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Research Paper

BAIAP2L2 promotes the proliferation, migration and invasion of osteosarcoma associated with the Wnt/β-catenin pathway

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ABSTRACT

Background: Osteosarcoma is the most common bone cancer that significantly affects the quality of life of patients. Studies have shown that overexpression of BAIAP2L2 elevates the proliferation and growth of some types of cancer cells. However, the role of BAIAP2L2 in osteosarcoma is unclear. This study aimed to investigate the functions of BAIAP2L2 in the development of osteosarcoma.
Methods: We used immunohistochemical and Western blot analysis to determine the expression levels of endogenic BAIAP2L2 in osteosarcoma cells. Cell counting kit-8 assay and colony formation assay were performed to investigate cell proliferation of tumor cells. Transwell assay was performed to detect cell migration. Flow cytometry assay was used to analyze cell apoptosis. The role of BAIAP2L2 in tumor growth was further explored *in vivo*.
Results: We found that BAIAP2L2 was significantly upregulated in human osteosarcoma, and inhibition of BAIAP2L2 suppressed the proliferation of osteosarcoma cells. In addition, down-regulation of BAIAP2L2 could lead to osteosarcoma cancer cell apoptosis, inhibit cell migration and invasion, and induce the inactivation of the Wnt/β-catenin pathway. In addition, down-regulation of BAIAP2L2 inhibited tumor growth *in vivo*.
Conclusion: In conclusion, down-regulation of BAIAP2L2 inhibited the proliferation, migration, and invasion of osteosarcoma associated with the Wnt/β-catenin pathway.

1. Introduction

Osteosarcoma, usually discovered at metaphysis that produce immature bone, is one of the most common bone cancers [1-3]. A majority of people diagnosed with osteosarcoma are under the age of 25, including young adults, teenagers and children [4,5]. The treatments for osteosarcoma mainly include chemotherapy and radiation. However, therapy effectiveness is quite limited and the recovery is not easy[6,7]. Although therapies are available, this disease still significantly affects the quality of life of patients. In recent years, studies have focused on the investigation of new therapeutic targets based on tumorigenesis, such as genes and their functioning signaling pathways that regulate somatic disruptive variant tumors[8].

I–BAR family proteins, including BAI1–associated protein 2–like 2, IRSp53 and MIM, have been shown to be associated with the regulation of cell membrane formation. It has been reported that BAI1-associated protein 2-like 1 (BAIAP2L1) could possibly be a marker of ovarian cancer [9]. Meanwhile, brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 2 (BAIAP2L2) was reported to promote the generation of planar membrane sheets. A recent study reported that overexpression of BAIAP2L2 could promote the proliferation and growth of lung cancer cells [10]. However, the function of BAIAP2L2 in osteosarcoma is unclear. This study therefore aimed to explore the role of BAIAP2L2 in the development of osteosarcoma.

The Wnt signaling pathways are a family of signal transduction pathways triggered by extracellular proteins, which transduce signals to cells via cell surface receptors. The Wnt/β-Catenin pathway has been shown to be related to the development of various can-

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cers, including breast cancer [11], prostate cancer [12], ovarian cancer [13] and others [14]. Previous studies have revealed that activation or inactivation of the Wnt/β-Catenin pathway is largely related to bone cancer, including osteosarcoma[15]. It has further been reported that inactivation of the Wnt/β-Catenin pathway can promote the proliferation and differentiation of high-grade osteosarcoma [16]. Regulation of the Wnt/β-Catenin pathway could inhibit the development of bone cancer *in vitro* [16]. Previous study has also demonstrated that BAIAP2L2 promotes the progression of gastric cancer via AKT/mTOR and Wnt3a/β-catenin signaling pathways[17]. This study was interested in the associations between BAIAP2L2 and the Wnt/β-Catenin pathway, and their combinational role in osteosarcoma.

In this study, the functions of BAIAP2L2 and its interactions with the Wnt/β-Catenin signaling pathway in the regulation or promotion of osteosarcoma were investigated. Immunohistochemical and Western blot analysis were conducted to measure the expression levels of BAIAP2L2. In addition, cell proliferation of MG-63 and HOS cells was detected using cell counting kit-8 assay and colony formation assay. Cell migration, cell cycle, and cell apoptosis were further assessed through transwell assay and flow cytometry. In addition, the expression of specific proteins was also evaluated. Our findings may shed light on the functional mechanism of BAIAP2L2 on the development of osteosarcoma and provide novel therapeutic directions for the treatment of bone cancer.

2. Materials and Methods

2.1. Reagents

RPMI-1640 medium was obtained from HYCLONE, USA. Gibco fetal bovine serum (FBS), and lipofectamine 2000 were purchased from Thermo Fisher Scientific, USA. Penicillin-Streptomycin, 0.25% trypsin digestion, and CCK-8 reagent were purchased from Solarbio Life Sciences, China. Ultrapure RNA kit, HiFiScript cDNA synthesis kit, and UltraSYBR mixture were purchased from CwBio, China. The primers used in this study were synthesized by Genewiz. BAIAP2L2 interference sequence and its negative control (NC) were synthesized by RiboBio, China. Antibodies were purchased from Proteintech, USA. Matrigel was purchased from BD, USA, and transwell chambers were purchased from Millipore, USA. Cell culture ingredients were purchased from Eppendorf, USA. All cell lines were provided by the Chinese Academy of Sciences, China.

