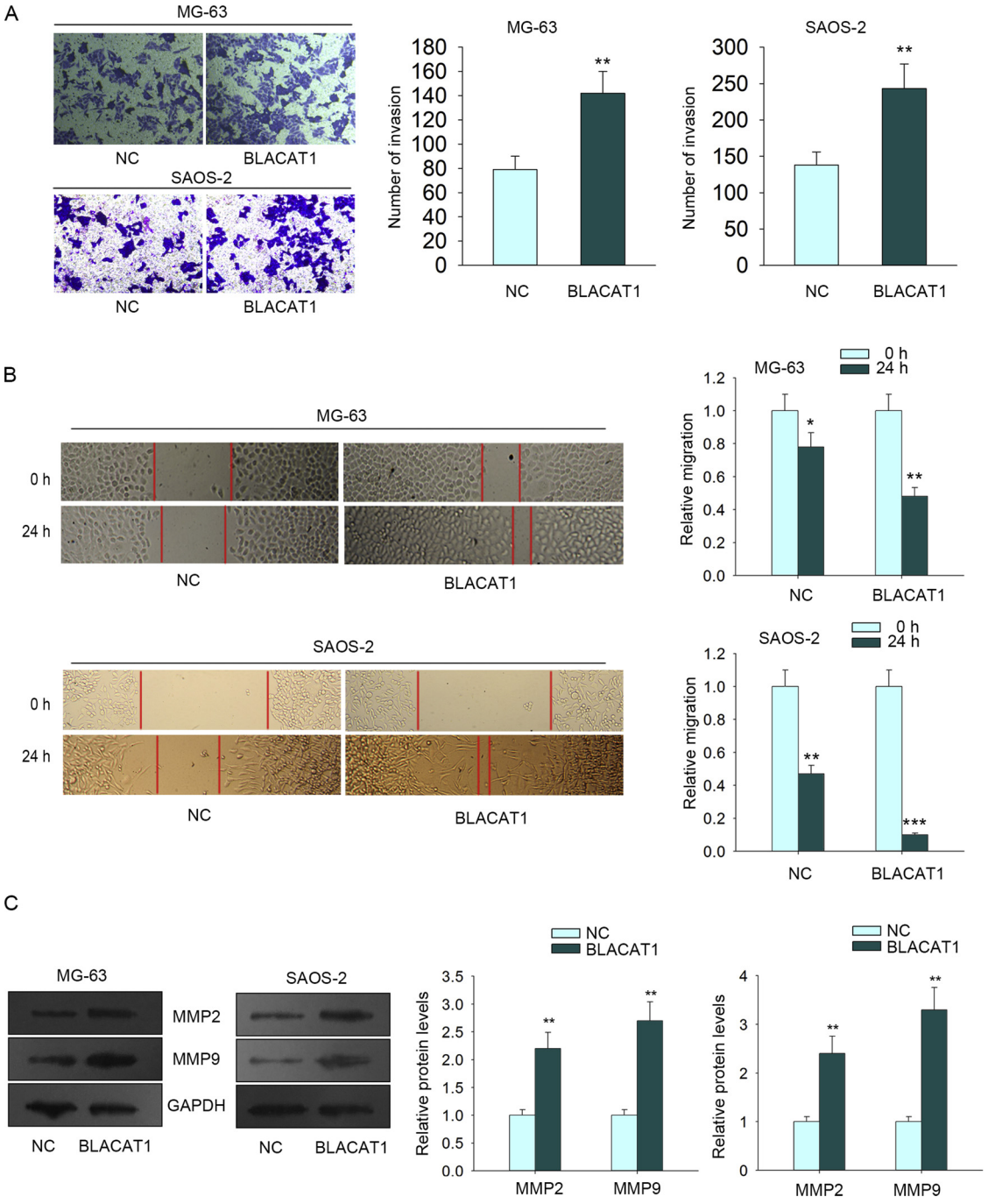

Fig. 5. Upregulation of BLACAT1 promoted OS cell migration and invasion.
(A–B) The invasion and migration of BLACAT1-downregulated OS cells were detected with transwell invasion assay and wound-healing assay. (C) The levels of migration-related proteins were examined in cells transfected with BLACAT1 expression vector or empty vector. $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Upregulation of BLACAT1 is associated with poor prognosis of OS patients

The expression pattern of BLACAT1 in OS was firstly examined and identified. According to the result of qRT-PCR analysis, BLACAT1 was expressed higher in OS tissues and cell lines compared with the corresponding normal controls (Fig. 1A–B). Dysregulation of lncRNAs is usually associated with the prognosis of cancer patients. To analyze the prognostic effect of dysregulated BLACAT1 on the OS patients, we classified 82 OS patients into two groups (BLACAT1 high expression group and BLACAT1 low expression group). The mean value of BLACAT1 in all 82 samples was used as the threshold. Kaplan-Meier analysis indicated that patients with high-BLACAT1 levels had lower overall survival rate than those with low-BLACAT1 levels (Fig. 1C). The relation between BLACAT1 expression and the clinicopathological features was analyzed. As illustrated in Table 1, the expression of BLACAT1 had a relation with TNM stage, tumor size and lymph node metastasis, not with age and gender. These findings revealed the potential involvement of BLACAT1 in OS progression.