2.2. Cell culture

The human osteosarcoma cell lines MG-63, U2OS, SOSP-9607, SAOS-2 and HOS, and the human osteoblast cell line hFOB were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultivated in a cell culture medium composed of RPMI-1640 with 10% FBS, 100 U/ml penicillin and 0.1 μg/ml streptomycin at 37 ℃ with 5% CO_2.

2.3. Transfection

MG-63 and HOS cells were seeded in 6-well plates. When cells were grown at logarithmic phase, the supernatant was replaced with fresh culture medium. Lipofectamine 2000 was used to transfect BAIAP2L2 siRNA and negative control siRNA (purchased from Santa cruz, Catalog NO. sc-72606 & sc-37007) into the cancer cells. After 6 h, culture medium was refreshed and cells were cultured for a further 48 h.

2.4. Fluorescence quantitative PCR for interference efficiency

Total RNAs were isolated using RNA extraction kit (Thermo Fisher, Catalog NO.12183018A). Precision nanoScript2 Reverse Transcription Kit (PrimerDesign) was used to perform reverse transcription to synthesize cDNAs. QuantiTect SYBR Green PCR kit (Qiagen) was utilized to measure the interference effect. Expression levels were normalized to the expression levels of β-actin. Annealing temperature for the primers was 55 ℃ and the extension temperature was 72 ℃. A total of 35 cycles were performed. The primer sequences were as following:

BAIAP2L2-F: 5′-CGGCACTTGAACTCTGACCT-3′,
BAIAP2L2-R: 5′-CTTGTCTCTCTTGCGCTCCA-3′;
β-actin-F: 5′-GCATGGGTCAGAAGGATTCCT-3′,
β-actin-R: 5′-TCGTCCCAGTTGGTGACGAT-3′.

2.5. Proliferation and colony assays

After 24 h of transfection, osteosarcoma cancer cells were digested. Cell numbers were then counted. Next, cells were plated into a 96-well plate with 1,000 cells in each well. Cell viability was measured every 24 h, and 10 μl of CCK8 reagent were added into the plate for incubation at 37 ℃ for 1.5 h. The absorbance at 450 nm was then measured. MG-63 and HOS cells were transfected with BAIAP2L2 siRNA or NC siRNA with 400 cells per well. Cells were cultured for 14 d. After this, cells were fixed with 4% paraformaldehyde and stained with crystal violet. Finally, cell colonies were photographed and cell numbers were counted.

2.6. In vitro invasion assay

In the transwell chamber, 100 μl of matrigel were added, followed by shaking and incubation at 37 ℃ for 4 h. After solidifying, 600 μl of serum-free 1640 medium were placed in the upper and lower chambers, and the membranes were hydrated for 30 min. Osteosarcoma cancer cells, which were transfected for 24 h, were utilized as cell suspensions in serum-free 1640 medium. Next, 100 μl of cell suspension (1×10^4 cells) were placed in the upper chamber, and 600 μl of 10% FBS were pipetted into the bottom chamber. After 24 h, the residual cells (upper chamber) were removed and the chamber was washed with PBS. Subsequently, the cells were fixed with paraformaldehyde for 15 min, dyed with 0.1% crystal violet for 5 min, and washed with PBS. A microscope was used to observe the cells and count the cell numbers. In addition, transwell migration assay was performed using the same protocol as the invasion assay, except without a Matrigel pre-coating.

2.7. Flow cytometry for apoptosis detection

Cells were first transfected for 48 h. Then, MG-63 and HOS cells were digested and suspended at a density of 5×10^6 cells/ml. Next, cells were incubated with Annexin V for 15 min and PI for 5 min in the dark. After pipetting out dye liquor, cell apoptosis was detected by flow cytometry (FACSCanto II, BD Bioscience, USA), and data were analyzed using Flowjo software.

2.8. Immunohistochemistry analysis

Osteosarcoma tumor tissues and *para*-carcinoma tissue were fixed with 10% of formalin, embedded in paraffin, and sectioned for immunohistochemical staining. Tissue sections were treated by dewaxing, antigen recovery, and hydrogen dioxide incubation to remove endogenous catalase. After blocking for 1 h, the sections were incubated with anti-BAIAP2L2 rabbit antibody (1:100; abcam, Catalog NO. ab224323). Next, 3,3′-diaminobenzidine

tetrahydrochloride was used to measure the stained cells in 4 randomly selected areas.

2.9. Western blot analysis

After transfection for 48 h, total proteins were isolated from MG-63 and HOS cells. Equal amount (20 μg) of protein samples were loaded for SDS-PAGE separation, followed by transferring to a PVDF membrane. The membrane was blocked in 5% skim milk for 1 h, and subsequently incubated with the primary antibody at 4 °C overnight. Next, secondary antibody was added and the membrane was incubated at room temperature for 1 h. After washing, ECL was performed to quantify the expression levels of proteins.

2.10. Lentivirus packaging

The lentivector-mediated short-hairpin BAIAP2L2 (sh-BAIAP2L2) and non-targeting plasmids (sh-control) were designed and synthesized into pCDH-CMV-MCS-EF1-Puro (System Biosciences #CD510B-1) lentiviral vector by Geneseed Biotech (Guangzhou, China). The pCDH-CMV-MCS-EF1-Puro empty plasmid was used as an empty vector control (EV). Lentivirus packaging was conducted by introducing the lentiviral expression plasmids plus lentivirus packaging vectors δ8.9 and VSVG into HEK293T cells (ATCC) using Lipofectamine 3000 (Life Technologies #L3000015). Lentiviral particles were collected by ultracentrifugation and stored at −80 °C.

2.11. Stable transfection cell model

Lentivirus was adjusted at MOI 5 to infect 1×10^6 MG-63 cells in the presence of Polybrene (8 ng/ml). After 24 h, selection media (containing 7 μg/ml puromycin) was used to screen the transfected cells. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Selection media was replaced every 2–3 days. Cells were grown in selection media for subsequent experiments to ensure that only stably transduced cells were cultured.

2.12. In vivo tumor formation model

MG-63 cells (5×10^6) stably transfected with sh-BAIAP2L2 or LV-NC (control) were subcutaneously injected into BALB/c nude mice (4–6 weeks old) at day 0 (n = 6 for each group, male and female was equally distributed in each group), housed under SPF conditions (temperature was 22 ℃, illumination time was 12 h, humidity was 45%). All animal experiments were performed under the institutional guidelines approved by the Use Committee for Animal Care. Before harvesting the tumors, mice were euthanized with isoflurane set at saturated vapor pressure. This study was approved by the Ethics Committee of Animal Use of the University Three Gorges Hospital. Mice were euthanized at 28 d, heart completely stopped and the dilation of pupil was used to judge the end point. Tumors were then excised and measured.

2.13. Statistical analyses

Data were analyzed using SPSS 10.0 and presented as the mean ± standard deviation (SD) of 3 independent replicates. Stu-

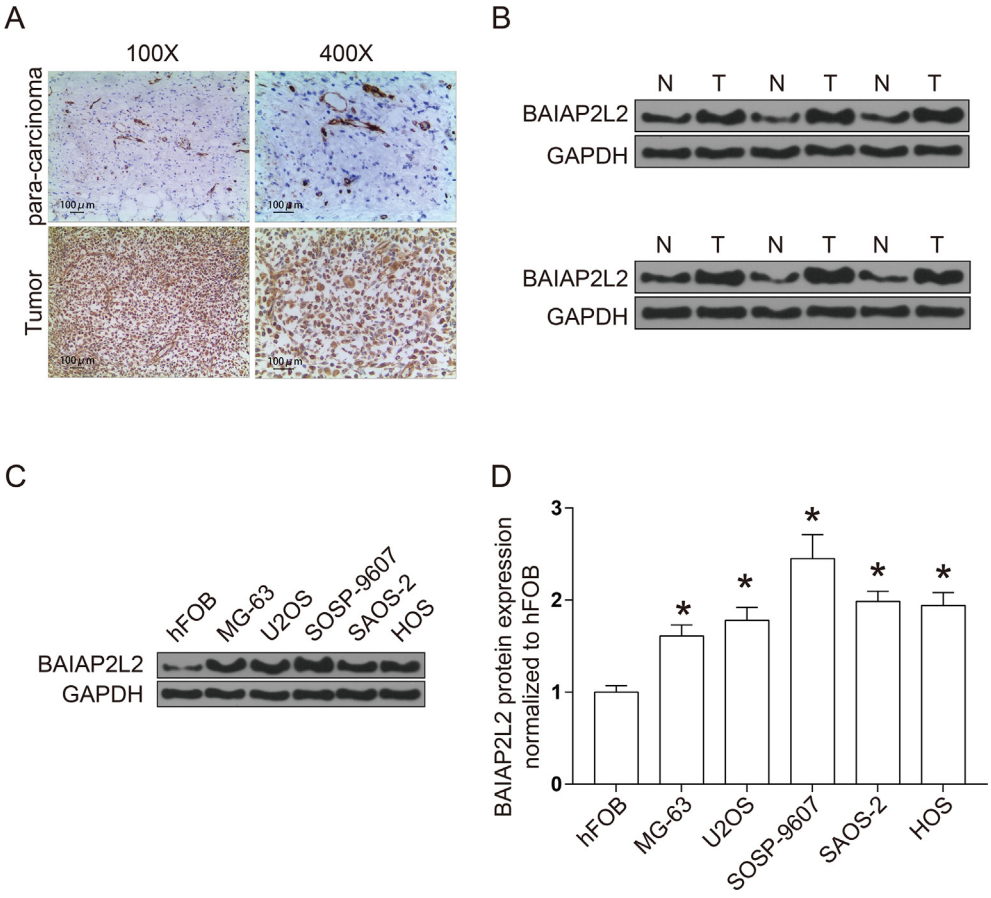

Fig. 1. BAIAP2L2 was upregulated in human osteosarcoma tissues and cells. A. Immunohistochemistry and B. Western blot analysis for the expression of BAIAP2L2 in tumor and *para*-sarcoma tissues. C. and D. Western blot analysis results for the expression of BAIAP2L2 in human osteosarcoma cell lines of MG-663, U2OS, SOSP-9607, SAOS-2 and HOS and human osteoblasts cell line of hFOB. T-test was used to perform statistic to compare each group, $^*p < 0.05$.

Table 1
The correlation between expression of BAIAP2L2 and clinic pathological parameters in osteosarcoma cancer patients.