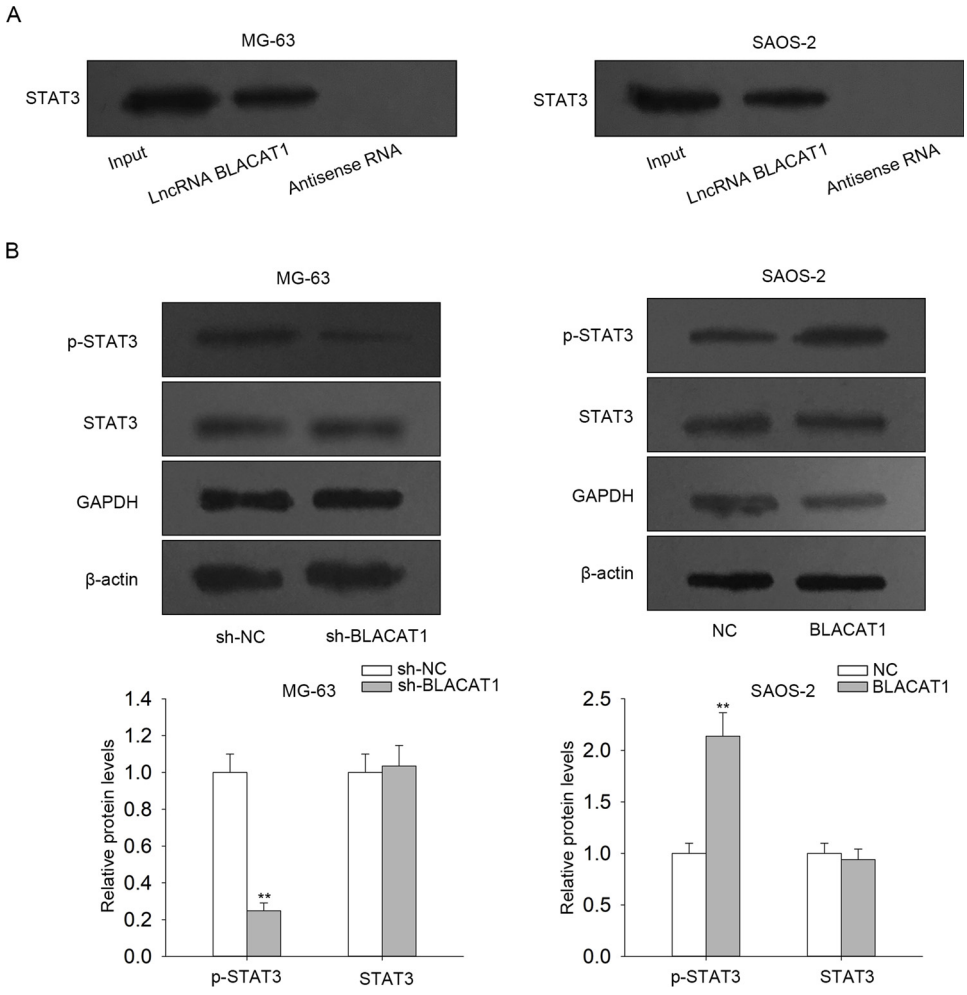

Fig. 6. BLACAT1 exerted its function in OS through regulating STAT3.
(A) The interaction between BLACAT1 and STAT3 was confirmed by RNA pull-down assay. (B) The relative protein levels of STAT3 and p-STAT3 were measured in BLACAT1-downregulated or BLACAT1-overexpressed OS cell lines by western blot assay. GAPDH was used as internal reference. $^{*}P < 0.05$, $^{**}P < 0.01$.

3.2. The effects of BLACAT1 knockdown or overexpression on cell proliferation, cell cycle progress and cell apoptosis

To analyze the effect of BLACAT1 on the biological processes of OS, we silenced and overexpressed BLACAT1 in MG-63 and SAOS-2 cells, respectively (Figs. 2A and 4 A). At first, cell proliferation assays such as CCK-8 and colony formation assays were conducted to determine the effect of BLACAT1 knockdown or overexpression on the OS cell proliferation. The results showed that proliferative ability of MG-63 and SAOS-2 cells was suppressed by the knockdown of BLACAT1 (Fig. 2B–C). BLACAT1 overexpression efficiently promoted OS cell proliferation (Fig. 4B–C). Moreover, cell cycle distribution and apoptosis condition were detected in cells transfected with sh-BLACAT1 or BLACAT1 expression vector. As presented in Fig. 2D–E, knockdown of BLACAT1 led to cell cycle arrest at G0/G1 phase and promoted cell apoptosis. However, overexpression of BLACAT1 promoted cell cycle progress and decreased cell apoptosis (Fig. 4D–E). In addition, the proteins involved in cell proliferation, cell cycle and cell apoptosis were examined in indicated OS cells. The decreased levels of PCNA and ki-67 (proliferation-related proteins) were examined in BLACAT1-downregulated OS cells. Moreover, the increased levels of Bax and p21 as well as the decreased levels of Bcl-2 and Cyclin D1 were tested in BLACAT1-downregulated OS cells (Fig. 2F). However, the opposite results were observed in BLACAT1-upregulated OS cells (Fig. 4F). All these results indicated the positive role of BLACAT1 in OS proliferation.