Clinic pathological parameters	N = 60	BAIAP2L2 Low (n%)	BAIAP2L2 High (n%)	P
Gender				
Male	38	15 (39.5)	23 (60.5)	0.498
Female	22	8 (36.4)	14 (63.6)	
Age (years)				
<18	26	9 (34.6)	17 (65.4)	0.379
≥18	34	14 (41.2)	20 (58.8)	
Tumor diameter (cm)				
<5	28	19 (67.9)	9 (32.1)	<0.001
≥5	32	4 (12.5)	28 (87.5)	
Tumor location				
Tibia/Femur	40	15 (37.5)	25 (62.5)	0.238
Other location	20	8 (40.0)	12 (60.0)	
Histological subtype				
Conventional	53	21 (39.6)	32 (60.4)	0.198
Special	7	2 (28.6)	5 (71.4)	
Surgical stage				
I-IIA	19	7 (36.8)	12 (63.2)	0.234
IIB	41	16 (39.0)	25 (61.0)	
Distant metastasis				
Yes	27	12 (44.4)	15 (55.6)	0.012
No	33	11 (33.3)	22 (66.7)	

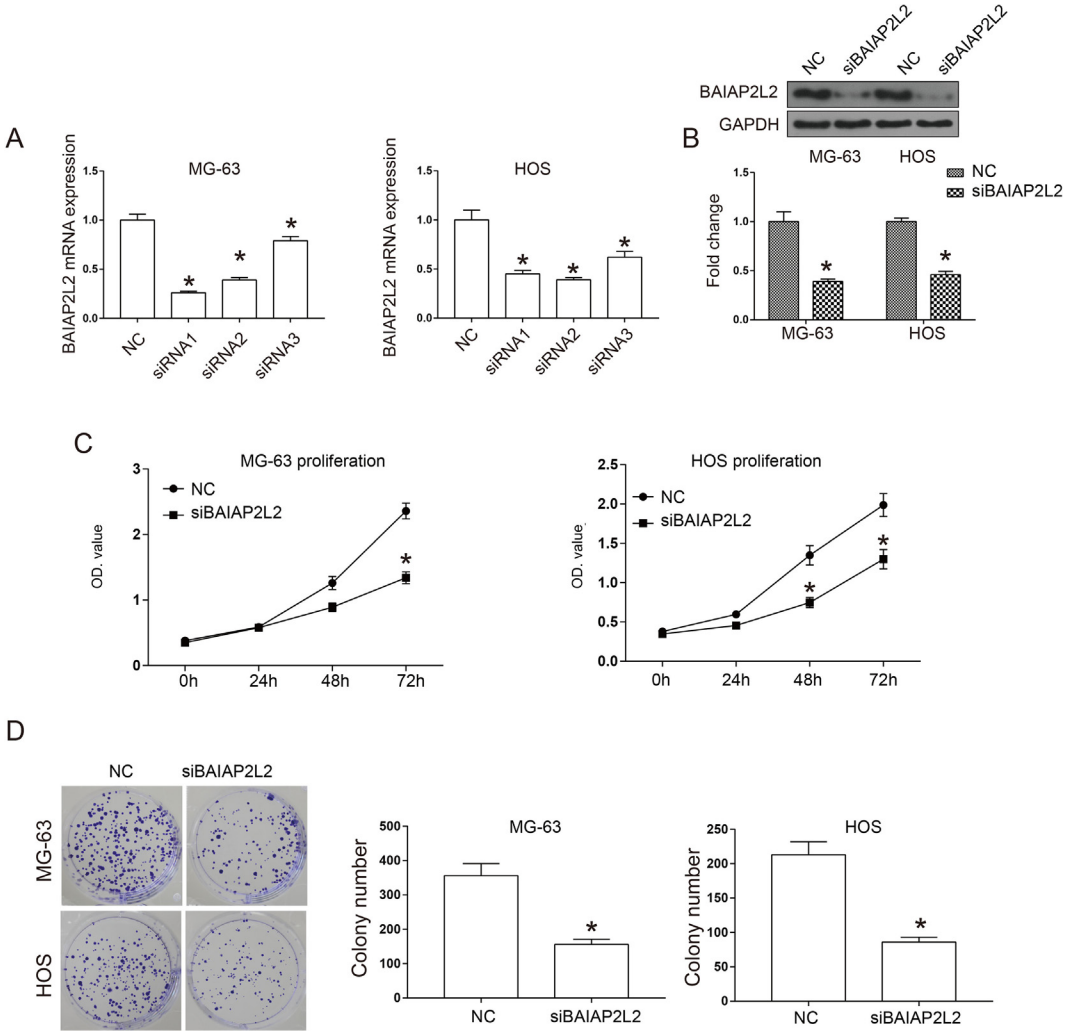

Fig. 2. Inhibition of BAIAP2L2 suppressed the proliferation of osteosarcoma cells (MG-63 and HOS). A. The mRNA expression of BAIAP2L2 via qRT-PCR. B. Western blot analysis for the measurement of the expression of BAIAP2L2 protein transfected with siRNA. C. CCK-8 assay for cell viability. OD value was used to indirectly indicate the cell numbers. D. Colony formation ability of MG-63 and HOS cells. $^{*}p < 0.05$.

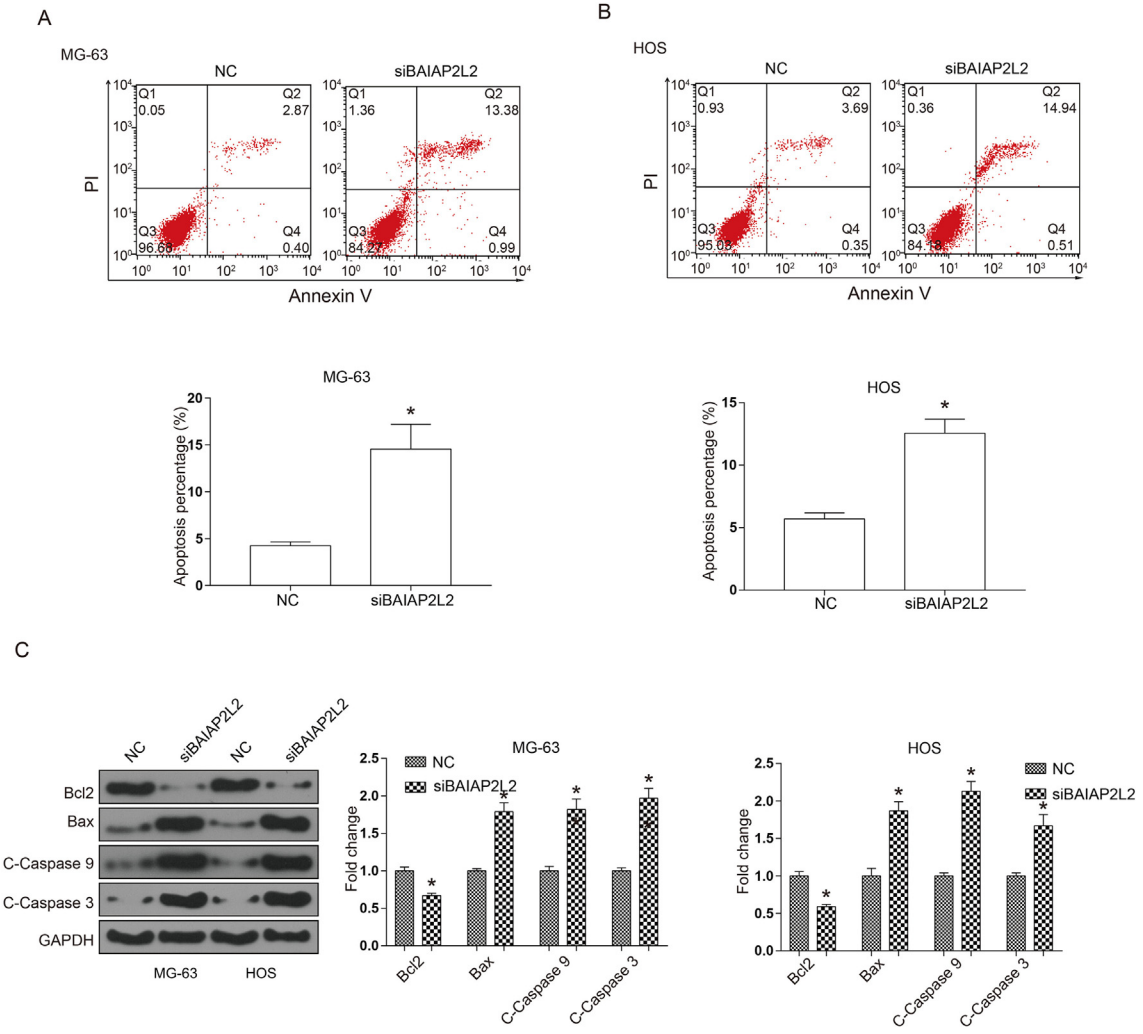

Fig. 3. Knockdown of BAIAP2L2 induced apoptosis of MG-63 and HOS cells via the Bcl2/Bax axis. A. and B. Flow cytometry results for cell apoptosis in MG-63, and HOS, respectively. C. The protein expression of apoptosis signaling pathway by western blot. $^{*}p < 0.05$.

dent's *t* test was used to compare the differences between 2 groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. BAIAP2L2 was greatly upregulated in human osteosarcoma

Immunohistochemistry and Western blot analysis showed that the expression of BAIAP2L2 was up-regulated in human osteosarcoma tumor tissues compared to that in *para*-sarcoma tissues (Fig. 1A and 1B). In addition, clinical pathological association analysis showed that the expression of BAIAP2L2 in sarcoma tissues was closely related to tumor diameter ($P < 0.05$) (Table 1). Subsequently, the protein expression levels of BAIAP2L2 in osteosarcoma cells and human osteoblasts cell line (hFOB) were detected. As shown in Fig. 1C and 1D, the expression levels of BAIAP2L2 were higher in MG- 63, U2OS, SOSP-9607, Saos-2 and HOS tumor cells compared to those in hFOB cells. The morphological analysis of U2OS, SOSP-9607 and Saos-2 showed all epithelial like cells, and MG-63 and HOS contained both epithelial and fibroblast cells. To investigate the role of BAIAP2L2 in a more general perspective, MG- 63 and HOS were selected for the following experiments.

3.2. Inhibition of BAIAP2L2 suppressed the proliferation of osteosarcoma cells

As shown in Fig. 2A, all three siRNAs could remarkably down-regulate BAIAP2L2 mRNA in both MG-63 and HOS cells ($P < 0.05$). Si-RNA2 displayed the most silencing ability for the expression of BAIAP2L2 in both MG-63 and HOS cells, and was therefore selected for the following experiments. Western blot analysis showed the interference effects of protein expression levels in siRNA1 (Fig. 2B). CCK-8 assay results showed that down-regulation of BAIAP2L2 resulted in a great reduction in cell viability of both MG-63 and HOS cells ($P < 0.05$) (Fig. 2C). In addition, colony formation assay results showed that knockdown of BAIAP2L2 significantly inhibited colony formation ($P < 0.05$) (Fig. 2D). These results indicated that knockdown of BAIAP2L2 could suppress the proliferation of osteosarcoma cells.