3.3. The effects of BLACAT1 knockdown or overexpression on OS cell migration and invasion

Furthermore, we detected the role of BLACAT1 in OS cell migration and invasion. According to the results of transwell invasion assay and wound-healing assay, BLACAT1 knockdown inhibited cell invasion and migration (Fig. 3A–B), while BLACAT1 overexpression led to the opposite results (Fig. 5A–B). The levels of proteins involved in migration and invasion (MMP2 and MMP9) were examined in indicated OS cells. The decreased levels of them were tested in BLACAT1-downregulated OS cells (Fig. 3C), while the increased levels of them were detected in BLACAT1-overexpressed OS cells (Fig. 5C). These results indicated the positive correlation between BLACAT1 expression and OS cell migration or invasion.

3.4. BLACAT1 exerted its function in OS through regulating STAT3

Above experiments revealed the oncogenic role of BLACAT1 in OS. Based on previous reports, we investigated whether BLACAT1 regulated OS progression by modulation of STAT3. The potential interaction between BLACAT1 and STAT3 was demonstrated in OS cells (Fig. 6A). More importantly, the phosphorylation of STAT3 at Tyr705 can regulate gene expression, thereby modulating cell proliferation and metastasis in human malignancies [1,20,43]. In our present study, we examined whether BLACAT1 regulated the phosphorylation of STAT3 in OS cells. Western blot assay revealed that knockdown of BLACAT1

positively regulated the phosphorylation of STAT3 at Tyr705 (Fig. 6B). Therefore, BLACAT1 exerted oncogenic function in OS potentially by interacting with STAT3 and regulating the phosphorylation of STAT3.

4. Discussion

Recently, lncRNAs have been demonstrated to be involved in the initiation and progression of osteosarcoma [6,11,45]. Upregulation of lncRNAs is associated with the poor prognosis of patients with OS [7,12,37]. In the current study, we identified the upregulation of lncRNA BLACAT1 in OS tissues and cell lines. Furthermore, the prognostic potential of BLACAT1 in OS patients was determined and analyzed. High expression of BLACAT1 indicated the poor prognosis for patients with OS. Thus, BLACAT1 might be a prognostic factor for OS patients. The expression of BLACAT1 was associated with several clinical features including TNM stage, tumor size and lymph node metastasis. Therefore, we further investigated the involvement of BLACAT1 in OS progression. It has been widely reported that upregulation of lncRNAs involved in cell proliferation, apoptosis and migration [5,16,42]. Considering the association between BLACAT1 expression and tumor size and lymph node metastasis, we further detected the effect of BLACAT1 knockdown or overexpression on OS cell proliferation and migration. Functionally, knockdown of BLACAT1 inhibited OS cell proliferation, led to cell cycle arrest at G0/G1 phase and promoted cell apoptosis. In addition, overexpression of BLACAT1 promoted cell proliferation, induced cell apoptosis and contributed to cell cycle progress. These findings revealed the oncogenic role of BLACAT1 in OS progression.

Based on previous studies, signal transducer and activator of transcription factors (STATs) family plays important roles in regulating cell proliferation and differentiation [21]. Among the STAT family, STAT3 has been certified as a critical regulator in tumor growth [19]. It has been reported that STAT3 regulated cell proliferation, migration and invasion by modulating the expressions of Bcl-2 (cell apoptosis-related genes) and CyclinD1 (cell cycle-related gene) [2,15,22,38]. Moreover, STAT3 can be activated frequently by certain cytokines and growth factors [41]. Phosphorylation of Try705 is followed by nuclear translocation, dimerization and gene transcription [10]. It was found that lncRNAs can regulate tumorigenesis and tumor progression by modulating STAT3 [31,34,44]. In this study, we explored whether BLACAT1 can interact with STAT3 and regulate the phosphorylation of STAT3. RNA pull-down assay revealed the interaction between BLACAT1 and STAT3 in OS cells. Moreover, the phosphorylation of STAT3 was positively regulated by the expression of BLACAT1. These findings suggested that BLACAT1 exerted oncogenic function in OS possibly by regulating STAT3. Collectively, this study revealed that lncRNA BLACAT1 predicted poor prognosis and promoted cell proliferation and migration in osteosarcoma. BLACAT1 can interact with STAT3 in OS cells and enhance the phosphorylation of STAT3. In conclusion, this study revealed that BLACAT1 exerted oncogenic function in osteosarcoma by regulating STAT3. Our findings may contribute to reveal a novel prognostic or therapeutic factor for osteosarcoma. We will make further investigation to reveal the mechanism between BLACAT1 and STAT3 in the future.

Conflicts of interest

Authors declared no conflicts of interest in this study.

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