3.3. Knockdown of BAIAP2L2 promoted osteosarcoma cancer cell apoptosis

As shown in Fig. 3A and 3B, the percentage of apoptotic cells was greatly increased by the knockdown of BAIAP2L2 in MG-63 and HOS cells. Western blot analysis results showed that knock-

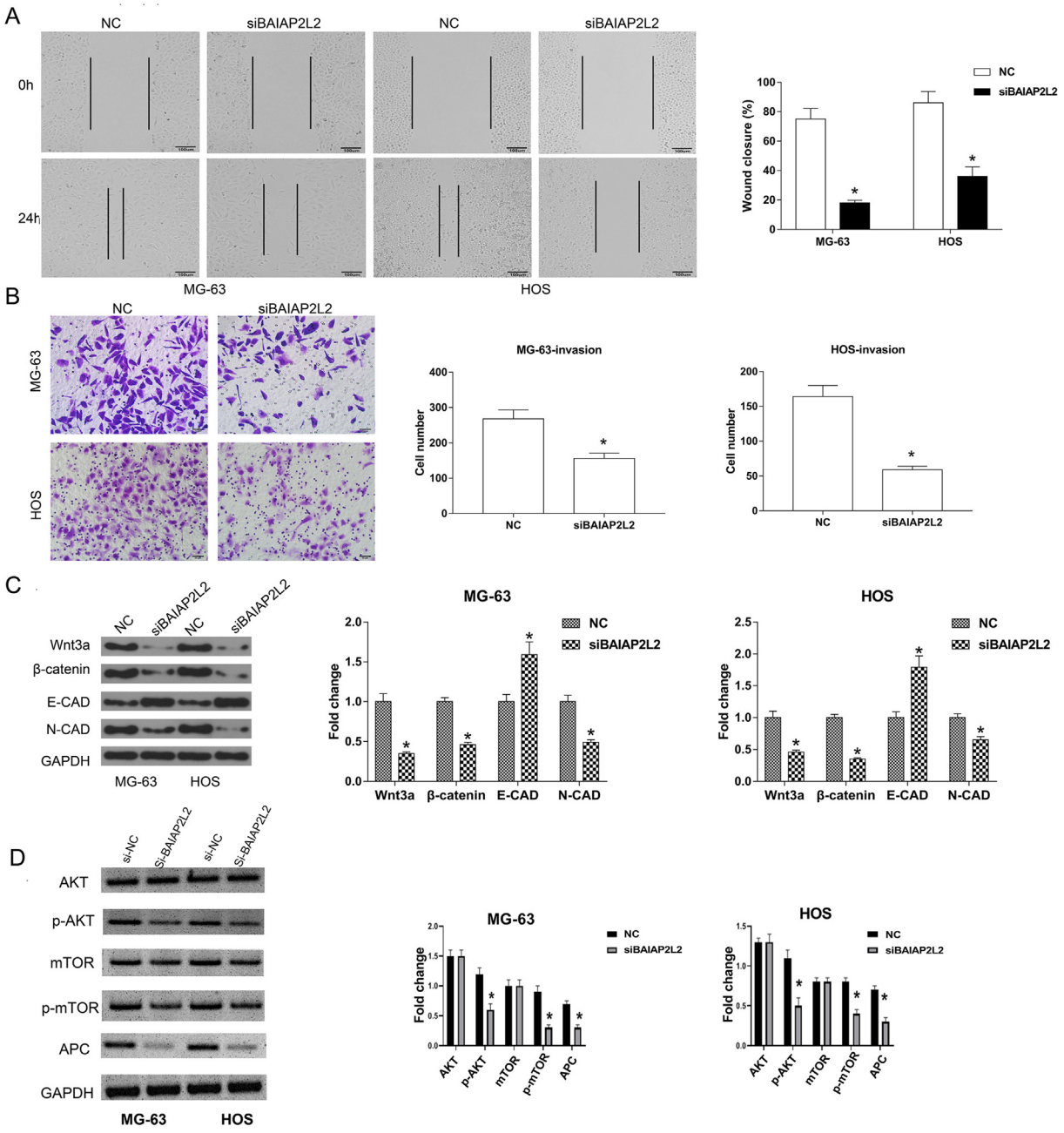

Fig. 4. Down-regulation of BAIAP2L2 inhibited cell migration/invasion and induced inactivation of the Wnt/β-catenin pathway. A. Scratch assay showed that down-regulation of BAIAP2L2 transfection suppressed cell migration in MG-63 and HOS cells. B. Transwell assay indicated that transfection of siRNA inhibited cell invasion in MG-63 and HOS. C. Western blot analysis for the measurement of the expression of the Wnt/β-catenin pathway. D. Western blot analysis for the measurement of the hub protein expression of AKT/mTOR pathway and Wnt/β-catenin pathway $^{*}p < 0.05$.

down of BAIAP2L2 increased the expression levels of the pro-apoptotic protein Bax and the apoptosis executor Cleaved-Caspase 3, and reduced the expression levels of anti-apoptotic protein Bcl2 (Fig. 3C). These results indicated that knockdown of BAIAP2L2 could induce cell apoptosis in MG-63 and HOS cells.

3.4. *Knockdown of BAIAP2L2 inhibited cell migration/invasion and induced inactivation of the Wnt/β-catenin pathway*

The transwell assay results showed that knockdown of BAIAP2L2 significantly inhibited the migration of MG-63 and HOS cells (Fig. 4A). In addition, knockdown of BAIAP2L2 negatively modulated the invasion of MG-63 and HOS cells (Fig. 4B). Given previous study revealed that BAIAP2L2 can regulate Wnt/β-catenin pathway and AKT/mTOR pathway to promote the gastric caner progression(17). Furthermore, knockdown of BAIAP2L2 resulted in the inactivation of the Wnt/β-catenin pathway, while reducing the expression levels of Wnt, β-catenin and N-CAD, and increasing the expression levels of E-CAD (Fig. 4C). There were complex cross- talk between AKT/mTOR pathway and Wnt/β-catenin pathway. For the detailed molecular mechanism, the expression of p-AKT and p-mTOR were also attenuated after silencing BAIAP2L2(Fig. 4D).

3.5. *Knockdown of BAIAP2L2 inhibited tumor growth in vivo*

Monitoring of tumor volume showed that knockdown of BAIAP2L2 remarkably suppressed tumor growth of the osteosar-

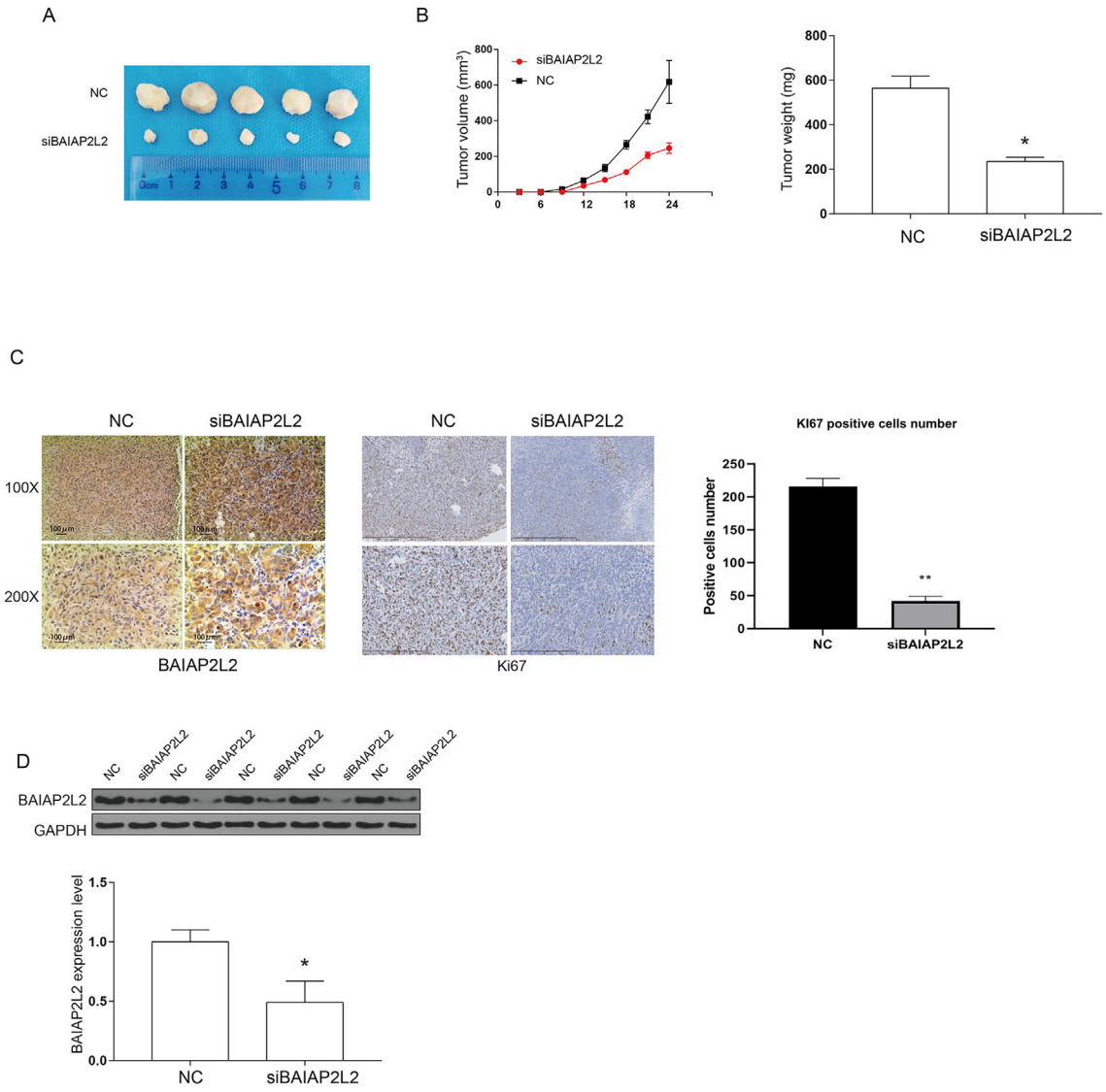

Fig. 5. Down-regulation of BAIAP2L2 inhibited tumor growth *in vivo*. A. Tumor comparison pictures. B. Tumor growth curve. The red line indicates the sh-BAIAP2L2 transfected cells, and the black line indicates the sh-NC transfected cells. The average tumor weights in the silencing of BAIAP2L2 and control group. C. The expression of BAIAP2L2 and Ki67 in xenograft tumors. Scale bar = 100 μm. D. Western blot analysis for the measurement of the expression of BAIAP2L2 protein in tumors transfected with shRNA. *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

coma cells MG-63, compared to the siNC group ($P < 0.05$) (Fig. 5A, 5B, and 5C). Immunochemistry analysis results suggested that the expression levels of BAIAP2L2 and Ki67 were greatly lowered by knockdown of siBAIAP2L2 (Fig. 5D). These results indicated that knockdown of BAIAP2L2 promoted osteosarcoma tumor growth *in vivo*.

4. Discussion

Osteosarcoma is the most typical bone cancer that usually occurs in young people under 25 years old[14]. Treatment for osteosarcoma includes chemotherapy and physical radiation, but the efficacy and prognosis are hindered by the natural properties of bone cancer[6,7]. Extensive studies have been conducted to explore the mechanism of tumorigenesis underlying the regulation and proliferation of human osteosarcoma tissues and cells, such as somatic disruptive genes. In this study, we investigated the molecular mechanisms of BAIAP2L2 and its association with the Wnt/β-Catenin signaling pathway in the development of osteosarcoma.

Previous studies have revealed that BAI-associated proteins maintain their regulatory roles in inflammation, tumorigenesis, and phagocytosis [18]. BAIAP2L2, an important member of the I–BAR protein family, has been identified as a possible biomarker for some diseases. Xu *et al.* reported that knockdown of BAIAP2L2 could lead to the suppression of the estrogen mediated s–phase entry pathway, suggesting that BAIAP2L2 serves as a marker for lung cancer [9]. To the best of our knowledge, we are the first to investigate the role of BAIAP2L2 in osteosarcoma. Our immunohistochemistry and western blot results showed that expression of BAIAP2L2 was up-regulated in human osteosarcoma tumor tissues compared with that in normal tissues. Our findings further suggested that BAIAP2L2 was positively correlated with the development of osteosarcoma. In addition, CCK-8 assay and colony formation assay confirmed that inhibition of BAIAP2L2 suppressed the proliferation of osteosarcoma cells. These results further proved that BAIAP2L2 participated in the development of human osteosarcoma.

It has been reported that BAI1 protein family could act as versatile and promising markers for human cancers and host immune response. Abnormal expression of BAI1 protein was linked to the development of several malignant tumors [18]. In our study, flow cytometry results revealed that down-regulation of BAIAP2L2

resulted in osteosarcoma cancer cell apoptosis. The knockdown of BAIAP2L2 increased the expression levels of the pro-apoptotic protein Bax and apoptosis executor Cleaved-Caspase 3. Our results were in agreement with previous findings that BAI1 activity was altered during the development, proliferation and apoptosis of tumor cells.

The Wnt family functions in the secretion of glycolipid proteins and initiates a cascade of signaling pathways [19]. Aberrant regulation of Wnt signaling pathways was found to be related to a range of diseases [20]. Extensive studies have shown that modulation of some specific RNAs could result in activation or inactivation of the Wnt/β-catenin signaling pathway in human cancers, including gastrointestinal cancers [21], prostate cancer [22], breast cancer [11] and many others [23,24]. Recently, two reports have also revealed the activated status of Wnt/β-catenin signaling pathway in Osteosarcoma[25,26]. Our results from transwell assay, migration assay, and invasion assay confirmed that down-regulation of BAIAP2L2 inhibited cell migration/invasion and induced the inactivation of Wnt /β-catenin pathway. According to previous reports [27,28], the suppression of this pathway may also have a negative impact on the proliferation of osteosarcoma cells.

It has been reported that regulation of BAIAP2L2 could affect the expression of many genes, including those that are closely involved in the development of tumors [10]. In this study, we found that knockdown of BAIAP2L2 led to decreased cell viability and colony formation ability in human lung cancer cell line [10]. However, overexpression of BAIAP2L2 promoted proliferation and growth of lung cancer cells [10]. These studies revealed that down-regulation of BAIAP2L2 may suppress tumor growth in osteosarcoma tissues and cells. In this study, we investigated tumor growth by monitoring the tumor weight, volume and growth curve with the knockdown of BAIAP2L2. We found that the expression of BAIAP2L2 and Ki67 was greatly down-regulated by knockdown of BAIAP2L2. In addition, tumor growth *in vivo* was also inhibited by down-regulation of BAIAP2L2. These findings supported our hypothesis that knockdown of BAIAP2L2 could suppress tumor growth. Wnt signaling has been shown to contribute to osteoblastogenesis[29], but sustained activation would likely result in Osteosarcoma[30]. BAIAP2l2, as a negative regulator for Wnt signaling, may be a new therapy target for Wnt signaling abnormal activated Osteosarcoma. Since there are too many obstacles for siRNA used in human, the antibody based drugs may be more proper for this protein.

5. Conclusion

In conclusion, BAIAP2L2 promoted the proliferation, migration and invasion of osteosarcoma associated with the Wnt/β-catenin signaling pathway.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability

The data that support the findings of this study are available on request from the corresponding author: Youhao Chen*, Department of Orthopaedics, Chong Qing University Three Gorges Hospital, Chongqing City, 404100, PR.China. Email address: youhaochenchongqin@163.com.

The data are not publicly available due to the fact that they contain information that could compromise the privacy of research participants.

Ethical Approval and Consent to participate

Informed consent was obtained from all individual participants included in the study. All experiments were approved by the Ethics Committee of Chong Qing University Three Gorges Hospital.

Consent to publish

Not applicable.

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